BIOMARKERS IN ACUTE KIDNEY INJURY DUE TO CONTRAST INDUCED NEPHROPATHY

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of

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DECLARATION

I, Justor Banda declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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Signed

Date: _____

In memory of my mother

Mary Goretti Mukelabai

1947-1996

PUBLICATIONS AND PRESENTATIONS ARISING FROM THE STUDY

Publications:

1. **J Banda**, R Duarte, C Dickens, T Dix-Peek, V Mngomezulu, P Manga, S Naicker. Risk Factors and Outcomes for Contrast Induced Nephropathy among Hospitalized South Africans. S Afr Med J. 2016; 106 (7): 699-703

2. **J Banda,** S Naicker. Contrast Induced Nephropathy among Hospitalized South Africans-Impact of Serum Albumin. J Am Society Nephrol 2015. Abstract Supplement, Vol 26; page 901A

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ABSTRACT

Background: Despite preventive guidelines, iatrogenic contrast-induced nephropathy (CIN) ranks third as a cause of hospital acquired acute kidney injury (AKI), and impacts significantly on morbidity and mortality and is associated with high hospital costs. In Sub-Saharan Africa, the rates and risk factors for CIN remain unexplored. Despite the positive association of genetic polymorphisms in the TNFα and IL10 genes with CIN in Asian populations, the CIN genetic susceptibility in other races is unknown. Serum creatinine is a sub-optimal biomarker for the early diagnosis of CIN resulting in delayed interventions. This study investigated rates, risk factors and outcomes of CIN, the influence of genetic susceptibility to CIN in the black population and lastly, the accuracy of novel biomarkers in the early diagnosis of CIN and prognosticating patient outcomes.

Methods: This was a prospective case-controlled study conducted at Charlotte Maxeke Johannesburg Academic Hospital, in South Africa from January 1, 2014 to December 30, 2015.Hospitalized patients undergoing enhanced computed tomography and angiography were consecutively recruited to the study and followed up for development of CIN. CIN was defined as an increase in serum creatinine >25% or an absolute increase of >44 µmol/l from baseline at 48-72 hours after exposure to contrast media. In the second part of the study, a nested case-controlled cohort that included 30 CIN patients and 60 controls (those undergoing contrast administrations and not meeting CIN criteria) were ethnically matched for gender, and age in a case: control ratio of 1:2 at all-time intervals. Sera for neutrophil gelatinase-associated lipocalin-2 (NGAL), cystatin C, beta-2 microglobulin (β2M), interleukin 18 (IL18), IL10, and tumor necrosis factor alpha (TNFα) were collected at four time points: baseline (pre-contrast), 24 hours, 48 hours and ≥5-7 days after contrast administration and their concentrations were determined using luminex assays and an enzyme linked immunosorbent assay for β2M as per manufacturer's instructions. The areas under receiver operating characteristic curves (AUROC) were generated to determine accuracy of novel biomarkers to diagnose CIN and CIN mortality.

Genomic DNA was extracted from peripheral blood samples of 208 black South Africans using the Maxwell DNA purification kit (Promega AS1010, USA) and their genotypes for -308(rs1800629) and -857(rs1799724) in the TNFα gene and -592(rs1800872), -819(rs1800871), -1082 (rs1800896) and +1582(rs1554286) in the IL10 gene were determined by restriction fragment length polymorphism (RFLP).

Results: We recruited 371 hospitalized patients (mean age 49.3±15.9); the rates of CIN were4.6% and 16.4% respectively, using an absolute or relative increase in serum creatinine from baseline. Anaemia was an independent predictor for the development of CIN (RR 1.71, 95% 1.01-2.87; p=0.04). The median serum albumin was 34 g/l (IQR: 29-39.5) vs. 38 g/l (IQR: 31-42), p=0.01 in the CIN and control groups respectively.Mortality was significantly increased in the CIN group (22.4% vs. 6.8%; p<0.001), and CIN together with anaemia predicted mortality with a 2-fold (p=0.01) and a 3-fold (RR p=0.003) riskrespectively. The median cystatin C at 24 hours (p<0.001) and β2M(at all-time points)levels were significantly higher in the CIN group compared to controls. The median cystatin C at 24 hours and β2Mlevels at 48 hours were 856.59 ng/ml (IQR 620.75-1002.96) vs. 617.42 ng/ml (IQR 533.11-805.20); p<0.001 and 5.3 µg/ml (IQR 3.8-6.9) vs. 3.3 µg/ml (IQR 2.7-4.5); p<0.001 with AUROCs of 0.75 and 0.78 respectively for early CIN discrimination.Pre-contrast IL18 (p <0.001), β2M (p=0.04) and TNFα (p<0.001) levels were significantly higher in the non-surviving group and their AUROC were 0.83, 0.82 and 0.94 for CIN+ mortality. Baseline NGAL was a better marker for excluding patients at higher risk of developing CIN with negative predictive and positive predictive values of 0.81 and 0.50 respectively. The frequency of TNFα -308 AA genotype was significantly increased in the CIN group compared to controls (13.3% vs.1.82%, p=0.016) and the presence of the TNFα-308 AA (high producer) vs. GA genotypes was associated with a 9-fold CIN risk (9.24, 95% CI, 1.88-45, p=0.006). The IL10-1082 AA-allele (low producer) was significantly higher in the non-surviving CIN+ patients compared to controls (p=0.01).

Conclusions:CIN occurred at a relatively high rate in our study and predicted poorer clinical outcomes. The presence of CIN and anaemia positively predicted mortality. Caution should be exercised in patients with anaemia and hypoalbuminaemia undergoing contrast studies. Serumcystatin C was the best novel biomarker for the early diagnosis of CIN and while baseline NGAL is superior as a biomarker for excluding patients at higher risk for CIN. IL18, β_2M and TNFα are the best novel biomarkers for predicting the prognosis of patients with CIN. Increased frequency of the TNFα-308 AA genotype is a predisposing factor for CIN development. The low producer IL10-1082 AA genotype decreases survival in patient with CIN.

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ADP	Adenosine diphosphate
ADQI	Acute Dialysis Quality Initiative
AKI	Acute Kidney Injury
AKIN	Acute Kidney Injury Network
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
AUROC	Area under the receiver operative characteristics curves
B2M	Beta 2 microglobulin
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiological Collaboration
CI	Confidence Interval
CrCl	Creatinine clearance
CRP	C reactive protein
CT	Computerized tomography
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
EDTA	Ethylenediaminetetraacetic acid
EP	Prostaglandin E receptor

eGFR	estimatedGlomerular filtration rate
ESRD	End stage renal disease
EtBr	Ethidium bromide
ESUR	European Society of Urogenital Radiology
HORISONS-AMI	Harmonizing Outcomes and Revascularization in Acute Myocardial Infarction
HOCM	High osmolar contrast media
HTN	Hypertension
HIV	Human immunodeficiency virus
ICAM	intracellular cell adhesion molecules
IL10	Interleukin 10
1L18	Interleukin 18
IOCM	Iso-osmolar contrast media
IVC	Inferior vena cava
HAART	Highly Active Antiretroviral Drugs
KDa	Kilo Dalton
KIDGO	Kidney Disease Improving Global Outcomes
LOCM	Low osmolar contrast media
MI	Myocardial infarction
mTAL	medullary thick ascending limb

MPO	Myeloperoxidase
NAC	N acetyl cysteine
NGAL	Neutrophil Gelatinase-Associated Lipocalin
NO	Nitric oxide
NPV	Negative predictive value
PCI	Percutaneous coronary intervention
PG	Prostaglandins
PPV	Positive predictive value
PTID	Participant's identification number
RCT	Randomized Controlled Trial
RIFLE	Risk Injury Failure Loss End stage
ROS	Reactive oxygen species
RRT	Renal replacement therapy
SCr	Serum creatinine
SBP	Systolic blood pressure
SD	Standard deviation
SSP	Serum separating tubes
SNP	Single nucleotide polymorphism
TNFα	Tumor necrosis factor α
TNFR	tumor necrosis factor alpha receptor

PREFACE

CIN is linked with increased morbidity and mortality; however, in South Africa (including Sub-Saharan Africa) there is dearth of data on rates and patient outcomes due to CIN. Despite recent reporting on positive associations of genetic polymorphisms in TNFα and IL10 with contrast induced nephropathy (CIN) among Asian populations, its role in other populations remains unexplored. Treatment of CIN is non-specific and limited to supportive care only and early diagnosis and interventions are delayed due to the existing suboptimal traditional biomarkers (serum creatinine).

It is for these reasons that this study was undertaken to identify prevalence of CIN and patient outcomes, the novel biomarkers for early diagnosis of CIN and prognostication and the genes associated with susceptibility to CIN in black South Africans. This will help in implementing targeted therapy among high risk populations with polymorphisms in these inflammatory cytokines genes (IL10 and TNFα genes). Identification of higher risk individuals to avoid exposure and earlier diagnosis of CIN using new biomarkers and identification of susceptibility genes will not only reduce morbidity and mortality but will also add knowledge to renal medicine.

This PhD is presented in an integrative format consisting of 6 chapters:

Chapters 1-2; literature review and the methods

Chapter 3; Chapter 3; are the results on the prevalence and risk factors of CIN including patient outcomes (Chapter 3 was accepted for publication in the South African medical Journal on 20.2.2016).

Chapter 4; are results on accuracy of novel biomarkers in predicting and prognosticating CIN

Chapter 5; are the results on influence of genetic susceptibility in the TNF and IL10 genes to CIN

Chapter 6 is summary of study findings, recommendations and limitations of the study.

1. CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Despite preventive guidelines, iatrogenic contrast-induced nephropathy (CIN) ranks third as a cause of hospital acquired acute kidney injury (AKI) and impacts significantly on morbidity and mortality and is associated with longer duration of hospitalization and higher medical costs (1-4).Globally, the number of iodinated contrast media procedures exceeds 80 million per year and is administered for both diagnostic and therapeutic indications (5, 6). In developed countries, rates of computed tomography procedures using iodinated contrast media are almost 30 million per year (7) and rates of angiography performed in developed countries also exceeds 2 million per year (7). Hence, CIN is a complication and an outcome of increased iodinated contrast media administration (8).

1.2 Incidence and prevalence of CIN

The rates of CIN vary, depending on study definitions employed and underlying risk factors, and ranges from 0.6% to 50% or higher among risk groups (9-13). Shema *et al*. reported a 4.5% incidence of CIN among hospitalized Israeli patients; CIN was defined as an increase in serum creatinine of more than 44 umol/l five days post contrast media administration (1). Chang *et al*. found 10.3% prevalence of CIN in a prospective study of cardiac patients undergoing percutaneous coronary intervention (PCI) in Taiwan (14).In this study, CIN was defined as an increased serum creatinine ≥ 44 umol/l from baseline. In other studies, Mitchell *et al*. reported a 14% prevalence of CIN among patients with suspected pulmonary embolism in Northern Carolina, USA (3, 4). A recent randomized clinical trial (RCT), the harmonizing outcomes with revascularization in acute myocardial infarction (HORIZONS-AMI) study, reported a 16.1% rate of CIN among PCI patients (12).

1.3 Risk factors associated with CIN

Patient factors that include age, comorbidities and contrast media related factors are associated with development of CIN in different settings (12, 15, 16) [Table 1.1].

Table 1.1 Risk factors for contrast induced nephropathy

Fixed (non-modifiable) risk factors	Modifiable risk factors
Older age	Volume of contrast media
Diabetes mellitus	Hypotension
Pre-existing renal failure	Anaemia and blood loss
Advanced CHF	Dehydration
Low LVEF	Low serum albumin (<35g/l)
Acute myocardial infarction	ACE inhibitors
Cardiogenic shock	Diuretics
Renal transplant	Non-steroidal anti-inflammatory drugs
	Nephrotoxic antibiotics
	IABP

ACE, angiotensin-converting enzyme; CHF, congestive heart failure; IABP, intra-aortic balloon pump; LVEF, left ventricular ejection fraction. Reproduced with permission from Mehran *et al.*2006 (16).

1.3.1 Association of age and CIN

In previous retrospective and observational studies, age independently predicted development of CIN (1, 17). Marenzi *et al*. in an observational studyof patients undergoing PCI, reported that age exceeding 75 years predicted a 5-fold risk for developing CIN (OR 5.28, 95% 1.98-14.05, p<0.001) (17). In a prospective observational study of patients undergoing iodinated computed tomography in Greece, the rates of CIN were 38.5% vs. 0% (p=0.02) in patients aged ≥ 65 years and < 65 years respectively (18). Sherma *et al*. in a retrospective study found age a significant risk factor for CIN development; patients aged 64.4±17 years had a significant risk for CIN compared to those of a younger age (59±19 years, p=0.04) (1). Various factors are implicated in age causing CIN and these include decreasing glomerular filtration rate following structural changes due to fibrotic changes to the kidneys with advancing age, calcification and likely increased presence of comorbidities in elderly patients (17, 18). However, Diogo *et al.* found a non-significant association between ages> 65 years and risk for CIN (19).

1.3.2 Association between comorbidities and CIN

The presence of comorbidities such as diabetes mellitus (DM), underlying renal and cardiac diseases are linked with high rates of CIN development (20).

In several observational and retrospective studies, presence of underlying renal disease positively predicted CIN development (21, 22). In a retrospective analysis of 322 patients undergoing PCI in France, underlying renal disease was associated with a 9-fold odds of developing CIN (OR 8.8, 95% CI 2.61-9.74; p=0.002) (21).In this study, renal disease was defined as estimated glomerular filtration (eGFR) <60 ml/min per 1.73m^2(21). Underlying renal disease defined by either decreased eGFR or increased serum creatinine are dominant risk factors for CIN in both retrospective and observational studies (10). An even smaller increase in serum creatinine 48 hours post contrast media administration was associated with a 14% mortality increase (10). Serum creatinine exceeding 132.6 µmol/l was an independent predictor for CIN in a retrospective study of patients who underwent angiography (23).

Diabetes mellitus is reportedly a significant risk factor for CIN with a rate as high as 30% (16). In an observational study of patients undergoing computed tomography with contrast in Brazil, Diogo *et al.* found a 2-fold odds of CIN in DM patients (OR 2.15; 1.13-4.06, p=0.02), while cardiac and underlying renal diseases were associated with a 2-fold and 3-fold odds for CIN risk (19). Evola *et al.* also reported a 3-fold presence of CIN in DM patients compared to controls (2). The increased risk of CIN in DM patients is linked to two factors; exacerbated hypoxia following overproduction of mediators of vasoconstriction (endothelins and angiotensin levels) and overwhelming production of reactive oxygen species (ROS) due to multivessel disease (24, 25). In animal studies, medullary partial pressure of oxygen (pO2) was 36 mmHg vs. 50 mmHg in DM rats and controls respectively (25).

Presence of cardiovascular diseases,that include congestive heart failure, myocardial infarction (MI) and cardiogenic shock, is associated with increased risk for CIN in both observational and RCT studies (22, 26). In a prospective study of PCI patients, low ejection fraction was associated with a 0.94 odds for developing CIN (p=0.007) (22).

Rosenstock*et al.*in a RCT in New York, USA found almost 5-fold odds of developing CIN in patients with cardiac disease (defined as low ejection fraction <40%) (26), while Bouzas-Mosquera*et al.* in Spain reported a 5-fold odds for CIN in patients with cardiogenic shock (OR 4.56, 95% CI 1.08-19.23, p=0.03) (23). In the majority of cases, haemodynamic compromise that characterizes cardiovascular diseases is the strong determining mechanism underlying CIN risk in these patients (26, 27). Presence of circulatory compromise positively activates renal vasoconstrictors that include sympathomimetics and angiotensin and thereby exacerbating medullary hypoxia occurring during contrast administration (26).

Recently, Li *et al.*2016 demonstrated that hypotension following contrast media administration was a significant predictor for CIN (28). In this study, a decrease >10 mmHg in systolic blood pressure (SBP) was associated with a 2-fold odds for CIN risk (2.37, 95% CI 1.04-5.38; p=0.04) (28). The mean decrease in SBP pressures were 16.2±19.1 vs. 5.9±18.9 mmHg, (p=0.001) in the CIN group compared to controls respectively (28).

Despite limited studies looking at the impact of anaemia on CIN, anaemia is a significant risk factor for CIN (16). Anaemia worsens the existing renal medullary hypoxia post contrast media administration (29). Li *et al.* reported 2-fold odds for developing CIN in patients with anaemia post PCI (29). In this study, when eGFR was 30-59 ml/min per 1.73m2; the rates of CIN were 7.9% vs. 3.8% in anaemic patients compared to controls (29).

1.3.3Impact of contrast media osmolality and administration

Regarding contrast media, type, volume and mode of administration of contrast determine the development of CIN e.g. patients receiving a higher than maximal recommended dose of contrast media were more likely to develop CIN or require renal replacement therapy (16, 30). Contrast media utilized in computed tomography and angiography is derived from tri-iodinated benzene (31). The iodine functions to provide radio opacity which is provided by the iodine component and therefore better visualization is associated with administration of higher volumes of iodinated contrast media (31).

Based on osmolality, three categories of contrast media are used; the high osmolar contrast media (HOCM), low osmolar contrast media (LOCM) and iso-osmolar contrast media (IOCM) (31). The HOCM has an osmolality above 1000 mOsmol/kg of water compared to plasma osmolality. The LOCM that include iopamidol and iohexol have an osmolality of 2-3 times higher (32) than plasma while the IOCM osmolality are comparable or lower than plasma (31). Despite the low osmolality, the IOCMs are extremely hyperviscous (31).

Previous studies have linked contrast volume and osmolality to increased CIN risk. In patients undergoing PCI recently, Shams-Eddin Taher *et al.*found a 4-fold and a 1-fold odds of developing CIN in patients using HOCM and contrast volume exceeding ≥ 400mls respectively (33). In this study, the odds for CIN in using a HOCM and higher contrast media volumes were OR 4.08, 95% CI 1.1-15.1, p=0.03 and OR 1.1 95% CI 1.00-1.01, p=0.01 respectively (33). In other studies of patients undergoing PCI, exceeding the maximum allowable dose was positively linked with a 2-fold odds (OR 2, 95% CI 1.67-2.40; p<0.001) for developing CIN and 6-fold odds (OR 6.41, 95% CI 3.3-12.1; p<0.001) for undergoing renal replacement therapy (30).

In other studies, increased volumes above 200 mls of contrast media were associated with a 2-fold odd of developing CIN (16). When the contrast media volume exceeded 600 ml, the odds ratio was almost 5-fold (adjusted OR 4.86, 95% CI 1.73-13.67) (16). Marenzi *et al.* found an almost 3-fold odds of developing CIN when contrast volume exceeded 300 mls (OR 2.80, 95% CI 1.17-6.68, p=0.02) (17).

1.4 Impact of CIN on early and late outcomes

In several studies, development of CIN is positively associated with increased hospitalization and early and late adverse clinical outcomes (13, 16, 34, 35) [Table 1.2].

Various mechanisms are implicated in causing increased mortality in patients with CIN (34). In animal studies with ischaemia-induced AKI, the vasoconstriction that characterizes the renal medulla is experienced in distant organs as well (34, 36).

In this study, the coronary vessels experienced severe narrowing and thereby severely affecting cardiac outcome post ischaemia (36). A similar mechanism might occur in patients with ischaemia-induced CIN and hence increasing mortality (34). However, other studies support the influence of comorbidities on outcomes following contrast media administration (34). However, after adjusting for these comorbidities, CIN still remained an independent predictor for mortality (34).

1.4.1 Morbidity due to CIN

Due to increased comorbidities, the rates of CIN are higher among hospitalized patients compared to ambulatory patients (17) and several studies have reported both renal and extra-renal complications following contrast media administration (34).

1.4.1.1 Extra renal complications due to CIN

CIN is associated with significant complications that include increased duration of hospitalization, renal and extra-renal adverse outcomes (1, 2, 23). In a study cohort of chronic kidney disease (CKD) and non CKD patients undergoing PCI patients in USA, Neyra *et al*. observed a longer duration of hospitalization in patients with CIN (37). The length of hospitalization in the non CKD group with CIN was 8.3±13.3 days vs.4.1±5.6 days, $p<0.001$ and CKD with CIN was 9±8.5 days vs. 6.1 ±12.6 days, $p<0.001$ in patients with CIN compared to controls (37).Shema *et al.* also reported increased duration of hospitalization among patients with CIN compared to controls (24 days vs.13 days, $p<0.001$) (1).

The rate of cardiovascular events is also higher in patients with CIN (17). Marenzi *et al*. found increased rates of cardiogenic shock and atrial fibrillation in CIN patients compared to controls (17). The presence of respiratory compromise was also higher in the CIN group compared to controls in this study (17). CIN was associated with a 2-fold risk of developing late myocardial infarction (MI) compared to controls 24.0 % vs. 11.6%, $p<0.005$ (16).

In a prospective single center study, Wi *et al.* reported higher rates of major adverse cardiovascular and cerebral vascular events together with mortality in the CIN group compared to controls (38).

In another retrospective study, Bouzas-Mosquera*et al.* in Spain reported increased rates of respiratory compromise associated with ventilation, increased duration of hospitalization and cardiogenic shock and cardiac arrhythmias in CIN patients compared to controls (23). The rates of respiratory compromise, cardiogenic shock and duration of hospitalization were 0.7% vs. 13.9%, 3.6% vs. 36.1%, 1.8% vs. 11.4% and 6 days vs. 12 days respectively, (with a $p < 0.001$) (23).

1.4.1.2Intrinsic renal complications due to CIN

The rates of dialysis requiring CIN are less than 1% (39); however in patients with underlying renal dysfunction, it occurs in up to 12% (when baseline serum creatinine exceeded 124 µmol/l prior to contrast) and is associated with adverse clinical outcomes (39). In a retrospective study of 161 patients with underlying renal dysfunction prior to radio contrast administration, almost 20% of the CIN patients underwent renal replacement therapy and progressed to CKD (40).

1.4.2 Mortality due to CIN

CIN is strongly linked with both early and late mortality in several observational and retrospective studies (34, 39) [Table 1.2]. In-hospital mortality due to CIN ranges from 7-22% or higher.McCullough *et al.* reported early mortality of 1.1% in controls, 7.1% in CIN patients, and 35% in CIN patients that required dialysis(34, 41). Sedeghi *et al*. in a multicentre study found that 30 day mortality was significantly higher among patients with CIN compared to controls (16.2 vs. 1.2; $p < 0.001$) (42).

Shema *et al.* found ten times higher mortality in CIN patients in a Western Galilee Hospital in Israel (31.4% vs. 3%, p <0.001)(1) while Mitchell *et al.* in the USA, reported a one year mortality rate of 18% vs. 6% in CIN compared to controls, and a 3-fold risk for mortality (RR 3.1, 95% CI; 1.7-5.4)(5).

Neyra *et al*. reported increased early and late mortality in CKD and non CKD patients who developed CIN (37). In the non CKD group, CIN was associated with a 2-fold adjusted odds for mortality (2.2 95% CI; 1.2-4.1, p=0.02) and a 9-fold adjusted odds for mortality in the CKD group with CIN (8.95; 95% CI 1.9-34.5, p=0.005) compared to controls. In the RCT, CIN was an independent predictor for mortality (8% vs. 0.9%, p<0.0001) (12).

Table 1.2 Mortality rates associated with contrast-induced nephropathy

Reference	No. of Patients and Type of Contrast Procedures	CIN Definition (Size of Increase in SCr from Baseline)	In-Hospital Mortality Rates: CIN vs. No CIN	Long-Term Mortality Rates: CIN vs. No CIN
McCullough *et al*., (41).	1826 in a derivation set, 2251 in a validation set; PCI	>25% during first 5 days	7.1% vs. 1.1%;35.7% for dialysis-dependent (P< 0.0000001)	-
Rihal *et al*., (43).	254 with CIN, 6890 without CIN; PCI	>0.5 mg/dl during first 48 hours	22.0% vs. 1.4% (P< 0.001)	12.1% vs. 3.7% (P< 0.0001) (1-yr hospital survivors);44.6% vs. 14.5% (P< 0.0001) (5-yr hospital survivors)
Gruberg *et al*., (40).	439 with CKD (SCr ≥1.8 mg/dl) not dialysis-dependent; PCI	≥25% during first 2 days or needing dialysis	14.9% vs. 4.9%;22.6% for dialysis-dependent (P< 0.001)	37.7% vs. 19.4% (P = 0.001) (1-yr cumulative rate)
Gruberg *et al*., (44)	7741; PCI	Requiring dialysis	27.5% vs. 1.0% (P< 0.001)	54.5% vs. 6.4% (P< 0.0001) (1-yr cumulative rate)
Dangas *et al*., (45)	5250 CKD(+), 1980 CKD(−); PCI	≥25% or ≥0.5 mg/dl during first 48 hours	6.3% vs. 0.8% (CKD(+), P< 0.0001);2.5% vs. 0.1% (CKD(−), P< 0.0001)	22.6% vs. 6.9% (CKD(+); P< 0.0001) 8.0% vs. 2.7% (CKD(−); P< 0.0001) (1-yr cumulative rate)
Levy *et al*., (46)	183 with CIN, 183 matched control subjects; various procedures (about half angiography)	≥25% to ≥2 mg/dl during first 2 days	34% vs. 7% (P< 0.001)	

CIN, contrast induced nephropathy, CKD, chronic kidney disease, SCr, serum creatinine, PCI, percutaneous coronary intervention.

Reproduced with permission from Rudnick *et al.*2008(34).

1.4.3. Correlation between CIN risk score with CIN and mortality

The presence of multiple and additive risk factors are associated with a higher frequency for CIN and mortality risk (16, 47) [Figure 1.1]. Mehran *et al.* included eight CIN variables, and assigned a scoring system; a higher score predicted poor outcomes (47). In another study, Sgura *et al.* recruited 891 patients with segmental elevation myocardial infarction (STEMI) undergoing angiography and classified them into low to high risk groups based on the number of CIN risk factors present (48). The odds for CIN was 3.82 vs. 1.73, $p<0.001$, in the high score vs. low score respectively (48). Similarly, zero mortality was experienced in the low risk group compared with 10% mortality in the high risk group (48). In other studies, the frequency of CIN were 1.2%, 11.2% and >20% in patients with zero, one and >2 CIN risk factors respectively (49).

Despite retrospective and observation studies including RCT studies from developed countries demonstrating increased morbidity and mortality associated with increased CIN, Sub-Saharan Africa has limited data on rates, risk factors and outcomes of CIN.

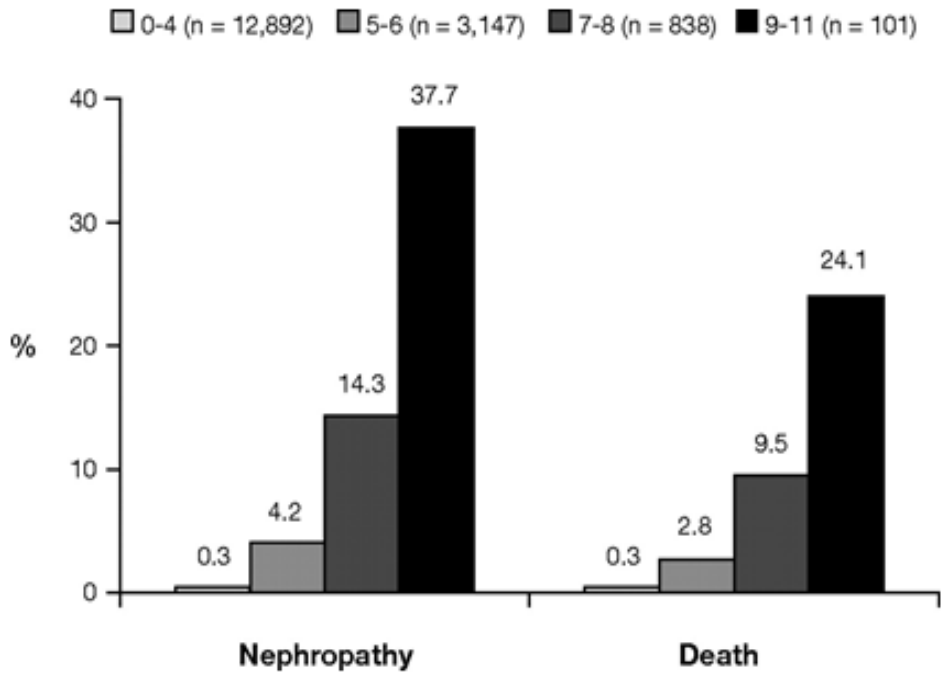

Figure 1.1 Association of CIN risk score with CIN and mortality

Increase in number of risk factors (CIN risk score) is associated with direct increase in CIN and mortality. For determination of risk score, variables eGFR < 60 ml/min per 1.73m^2, an urgent PCI, use of an intra-aortic pump device were scored 2 CIN risk points while presence of DM, cardiac disease, hypertension and peripheral vascular disease including iodinated contrast volumes exceeding 260 mls were scored 1 CIN risk point.

Reproduced with permission from Rudnick *et al.* 2008 (34).

1.5 Definition of CIN

CIN is defined as an acute decline in renal function that occurs following the administration of contrast media (14, 50-52). The definition is based on three factors; increased serum creatinine from baseline, exposure to contrast media and exclusion of other confounders for renal disease. Therefore in several studies, CIN is mostly defined as a rise in serum creatinine of >44 umol/l or a 25% increase from baseline assessed at 48-72 hours after a radiological procedure in accordance with the European Society of Urogenital Radiology (ESUR)(12, 16, 35, 50, 53). Additionally the above definition positively predicted adverse cardiovascular events and early and late mortality (12, 16, 50). The updated Canadian Association of Radiologists (CAR) guidelines also defines CIN as >44 µmol/l serum creatinine increase over a 72 hour period (54). Classically CIN occurs within 1-3 days, peaking at 3-5 days after contrast administration (53, 54). In comparison between using an absolute (>44 µmol/l) or relative increase (>25%) in serum creatinine, the Contrast Induced Nephropathy Consensus Working Panel approved the later definition (55). However the Kidney Disease Improving Global Outcomes (KDIGO) defines CIN as "an increase in serum creatinine by 50% within 7 days or increase in serum creatinine >26.5µmol/l from baseline and occuring within 48 hours or presence of oliguria" (56, 57).

1.6 Pathogenesis of CIN

In spite of an unclear understanding of the mechanisms underlying CIN, tubular toxicity and endothelial vasoconstriction, together with reactive oxygen species (ROS), are implicated in the pathogenesis of CIN (11, 13, 58-62) [Figure 1.2].

1.6.1 Contrast induced tubular toxicity

Iodinated contrast media directly injures the renal tubular epithelium by producing ROS radicals that cause intra-renal vasoconstriction leading to ischaemia and death of tubular cells (3, 13). Contrast media is characterized by high osmolality and increased viscosity (thickness) (32), even the iso-osmolar contrast media is extremely hyperviscous compared to plasma (31). Following glomerular filtration, contrast media is localized to the tubules due to impaired

absorption (31). With increased water reabsorption in the proximal tubules, intratubular viscosity and pressure are increased and consequently, cause injury to the surrounding interstitium and descending vasa recta (31). Increased hyperviscosity and osmolality cause direct damage to renal tubules and with increased intratubular pressure consequently lead to compromised renal blood flow and decreased glomerular filtration rate. (31). Previous studies have reported a positive association between contrast media administration and tubular cell vacuolation. (4, 59).

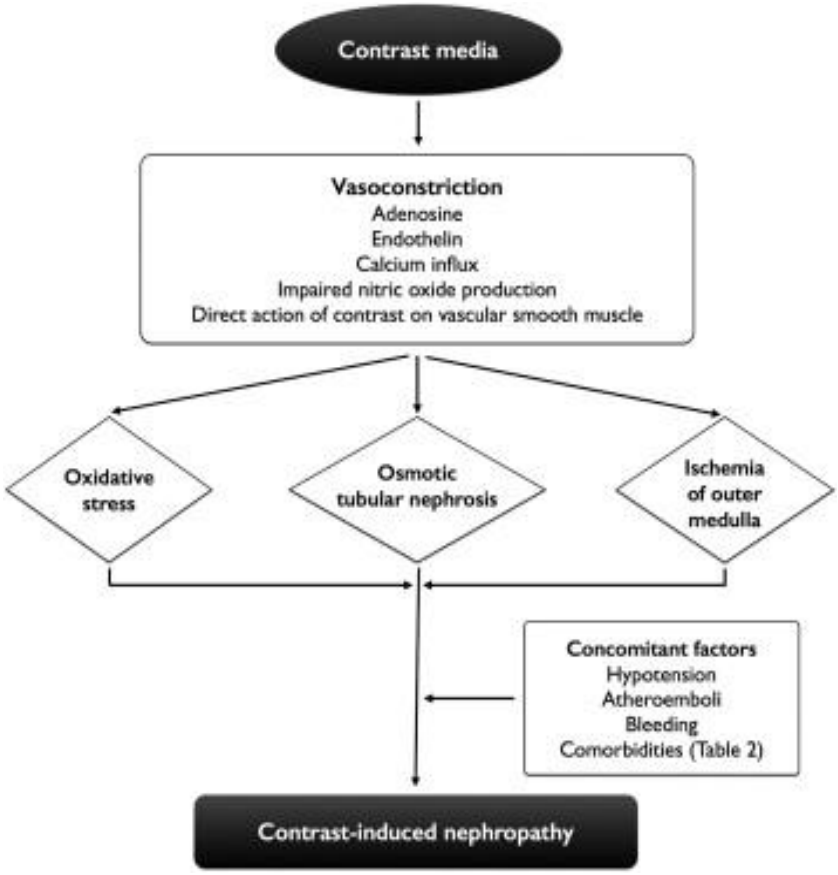

Figure 1.2 Pathophysiology of CIN.

An interaction of vasoconstriction and contrast media leads to oxidative stress and ischaemia. Reproduced with permission from Azzalini *et al*. 2016(61).

1.6.2 Endothelial dysfunction in CIN

An imbalance of vasoconstrictors and vasodilators plays a critical role in mediating CIN [Figure 1.3]. Contrast media suppresses intra-renal vasodilators i.e. nitric oxide (NO) and prostaglandin E2 (PGE2) and increases intra-renal vasoconstrictors that decrease blood flow to the renal medulla leading to hypoxic ischaemia, production of ROS and death of tubular cells (59-61, 63). Vasoconstriction in CIN is mediated by adenosine and endothelins acting on A1 and E1 receptors respectively (58-60, 62, 63). Endothelin receptor stimulation is associated with decreased GFR (64). Microcirculation in the renal medulla is provided by the descending vasa recta whose diameter ranges from 12-18 μm (65) compared to 7.5 μm for red blood cells (66). After radio contrast administration, the diameter of the descending vasa recta is decreased by almost 50% due to vasoconstriction and loss of NO(65). The vasoconstricted descending vasa recta diameter which approximates the red blood cell diameter after contrast media further impedes circulation in the renal medulla (65).

1.6.3 Medullary hypoxia and CIN

In CIN, the presence of hypoxia in the outer medulla of the kidney is essential in the pathogenesis of CIN (27, 65). For several reasons, the renal outer medulla is susceptible to hypoxia following CIN; firstly only 10% of renal blood flow is directed to the medulla and secondly the medulla is normally characterized by low oxygen pressures ranging from 30-40 mmHg (65). Lastly the medulla is actively involved in adenosine triphosphate (ATP) mediated transportation of salt in the medullary thick ascending limb (mTAL) of the loop of henle and therefore exhibits high metabolic activity (31, 65). Following contrast administration, the outer medullary renal blood flow exhibits two phases; a brief period characterized by increased flow that is later followed by an almost 25% sustained decrease in renal blood flow that ultimately impacts poorly on oxygen delivery to the medulla (65).

The partial pressure of oxygen decreases to as low as 10-15 mmHg post contrast administration (25, 31, 65).

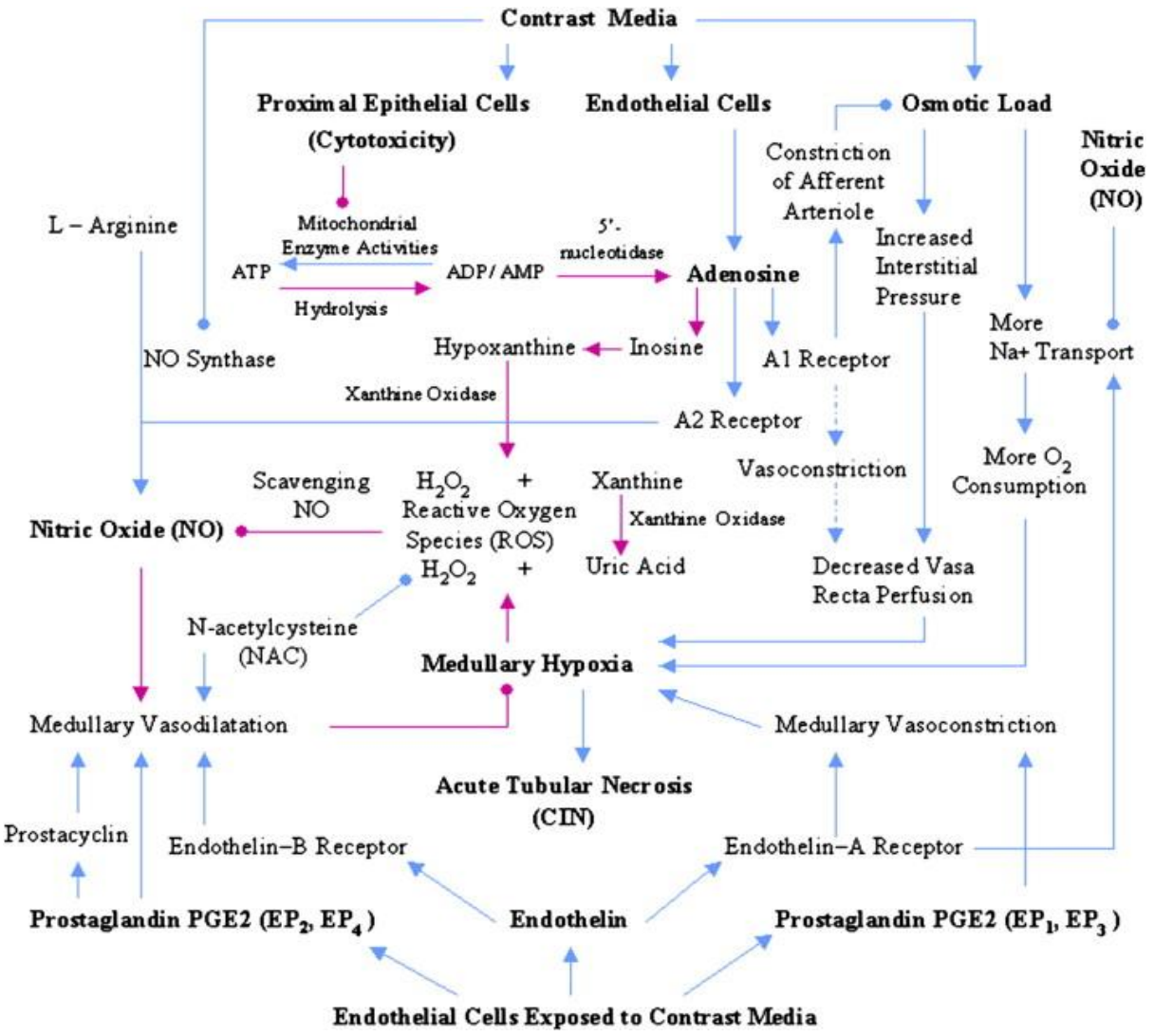

Figure 1.3Vasoconstrictors mediating CIN

Presence of contrast media down regulates action of vasodilating NO synthase and also mitochondrial enzyme activities and the later leads to increased ATP hydrolysis production of adenosine. The contrast media also increases interstitial and intratubular pressures and sodium uptake. This increases oxygen consumption and production of damaging ROS. Endothelins together with adenosine leads to CIN (ATN). Reproduced with permission from Wong *et al.* 2012 (58).

1.7 Cytokine induced inflammation in CIN

In recent studies, evidence strongly implicates inflammation in the mechanism of CIN and other forms of ischaemic AKI (63, 67-69). Cytokine induced inflammation plays a significant role as predictive biomarkers of disease and prognosis (67). Increased levels of cytokines are associated with adverse outcomes in patients with AKI (68). Polymorphisms in these inflammatory cytokines genes are also strongly implicated in renal disease including CIN and studies have shown individual variation in genetic regulation of cytokine levels in plasma following insults (14, 67).Therefore, knowledge of the key roles played by inflammatory cytokines in CIN is critical in laying down workable interventions for CIN.

Cytokines exist as soluble molecules produced locally and exert their actions at the injury and distant sites(67). In the circulation and tissues, cytokines generally remain in insignificant amounts and are sometimes beyond detection limit in tissues (67). Due to their damaging effects, various systems exist to regulate and neutralize pro-inflammatory cytokinese.g. TNFα release is linked with production of IL10 by similar stimulating agents (67).

Iodinated contrast induced vasoconstriction leading to ischaemia is a central mechanism for CIN due to decreased renal blood flow (13, 69). Like other forms of ischaemia induced-AKI, studies have found a strong interaction of inflammation, tubular and endothelial injury as mediators in the pathogenesis of ischaemic-induced AKI including CIN (14, 63, 68, 70) [Figure 1.4]. Impairment of renal blood flow and ischaemia to the outer medulla is linked with injury to the endothelium and renal tubules and this leads to structural and pathological changes in these cells (68). Renal ischaemia to the medulla is associated with increased metabolism and depletion of adenosine triphosphate (ATP) and production of adenosine (67). The latter is a potent vasoconstrictor implicated in pathogenesis of CIN (63). ATP depletion is associated with increased renal tubular injury and endothelial injuryand this leads to cytoskeletal structural deformities and functional derangements of these cells (67, 69).

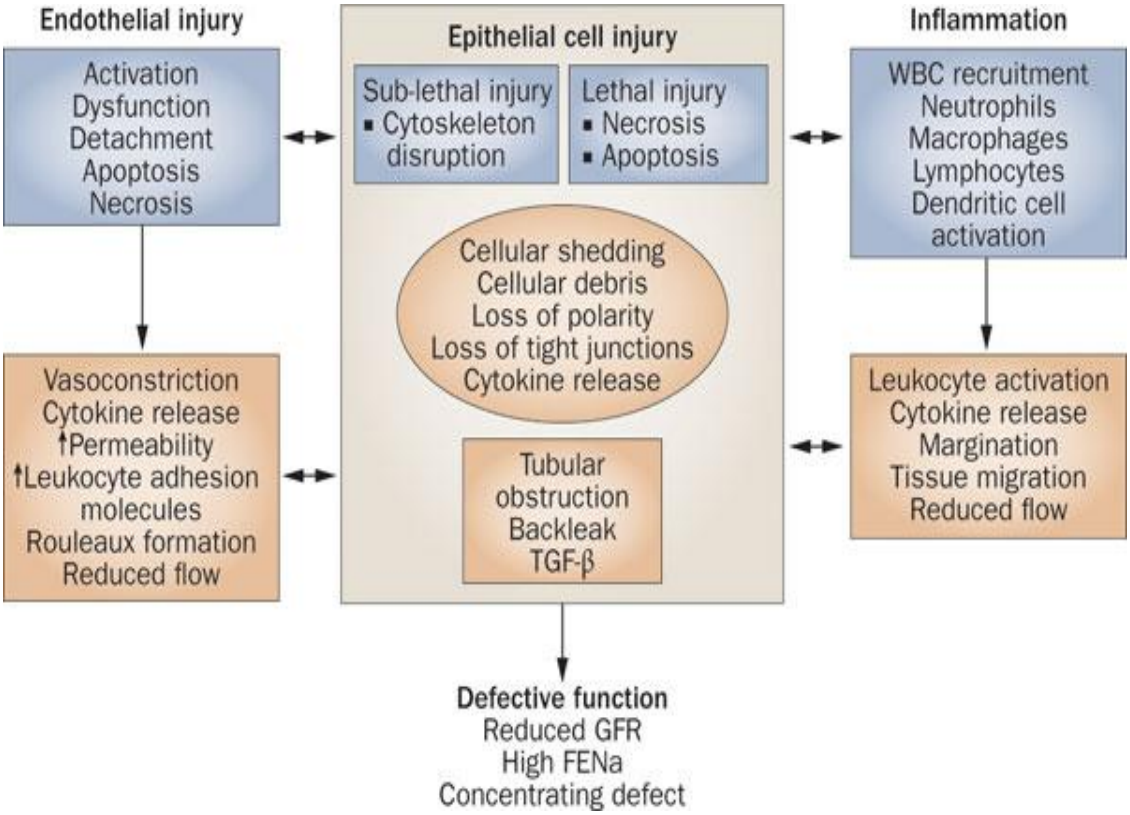

Figure 1.4 Renal ischaemia and AKI

In ischaemic AKI, an interaction of endothelial injury, epithelial injury and inflammation occurs and leads to ATP depletion and cell death. Inflammation leads to upregulation of cell adhesion molecules and secretion of cytokines. Resulting vasoconstriction and inflammation cause decreased filtration (ischaemic-AKI). Reproduced with permission from Sharfuddin *et al*. 2011 (69).

The injured endothelial and renal tubular cells following ischaemia respond by initiating an inflammatory cascade and the microvascular injury remains critical in initiating this event (68, 71). The endothelial and tubular injury performs two important functions that initiate inflammation; the endothelium in close proximity to the insult is characterized by increased permeability and upregulationof intracellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1) and P and E-selectins and together with injured renal

tubules, function in recruiting inflammatory cells (67, 68, 71). The recruited inflammatory cells include neutrophils, macrophages and lymphocytes(68, 69). Post recruitment, the inflammatory cells and injured endothelial and epithelial cells secrete various cytokines and chemokines that mediate renal injury (68, 69) [Figure 1.5]. Cytokines of significance includingtumor necrosis factor alpha (TNFα), interleukin 6 (IL6), IL10, IL18 and interferon-1 beta (*IL*-1β) are upregulated by injured epithelial and endothelial cellsand also infiltrating neutrophils and microphages (67, 68, 70, 71). The presence of cytokines and hypoxia leads to cytokine induced inflammation in AKI (67).

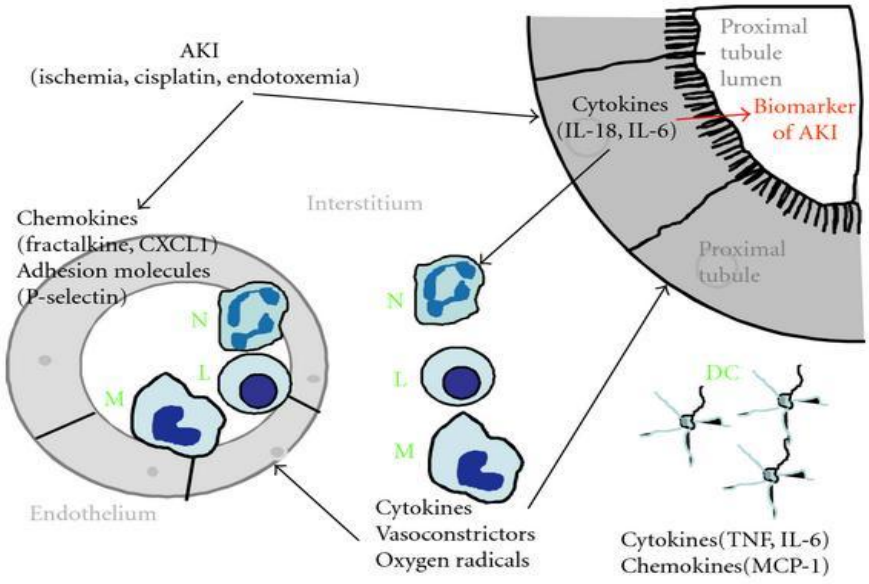

Figure 1.5 Inflammatory cytokines in pathogenesis of AKI

Ischaemia induced AKI leads to upregulation of inflammatory cytokine including IL18, IL6, and TNFα that serve as AKI biomarkers. Infiltrating neutrophils and macrophages secrete adhesion molecules and produce oxygen free radicals. Reproduced from Akcay *et al.* 2009 (68) under the Creative Common Attribution License.

1.7.1 The influence of TNFαon CIN

TNFα positively mediates inflammation in the kidneys by recruiting other pro-inflammatory cytokines such as IL6 and IL8 and also increases expression of cell adhesion molecules (63, 72). Excessive production of TNFα is linked to the development of several inflammatory diseases, including CIN (3, 63, 72, 73).

In previous animal and human studies, TNFα and Interleukin 6 (IL6) were upregulated following contrast media administration and potentiated CIN (14, 70, 74). Recently Deng *et al.* reported almost 2-fold increased levels in the TNFα and IL6 cytokines in diabetic rats with CIN compared to controls (48.6±5.2 vs. 27.1± 2.3 pg/ml, $p<0.01$) and (15.1±1.2 vs. 7.6±0.7 pg/ml) respectively (63). Hudzik *etal.*found increased levels of IL6 in the CIN group compared to controls (8.3 vs. 2.7 pg/ml, $p < 0.001$) among patients undergoing PCI (70). Chang *et al*. also reported increased expression of TNFα in the patients with CIN (70). In other studies, serum and urine levels of TNFα correlated with severityof renal disease (72).

TNFα functions as a mediator of inflammation and it initially occurs as a large membrane bound molecule; however, it is split into its functional and soluble 17 kDa protein. The tumor necrosis factor receptors TNFR1 and TNFR2 both mediate function of TNFα with varying affinities (72).

The TNFα cytokine is undetectable in normal kidney tissue; however post renal injury, the injured endothelium and renal tubules together with infiltrating inflammatory cells secrete significant amounts of it (69, 72). Despite the absence of TNFR2 in uninjured kidneys, it is over expressed in renal tubules, endothelium and interstitium post renal injury (72).

1.7.2Association of TNFα gene polymorphisms with CIN

The central positioning of the TNFα gene on class III of the major histocompatibility complex makes it a highly polymorphic gene and polymorphisms of the gene are associated with disease risk and poorer outcomes (72, 73).

Within the promoter region, TNFα has several single nucleotide polymorphisms (SNPs) that include -308G/A and -857C/T; however, the former is the commonest genotype linked with disease susceptibility (72).

The TNFα -308 genotype exists in two forms; the predominant guanine (G-allele) and lesser adenine (A-allele) (75); in previous studies, the A allele was positively associatedwith a 6-fold higher transcription rate (72, 75). The A allele also predicted poor clinical outcomes in post-transplant kidney patients (72).

Among the Han Chinese populations with CIN, Chang *et al*. found a 2-fold increase in frequency of the A-allele (rs1800629) compared to controls (OR 2.01, 95% CI 1.13-3.35, p=0.02) (14).

1.7.3Influence of Interleukin 10 on CIN

A decreased level of IL10 positively predicts development of AKI (14, 76, 77). Chang *et al*. reported reduced baseline IL10 levels in CIN patients compared to controls (1.02±1.14 vs. 2.78±4.73 pg/ml, p=0.008) (14).

The IL10 cytokine is involved in suppression of many inflammatory cytokines and therefore protects the kidney following insult and hastens renal recovery (76, 78). IL10 occurring as a 37 kDa homodimer molecule (78) is located on chromosome 1(14, 78). IL10 is mostly produced by inflammatory cells, however, the mesangial and endothelial cells in presence of renal injury produce IL10 (72, 76, 78).IL10 performs various functions through its linked IL10 receptors 1 and 2 that include down regulation of cytokines TNFα andIL12 and also inhibition of T helper cells (77).

1.7.4 Association of IL10 gene polymorphism with CIN

IL10 contains several SNP in its promoter region that include -1082 G/A, -819 C/T, -592C/A (78), and these influence the production of IL10 levels (77). In previous studies, SNPs in the IL10 gene were positively associated with CIN risk (14).

Recently, Chang *et al.* reported a positive association between individuals with genetic polymorphisms in rs1800896 in the IL10 gene with CIN (14).In this study, increased frequency in the –IL10 -1082 G-allele (rs1800896) was associated with an almost 3-fold increase in development of CIN (14).

In summary, CIN is characterised by an imbalance of inflammatory cytokines namely TNFα and IL10 whose genetic polymorphisms are known to influence disease. TNFα as a proinflammatory cytokine accelerates disease such as CIN and therefore poorer clinical outcomes, while IL10 accelerates recovery. Identification of protective cytokine genetic polymorphisms is critical in implementing targeted modalities that will lessen the impact of CIN among patients with decreased IL10 and high TNFα levels.

1.8 Accuracy of biomarkers in predicting and prognosticating CIN

1.8.1 Diagnostic limitation of serum creatinine

Treatment of CIN including AKI in general remains supportive,non-specific and diagnosis is based on serum creatinine based definitions(9). Earlier diagnosis of CIN is paramount in offering timely interventions. However, early intervention is limited because serum creatinine,the golden diagnostic marker, is suboptimal in the early diagnosis of AKI (79-84). Previous studies have shown that almost 80% of CIN would be identified within 24 hours post contrast administration, however delayed detection of AKI due to shortcomings in serum creatinine lead to poorer outcomes in patients with CIN (82, 85).

For several reasons, serum creatinine is limited in the early detection of AKI; it is affected by extra renal factors that include an individual mass and extracellular volume irrespectiveof the level of glomerular filtration rate; it does not increase immediately post kidney injury and occasionally exhibits a downward trend post kidney injury(67-72 (86, 87). Increase in serumcreatinine is also noticed at least 72 hours after injury to the kidney and sometimes delay of up to 5 days occurs after an insult (56, 82).

Additionally, a significant loss of renal injury is accompanied by normal serum creatinine levels (88). Due to increased morbidity and mortality associated with AKI, the Acute Dialysis Quality Initiative (ADQI) recognized the development of early biomarkers of AKI(89).

There is the need of identifying biomarkers that will identify early AKI and also stage the severity of kidney disease in line with the available risk, injury failure loss and end stage (RIFLE) and also according to the Acute Kidney Injury Network (AKIN) severity grading systems for renal injury(89, 90)[Figure 1.6].

Increased serum creatinine to post RIFLE-I or AKIN-2 is associated with worse outcomes (89, 91) and hence the need of early biomarker that will identify subclinical AKI. Studies confirm that even small variations in levels of serum creatinine were associated with morbidity and mortality (48, 92, 93). AKI is classified into three stages as; increased serum creatinine ≥ 1.5, ≥ 2 and ≥ 3 as stages one, two and three respectively (57).

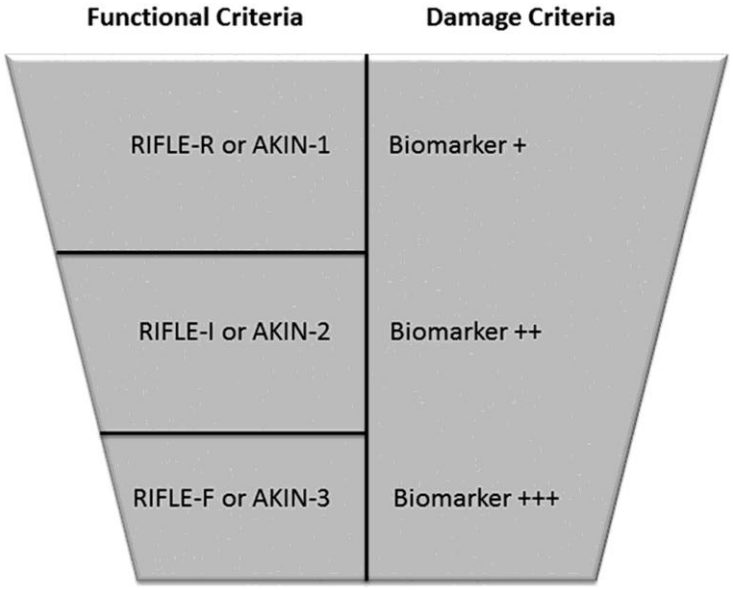

Figure 1.6Roles of novel biomarkers in RIFLE and AKIN criteria

The interaction of novel biomarkers with the current RIFLE and AKIN criteria will improve the diagnosis of AKI. Reproduced with permission from Charlton *et al.* 2014 (90).

1.8.2 Definition of a biomarker

A biomarker is defined as a“measurable characteristic that is indicative of normal biological processes, including pathological and pharmacological processes” (93). An ideal biomarker of AKI should be non-invasive by utilizing blood and urine and should demonstrate diagnostic accuracy for early risk, prediction and good functionality in recognizing stable disease and prognosis (93, 94).

Biomarkers (either single or multiple) are needed at several stages of renal disease or risk identification for the management of renal disease. Several major questions on biomarkers of AKI remain unexplored especially in heterogeneous populations (95);which biomarkers identify earlier risk, best timing, threshold to use for identification, which biomarkers prognosticate outcome and whether multiple biomarkers perform better compared to one [Figure 1.7]. Lastly, in a few studies that explored accuracy of AKI biomarkers in heterogeneous populations that are characterized by varying renal insults, the area under the receiver operating curves (AUC-ROC) results were conflicting (85, 89, 95).

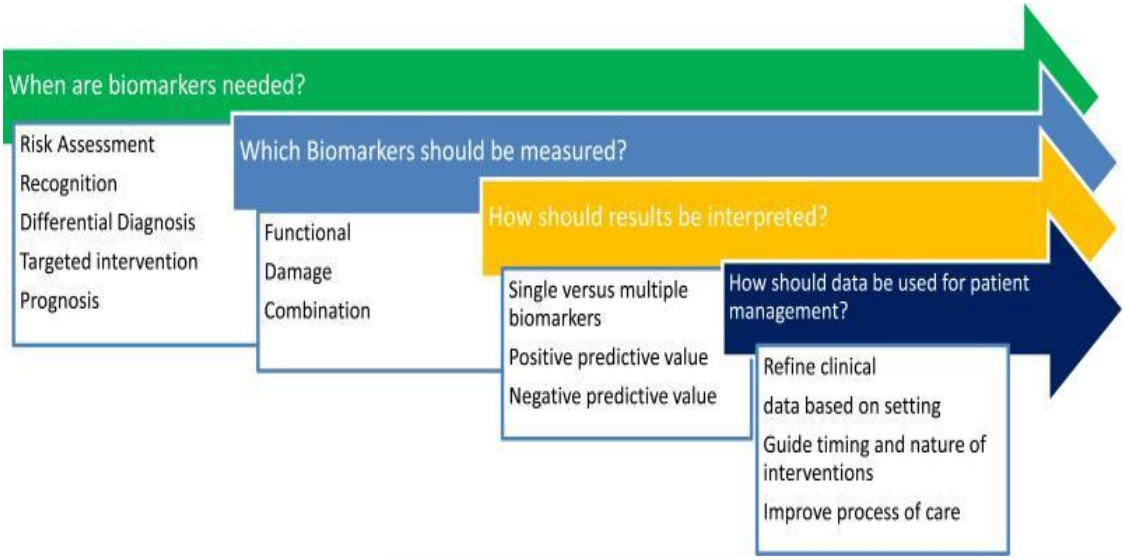

Figure 1.7 Clinical uses of biomarkers in AKI.

Reproduced with permission from Murray *et al.* 2014(89).

1.8.3 Classification of novel biomarkers

Sources of novel biomarkers are variable (96) and include renal and non-renal tissues (90) and are classified as those molecules reflecting glomerular function (cystatin C and β2M) and those that are upregulated in both plasma and urine (IL18, NGAL) and reflect renal injury, damage (tubular and endothelial) or repair (85, 89, 90, 96). AKI including CIN is characterized by impaired glomerular filtration and tubular dysfunction (85). The resultant kidney dysfunction leads to accumulation of these biomarkers in both plasma and urine (85, 96). Cystatin C and β2M including serum creatinine are considered as filtration biomarkers (85, 89). Extra renal production of biomarkers due to systemic inflammation leads to increased accumulation of these biomarkers in circulation and impairment in glomerular filtration or absorption worsens clinical picture (85).

1.8.4 Functions of novel biomarkers

In normal kidneys, novel biomarkers for AKI are barely detectable in blood or urine; however they are upregulated following injury such as contrast administration (97, 98). A good biomarker should potentially identify the risk and severity of acute renal injury and it should prognosticate AKI; that is recovery, renal replacement therapy in form of dialysis and patient outcomes such as mortality (92, 93, 99) [Figure 1.8]. However, as shown in Figure 1.8, serum creatinine is a traditional biomarker of significance in late stages of renal disease. Additionally, levels of the biomarker should reflect the degree of injury intrinsic to the AKI (94). The ideal AKI biomarker should be able to monitor progression of renal disease (94).

Either urine or plasma/serum is utilized for AKI biomarkers with significant variation in biomarker results (100). A recent observational study showed superiority of plasma/serum in biomarker assessment compared to urine (100). Schley *et al.*in a recent comparative study of novel biomarker performance in patients undergoing cardiopulmonary surgery in Germany reported that the plasma biomarkers demonstrated superior diagnostic accuracy for AKI (100).

There are several advantages of plasma/serum in biomarker assessment; unlike urine, serum is unaffected by bacterial contamination and can be collected despite presence of anuria in patients (100). Zhou *et al.* 2016 in a meta-analysis demonstrated similar diagnostic accuracy in plasma and urine (101).

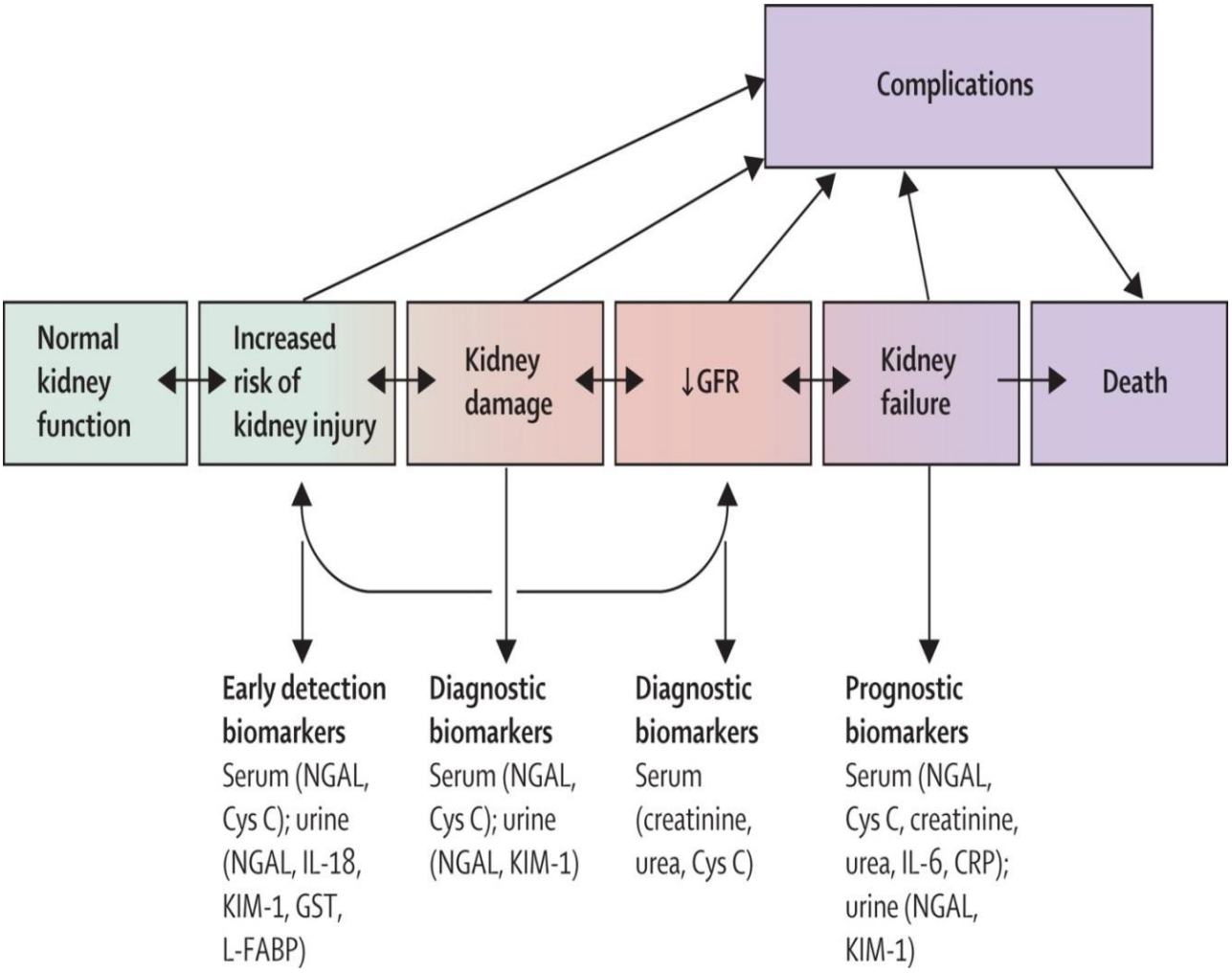

Figure 1.8 Timing of novel biomarkers in AKI progression

Injury to the kidney occurs prior to decreasing GFR or increasing serum creatinine and novel biomarkers identify early acute kidney injury and are useful in prognostication. Reproduced with with permission from Bellomo *et al.* (99).

1.8.5 Neutrophil gelatinase-associated lipocalin-2 (NGAL)

NGAL occurs as a 25 kDa glycoprotein and is composed of 198 amino acids belongs to the lipocalin super family (94). Unique to all lipocalins is the presence of eight beta strands that are folded together through hydrogen bonds and creates a site binding ligand (102, 103).

The NGAL gene called LCN2 is localized to chromosome 9q34.11 (103). Three forms of NGAL are described: a 25 kDa monomer, 45 kDa homodimer and 135 kDa heterodimer (94); however, the 25 kDa remains the most important. Homodimeric NGAL is processed by neutrophils from their myeloperoxidase (MPO) granules while in the kidney predominantly monomeric NGAL and to a limited extent heterodimer NGAL are expressed (94). The sources of NGAL in the kidneys are the proximal tubules and collecting ducts (93).

Normally NGAL is expressed at very low levels in many tissues including the kidneys (94). In the circulation and in the urine, the levels of NGAL are kept low, averaging 20 ng/ml(90). NGAL is filtered by the glomerulus andundergoesabsolute megalin mediated endocytosis in the proximal tubules (90) [Figure 1.9]. The main NGAL receptors are megalin located in the proximal tubular cells of the kidneys (102). Following internalization, NGAL is degraded in lysozymes into a 14 kDa product (90).

However, post-acute kidney injury NGAL is up regulated within 2-6hours(79, 81, 104, 105). The main stimulant to NGAL production is ischaemia-induced hypoxia to the kidneys (93) such as contrast induced hypoxia. In previous studies, the levels of serum NGAL were also increased in the presence of inflammation and in some malignancies (93). During injury, IL-1β positively activates nuclear factor kappaB (NF-kB) that leads to translocation of the latter into the nucleus and ultimately leads to increased transcription of NGAL promoters (93).

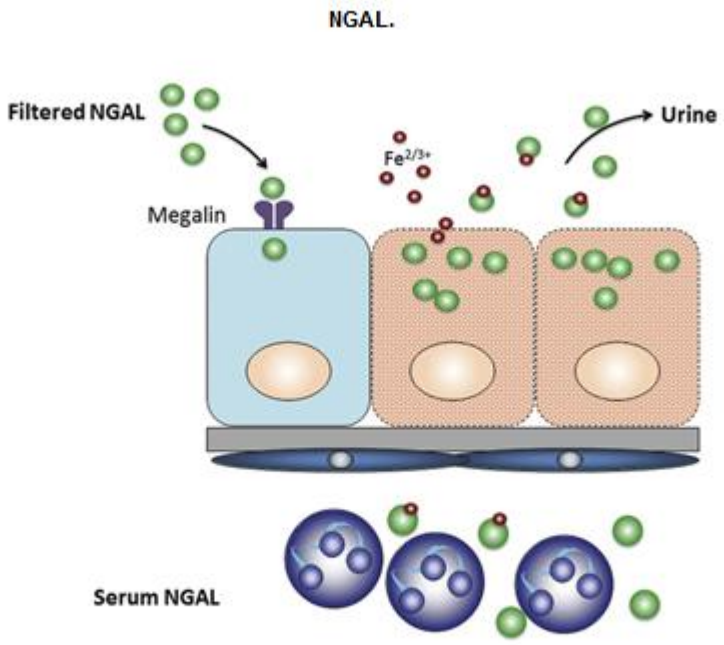

Figure 1.9 Metabolism of NGAL in the kidney

NGAL, predominantly produced by neutrophils and to a smaller extent by the liver and kidneys, undergoes filtration and megalin mediated endocytosis. After injury, NGAL increases in blood and urine. Reproduced with permission from Charlton *et al.* 2014 (90).

1.8.5.1 Functions of NGAL and lipocalin

Lipocalins are involved in immunological (bacteriostatic) function and transportation of smaller molecules that include retinol binding proteins and siderophores(90, 94). In humans, ferritin, transferrin and lactoferin serve as major iron binding molecules; however compared to siderophores in microorganisms, the latter has increased affinity for iron (103). NGAL serves an important immunological role by depriving microorganisms of iron and thereby inhibiting their proliferation (102, 103).

Microorganisms rely on the presence of siderophores for multiplication; however, once complexed to siderophores (iron binding molecules in microorganisms), iron is internalized by NGAL (102, 103).

1.8.5.2 NGAL as a biomarker of AKI

Studies support NGAL as an early marker for predicting AKI and its outcomes (106). In patients undergoing enhanced computed tomography, Filiopoulos *et al*. demonstrated a 10-fold increase in plasma NGAL in the CIN group compared to controls at six hours after contrast administration (106). Despite insignificant differences in NGAL values at baseline in the two groups, at 6 hours the levels of plasma NGAL were 779.25 ng/ml ±361.5 vs. 82.3 ng/ml ±40.6, p <0.001 in CIN group and controls respectively (106). In this study, the reported sensitivity and specificity at 6 hours after radio contrast was 100% with an area under the receiver operating characteristic curve (AUROC) of 1.00 when the cut-off value for NGAL of 200 ng/ml (106).

In hospitalised patients admitted to an intensive care unit, Cruz *et al.* demonstrated significantly high levels of plasma NGAL in the entire study cohort compared to a healthy population irrespective of the development of AKI (104). The median plasma NGAL in this study cohort was 117.0 ng/ml (68.2-213.8), P<0.001. Increased levels of plasma NGAL were observed in AKI patients compared to controls (185 ng/ml vs. 82 ng/ml, p<0.0001)(104). Vijayasimha *et al*. in India reported maximum rise in NGAL levels within 4 hours of contrast administration and the AUC was 0.96 (97). After 48 hours post contrast administration, plasma NGAL levels in the CIN group were not significantly raised (97).

Despite variations in cut-off values for NGAL in discriminating AKI (including CIN), a recent meta-analysis supported the accuracy of NGAL in the early diagnosis of CIN (83); however several studies on NGAL are predominantly limited to surgical and ICU based homogenous populations studies and few to CIN or heterogeneous populations (83, 101).

Tong *et al.* in a recent meta-analysis of ten studies that included patients undergoing contrast media administration, reported the overall diagnostic odds ratio (DOR) for NGAL to predict CIN of 20.56, 95% CI 9.67-43.74 with AUROC of 0.87, 95% CI 0.84-0.96) (83). NGAL sensitivity and specificity in this meta-analysis were 0.80, 95% CI 0.74-0.90 and 0.83, 95% CI 0.73-0.90 respectively and cut-off levels ranged from 52.4 ng/ml to 115 ng/ml (83). NGAL at 2-6 hours provided the best discrimination for CIN compared to 24 hours after contrast media (83). Despite being the only meta-analysis with ten studies that associated NGAL and CIN; only five studies from this meta-analysis were limited to AKI (with four looking at patients undergoing PCI and one in critical patients) (83).

Another meta-analysis found that the overall DOR for AKI was 18.6, 95% CI 9-38.1 and the AUROC; 0.81, 95% CI 0.73-0.89 across all settings using NGAL cut-off values of 190.2 ng/ml (122.8-257.2) (107). In this meta-analysis, the DOR for CIN was 92, 95% CI 10.7-794.1 and the AUROC was 0.83-0.95 using a NGAL cut-off of 100 ng/ml (80-100ng/ml) despite extreme wide confidence intervals (107). Using high cut-off values of 212 ng/ml (121-506.7ng/ml), the overall DOR in predicting mortality was 8.8, 95% CI 1.9-40.8 with an AUROC of 0.71, 95% CI 0.53-0.75(107). The mortality sensitivity and specificity were 65, 95% CI 51.2-80.8 and 82.6, 95% CI 51.8-95.5 respectively (107).

In a recent meta-analysis that included 46 studies of cardiac surgery associated-AKI, Zhou *et al*. observed significant variations in the predictive accuracy of NGAL for AKI post-surgery (101). The overall serum NGAL DOR of 13.05, 95% CI 7.85-21.70 was lower in this meta-analysis compared to the previous ones and so were the sensitivity (0.68, 95% CI; 0.65-0.70) and specificity (0.79, 95% CI 0.77-0.80 (101). This meta-analysis also showed similar diagnostic accuracy in using both serum/plasma and urine (101). The DOR for NGAL in urine was 13.09 with AUROC of 0.85 while in plasma/serum the DOR was 13.20 and AUROC was 0.88 (101). Only 2 studies from this meta-analysis showed superiority in using urine NGAL compared to plasma/serum (101).

The limitation of the NGAL meta-analysis is that the study populations were predominantly homogenous, included CKD patients, uncontrolled for sex and age and few of these studies included patients exposed to contrast media in which the insults to the kidneys may be heterogeneous.

1.8.6 Cystatin C and AKI

Cystatin C belonging to the cystatin superfamily is a non-glycosylated protein with a molecular weight of 13 kDa and 120 amino acids (108, 109).

The *CST3* gene for cystatin is mapped on chromosome 20 (109). Intracellularly, cystatin C is in an inactive form and is localized to the endoplasmic reticulum of cells as well as the Golgi apparatus, but prior to secretion, it is activated to an active monomer (109). Cystatin C occurring as monomers is linearly secreted and has wide distributed in body fluids and tissues (109).

Cystatin C, produced by all nucleated cells is freely filtered by the glomerulus and is completely reabsorbed by the tubules and therefore barely detectable in the urine (87, 98, 110). Up to 99% of the metabolism of cystatin C is via the kidney (109) [Figure 1.10].

Following reabsorption, cystatin C is complexed to proteases and internalized within lysozyme organelles where it functions to inhibit cystatin peptidases (109). In studies, cystatin C has been shown to down regulate chemotaxis by neutrophils and production of ROS (109).

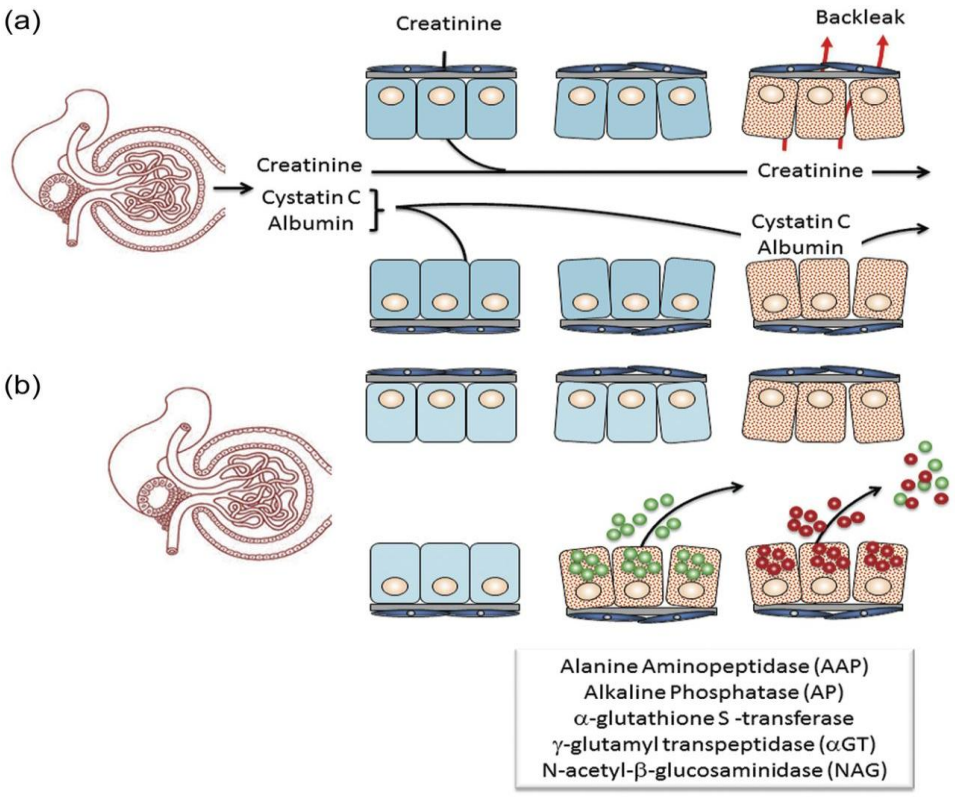

Figure 1.10 Metabolism of cystatin C in the kidney

As a filtration biomarker, cystatin undergoes freely filtration and reabsorption in the proximal tubules. In acute kidney injury due to decreased glomerular filtration or impaired proximal tubular absorption (b), cystatin C accumulates in circulation and urine. However the presence of albuminuria also increases urine cystatin C levels. Reproduced with permission from Charlton *et al*.2014 (90).

Levels of cystatin C in blood range from 0.8-1.2 mg/dl; however, this is increased in conditions of decreased glomerular filtration or reabsorption (109). Levels of cystatin C in blood or urine correlate directly with the development of AKI; however, cystatin C is negatively affected by proteolytic enzymes in the urine (109).

Cystatin C positively predicts AKI and poorer early and late clinical outcomes in patients with AKI (111, 112).

In previous studies, cystatin C predicted AKI earlier compared to serum creatinine; however the results were variable (111, 113). Ishibashi *et al.* reported significantly high baseline cystatin C levels in CIN patients compared to controls (1.08±0.42 vs.0 0.77±0.15 mg/l)(111). The sensitivity and specificity of cystatin C in the diagnosis of CIN were 81.8%, 95% CI 0.81-1.06 and 91%, 95% CI 0.81-1.06 respectively with a cut-off value above 1.18 mg/l (111).

In a prospective study of 374 post cardiopulmonary bypass patients under 18 years of age, cystatin C levels at 12 and 24 hours positively predicted AKI and patient outcomes (113). At baseline, Krawczeski *et al*. observed insignificant differences in cystatin levels at baseline between the two groups; however after 2 hours, cystatin C increased significantly until 24hours and independently predicted AKI (113). The AUROC at 12 hours was 0.81; 95% CI 0.74-0.88; $p<0.001$ and 24 hours was 0.84; 95% CI 0.78-0.91; $p<0.001$ (113). In CKD patients undergoing coronary angiography, the pre-contrast and 24 hours post contrast serum creatinine were not significantly different between the CIN group and controls until 48 hours (114). However, serum cystatin C increased significantly at 24 hours after contrast administration and the AUROC for serum cystatin C discrimination for CIN at 24 hours was 0.80 (114).

Evidence from meta-analysis strongly supports cystatin C as an early biomarker of AKI (115). In a meta-analysis that included 19 studies, Zhang *et al* found an overall DOR for cystatin C in predicting AKI of 23.5, 95% CI 14.2-38.9 (115). Cystatin C measured within 24 hours demonstrated good AUROC ranging from 0.86-0.88 (115). The studies included in this meta-analysis were uncontrolled, the majority looked a homogenous populations and only one study addressed patients with CIN (115). This is due to limited studies exploring the role of cystatin C and other biomarkers in patients undergoing contrast media administration.

1.8.7 Beta-2Microglobulin and AKI

Beta-2Microglobulin (β2M) with a molecular weight of 11.8 kDa occurs as a non-glycosylated functional polypeptide expressed on all nucleated cells (116-118). β2M is a component of the major histocompatibility complex (MHC) class 1 molecules and is linked non-covalently to the α-1 chain (116, 118).

Insignificant levels of β2M are seen in the circulation averaging less than 2 mg/l and less than 400 μl is reflected in urine per 24 hours (116, 117). When released into the circulation, β2M undergoes almost 95-100% metabolism in the kidneys; it is filtered by the kidneys and completely absorbed via megalin mediated proximal tubular endocytosis (116, 117, 119).

Increased serum levels of β2M are seen in patients with impaired renal function; either due to reduced glomerular filtration rate or decreased absorption by the proximal tubules (116). However, inflammatory conditions including malignancies and rheumatoid arthritis have been linked with increased levels of β2M (116).

Beta-2 microglobulin predicts AKI earlier compared to serum creatinine (119). Nozue *et al*. reported higher serum β2M levels among patients with CIN compared to controls and its sensitivity was 75% and specificity 80% at a cut off value of 2.8 mg/l (112). Studies have demonstrated positive association between β2M with cardiovascular changes and patient outcomes (116). In a case controlled study of CKD patients, Sedighi *et al*. found increased serum β2M in renal patients compared to controls 7.6 ±3.7 vs. 2.1mg/l ± 1.7 mg/l, $p < 0.001$ (116). In previous studies, increased levels of β2M are also positively associated with increased mortality (116).

Despite previous studies demonstrating positive association of high β2M in patients with impaired filtration (118) and also association with mortality (116), there are lack of studies that have looked at β2M as a biomarker for CIN and prognostication.

1.8.8 Interleukin 18 and AKI

IL18 is an inflammatory cytokine produced by both inflammatory and non-inflammatory cells (120). The primary inflammatory cells producing IL18 are the macrophages, neutrophils and lymphocytes while in the kidneys, it is produced by the proximal tubular cells, mesangial cells and podocytes (120) [Figure 1.11]. IL18, belonging to the IL1 family of cytokines, is initially produced as an inactive pro-IL18 consisting of a 24 kDa molecule of 192 amino acids, but is later cleaved into an active 18 kDa molecule with 157 amino acids by caspase-1 (90, 120).

IL18 performs its functions through two receptors; IL18 receptor alpha (IL18Rα) and IL18 receptor beta (IL18Rβ) (121). The β chain is linked to an accessory molecule. The α-chain is the binding ligand for IL18 while the β chain is involved in signaling pathways (90, 121). IL18 performs various functions linked to inflammation, increases transcription rate for chemokines, adhesion molecules and inflammatory cytokines, and also induces nitric oxide and NF-kB (120).

An insult to the kidney is associated with significant secretion of IL18 by the proximal tubular cells and endothelial cells (90). Sirota *et al.* reported increased IL18 in the urine in AKI patients compared to controls (884.0 vs.0 ng/ml, p=0.004) with an AUC of 0.75 in patients that underwent liver transplant (122). In other studies, IL18 was associated with almost 4-fold and 5-fold odds for AKI and mortality respectively (123). In this study, AUC-ROC for IL18 in discriminating AKI was 0.73 within 24 hours (123).

Few studies have explored the association of IL18 and other inflammatory cytokines including TNFα and IL10 as biomarkers of CIN. In a meta-analysis of IL18 (and limited to urine) that included 11 studies, Lin *et al.* observed significant heterogeneity in the included studies and the overall diagnostic odds of urine IL18 for AKI was lower than NGAL with a sensitivity and specificity of 0.51 and 0.79 respectively (124) compared to other biomarkers such as cystatin C or NGAL. In this meta-analysis, the diagnostic performance of urine IL18 in adults was suboptimal compared to paediatric populations (124).

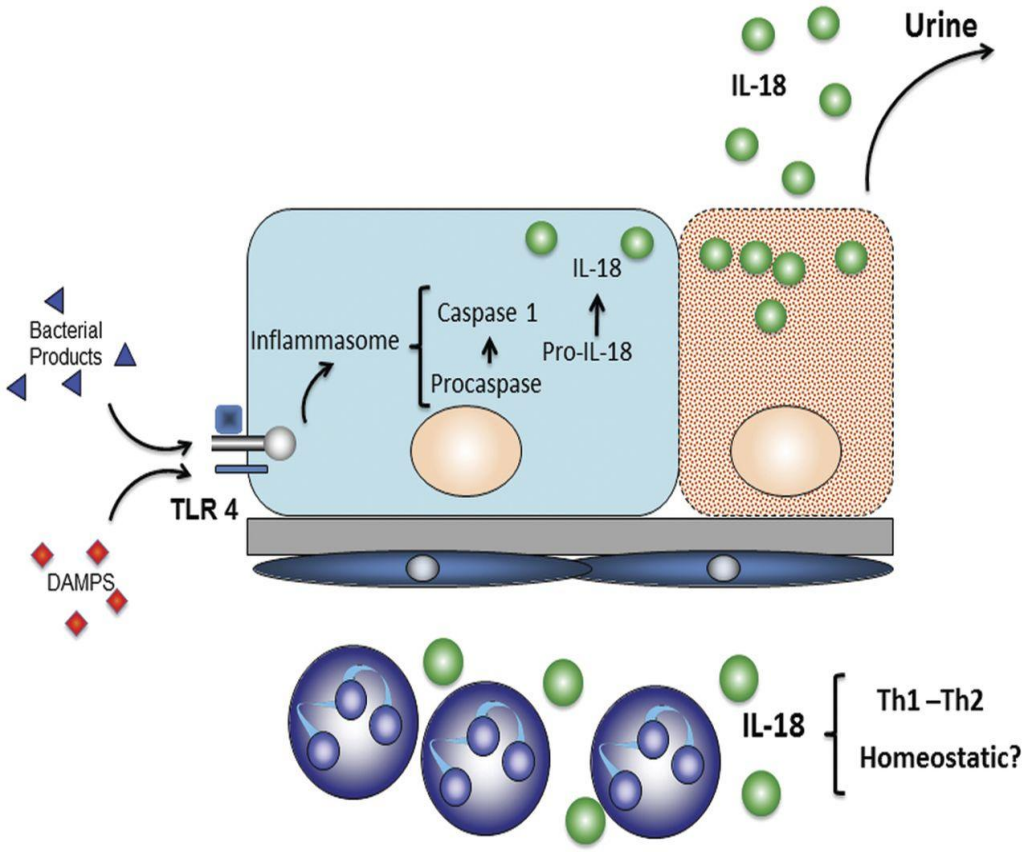

Figure 1.11Metabolism of interleukin 18 in acute kidney injury

An insult to the kidney leads to cleavage of the pro-IL18 to IL18 by Caspase 1 and secretion into blood and urine. The sources of IL18 are both inflammatory cells and injured renal tissue. Reproduced with permission from reference (90).

1.9 Correlation of novel biomarkers with morbidity and mortality

CIN biomarkers have been shown to predict short and long term outcomes in various studies, even in the presence of normal baseline serum creatinine values (114).Ishibashi *et al.* reported high short term mortality with increased cystatin C levels (OR 0.31; 95% CI 0.058-0.0538; P=0.03) (111). Patients with cystatin C levels exceeding ≥ 1.29 mg/dl had increased cardiovascular deaths, MI and strokes (111).

Among patients undergoing contrast studies for acute pulmonary embolism, increased baseline NGAL and cystatin C plasma levels positively predicted thirty day mortality (125).

In other studies, cystatin C predicted the development of heart failure and prognosis better than serum creatinine (126).Recently, Tong *et al*. reported NGAL cut-off values of 91 ng/ml as a predictor of major cardiovascular events (83).

1.10 Association of HIV withnovel biomarkers of AKI

The levels of serum creatinine shows variation in human immune deficiency virus (HIV) infected patientsdue to underlying reduced body mass and medications e.g. septrin, a recommended primary and secondary prophylactic treatment for *pneumocystis Carinii* pneumonia (PCP) in HIV patients, increases serum creatinine (127).

Studies have shown variable levels of new biomarkers among HIV infected patients (128). Cross sectional studies of 1008 HIV infected and 290 uninfected patients found significantly raised levels of cystatin C in the HIV infected subgroup (0.90± 0.22 mg/dl) compared to the uninfected group (0.76± 0.15) (127, 128). There were no differences in creatinine levels in the two groups. Presence of HIV infection was associated with an at least 10-fold increase in cystatin C after adjusting for other parameters and cystatin C predicted worse renal kidney dysfunction in the HIV subgroup (128).

In other studies, the levels of NGAL were low in highly active antiretroviral therapy (HAART) naïve patients and increased after commencement of HAART (110, 128, 129). Landro *et al.* in a 24 month follow up of HIV infected patients, demonstrated decreased levels of NGAL prior to HAART; however after commencement of HAART, the levels of NGAL were comparable to healthy controls. In this study, decreased NGAL levels in the presence of impaired immunity were probably a reflection of decreased neutrophils or impaired neutrophil chemotaxis (129). However, the association of novel biomarkers with HIV remains unexplored.

In summary, novel biomarkers produced from various sources are upregulated in blood or urine leading to their role in early AKI diagnosis and prognostication(53) [Table 1.3]

Table 1.3 Novel biomarkers proposed in AKI

Novel Biomarker	Use	Measured in	Sources
Filtration biomarkers			
Cystatin C	Early diagnosis severity (prognosis)	Serum, Plasma and urine	Belongs to a family of cysteine proteinase inhibitors, filtered and reabsorbed (53)
B2-microglobulin	Severity (prognosis)	Serum, plasma and urine	Present on all nucleated cells. Glomerular or tubular dysfunction leads to increased levels in blood and urine(53).
Renal tubular injury/damage			
Neutrophil gelatinase-associated lipocalin-2	Early diagnosis and severity (prognosis)	Serum, plasma and urine	Plasma NGAL is freely filtered and reabsorbed in the proximal tubules(53).
Interleukin 18	Early diagnosis and severity	Serum, plasma and urine	A proinflammatory cytokine secreted by injured renal cells(53).
Tumor necrosis factor alpha and interleukin 10	Severity(14, 76)	Plasma, serum and urine	Inflammatory cytokines upregulated after injured renal cells (72, 76)

The table on all biomarkers (excluding IL10 and TNFα) is reproduced from Chalikias *et al.*2016(53) with permission. Information on IL10 and TNFα from is from Chang *et al.* (14)and Jaber *et al.*(76)

1.11 Summary of the study and the research gaps

In developed countries, CIN ranks third as a cause of hospital acquired AKI impacting highly on morbidity and mortality (Chang, 2013). In sub-Saharan Africa, information on prevalence and risk factors for CIN including patient outcomes remains unexplored.

The diagnosis of CIN utilizes serum creatinine (a traditional biomarker) that peaks at 3-5 days (53) after an insult and therefore delays diagnosis by almost 48 hours post contrast media when compared to the new biomarkers (125).

Additionally, despite identification of the traditional risk factors for CIN and its pathogenesis from studies in developed countries, information on susceptibility genes contributing to development of CIN and its severity in Africans and other populations, and the influence of new biomarkers of CIN on patient outcomes is lacking. Treatment for CIN is limited to supportive care only (9).

There has been no study that has investigated the susceptibility genes for CIN in black populations including other races (except Han Chinese). Additionally, the roles of novel biomarkers in the diagnosis of CIN or prognostication remains unexplored among South Africans. Identification of higher risk and genetically susceptible individuals to avoid exposure and earlier diagnosis of CIN using new biomarkers and identification of susceptibility genes will not only reduce morbidity and mortality but will also add knowledge to renal medicine.

It is for these reasons that the study was undertaken to identify the rates of CIN, important novel biomarkers for diagnosis and prognostication and the genes associated with susceptibility to CIN.

1.11.1 Research hypotheses

Our research hypotheses were as follows;

1 The proportion of CIN is significantly higher in hospitalized South Africans compared to developed countries.

2 Novel biomarkers are more accurate in diagnosing and prognosticating CIN compared to serum creatinine

3. The TNFα and IL10 cytokine genotype single nucleotide polymorphisms (SNPs) are positively associated with CIN in black South African populations.

1.12 AIMS

The aim of this study was to determine the diagnostic and prognostic utility of biomarkers for CIN and genetic susceptibility for AKI in patients receiving contrast media

1.12.1 Specific objectives

1 To determine the prevalence of CIN and associated risk factors at Charlotte Maxeke Johannesburg Academic Hospital.

2 To determine the accuracy of biomarkers in identifying post contrast AKI

3 To evaluate the accuracy of non-traditional biomarkers in predicting morbidity and mortality in patients with post contrast AKI.

4 To determine the influence of genetic susceptibility in contrast induced AKI patients.

CHAPTER 2 MATERIALS AND METHODS

2.1 Study design, setting and population

A prospective case-controlled study was conducted at the Charlotte Maxeke Johannesburg Academic Hospital in South Africa from July 1, 2014 to July 30, 2015. Ethical approval was obtained from the human research ethics committee (HREC) of the University of the Witwatersrand (Appendix A) and informed written consent was obtained from all patients (Appendix C). In-patients undergoing computerized tomography and angiography from the Divisions of Radiology and Cardiology were recruited consecutively and followed-up for developing of AKI [Figure 2.1].

2.1.1 Inclusion criteria

Inclusion criteria were patients aged 18 years and above, written informed consent, undergoing computed tomography contrast media administration or angiography and stable chronic kidney disease (CKD). All included patients received contrast media administration.

2.1.2 Exclusion criteria

The following patients were excluded: participants below 18 years, evidence of pre-existing AKI (clinical or laboratory), end stage renal disease (ESRD) on renal replacement therapy (RRT), prior contrast media administration in the preceding 7 days, pregnancy and non-consenting participants.

2.1.3 Study outcomes

The primary end point was the occurrence of CIN, defined as a serum creatinine increase >25% from baseline or at least 44 umol/l assessed within 48-72 hours post contrast media administration. Patients included in the study but not meeting the CIN definition criteria where designated as controls. The secondary endpoints were death and duration of hospitalization obtained from discharge summaries.

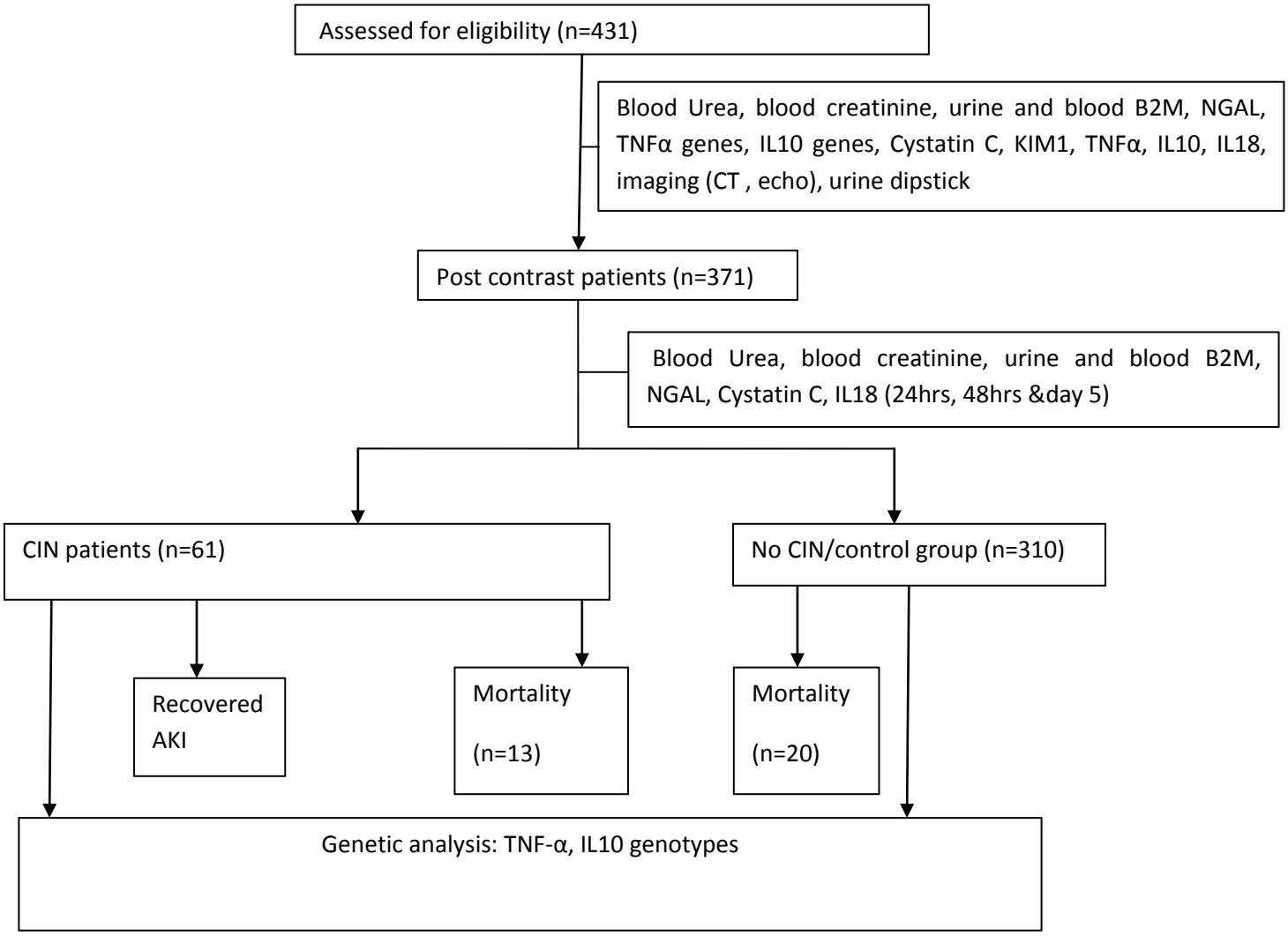

Figure 2.1 Participants and specimen flow chart

2.2 Study procedures

2.2.1 Clinical procedures

The study physician reviewed participants' medical records and examined the participants in order to determine pre-existing risk factors for CIN.Using a data collection sheet, demographic data was obtained in addition to information on factors associated with CIN i.e. hypertension, DM, heart failure, current medications and hypotension. Co-morbidities, presence of peripheral edema, HIV status, anaemia, and current medications and information on type and dose of contrast media were also recorded. The medical records were reviewed to determine patient duration of hospitalization, dialysis requirements and mortality.

2.2.2 Blood and urine sample preparation

Study phlebotomists' collected blood and urine samples prior to and post contrast media administration. Whole blood (5 mls) was collected into ethylenediaminetetraacetic (EDTA) tubes and stored at -20^{o}C for genomic DNA extraction. For biomarker studies, 5 mls whole blood collected in serum separating tubes (SST) prior to CT contrast administration and at 24 hours, 48 hours and 5 to 7 days post contrast media administration were left at room temperature over 10 minutes to clot and later centrifuged at 5000 rpm at 4^{o}C for 10 minutes (U-32012 Centrifuge, Boeco, Germany). Post centrifuge, 500 µl of obtained serum was aliquoted into 1500 ul microcentrifuge tubes and stored at -80^{o}C. Pre contrast urine was collected for determination of proteinuria and microalbuminuria.

2.3 DNA extraction and genotyping

2.3 1 DNA extraction

Genomic DNA was extracted using the Maxwell DNA purification kit (Promega AS1010, USA) as per manufacturer's protocol. DNA concentrations were determined by the NanoDrop^{TM} 2000 spectrophotometer (Thermo Scientific, USA) with A260/280 ratios. Samples that had suboptimal DNA concentrations <10 ng/ul were re-extracted using a standard salting out method.

For the salting out method, buffy coats of nucleated cells obtained via anti-coagulated blood (EDTA) were re-suspended in 15 mls polypropylene centrifugation tubes with 3 mls of nucleic lysis buffer (1% SDS; 2 mM EDTA) containing 2 mg/ml proteinase K.

The cell lysate was left for digestion overnight in a 37^0C water bath and post digestion; sodium chloride was added to each tube, mixed vigorously and incubated on ice for 5 minutes and thereafter centrifuged at 2300 rpm. In order to remove the salt used to precipitate DNA, the pellets were washed with ice cold 70% ethanol and centrifuged at 8000 rpm for 20 minutes at 4^0C. The DNA pellets were air dried at room temperature and later dissolved in 500 ul 1X TE (10 mM Tris-Cl; 2 mM EDTA, pH 8.0) buffer before undergoing quantification. The DNA samples were then stored at -80^0C until final genotyping analysis.

2.3.2 PCR reaction set up and amplification

PCR reactions consisted of water, 1 x master mix (KAPA2G Robust Hot Start Ready-Mix PCR kit, Kapa Bio systems, USA), 1.25 ul DNA primers [Table 1] and 100 ng DNA (diluted to 25 ng/ul). The reactions were amplified on the MJ Mini^{TM} Thermal cycler (Bio-Rad, USA) with initial denaturation at 95^oC for 3 minutes followed by a 40 cycles with denaturation at 95^oC for 15 seconds, annealing at 60^0C for 15 seconds and final extension at 72^0C for 20 seconds.

Table 2.1 Primers and restricting enzymes

Gene	Primer	Alleles (bp)	Enzyme	Annealing temperature
TNFα -308	F: 5'-GAG GCA ATA GGT TTT GAG GGC CAT-3' R: 5'-GGG ACA CAC AAG CAT CAA G-3'	GG: 126bp, 21bp, GA: 147bp, 126bp, 21bp and AA: 147bp	NcoI (5'-C/CATGG-3')	60^{o}C
TNFα -857	F: 5'-AAG TCG AGT ATG GGG ACC CCC CGT TAA-3' R: 5'-CCC CAG TGT GTG GCC ATA TCT TCT T-3'	CC: 106bp, CT: 131bp, 106bp, 25bp and TT: 131bp	HincII (5'-GTY/RAC-3')	60^{o}C
IL10 -592	F: 5'-GGT GAG CAC TAC CTG ACT AGC-3' R: 5'-CCT AGG TCA CAG TGA CGT GG-3'	CC: 412 bp, CA: 412 bp, 236 bp and 176 bp, AA: 176pb	RsaI (5'-GT/AC-3')	60^{o}C
IL10 -819	F: 5'-TGT ACC CTT GTA CAG GTG ATG TGA-3' R: 5'-ACC CCG ATT TCA TTA GGA TTC T-3	TT: 190bp, 25bp, TC: 215bp, 190bp, 25bp and CC: 219bp	EcorRV (5'-GAT/ATC-3')	60^{o}C
IL10 -1082	F: 5'- CCC TTA CCT TCT ACA CAC ACA C-3' R: 5'- TCC TCT TAC CTA TCC CTA CTT CC-3	MnlI (5'-CCTC(N_7)/- AA: 106bp, 11bp; AG:106bp,83bp,23bp,11bp and GG: 83bp,23bp,11bp.	MnlI (5'-CCTC(N_7)/-3'	60^{o}C
IL10 -1582F	F: 5'-CTG TGT AAG TAG CAG ATC AGA T-3 R: 5'-TGC CAG TCT GTG TCT TTG-3'	TT: 112bp, TC: 112bp, 91bp, 21bp and CC: 91bp, 21bp.	BglII (5'-A/GATCT-3')	60^{o}C

2.3.3 TNFα and IL10 restriction fragment length polymorphism

Genotypes for -308 (rs1800629) and -857 (rs1799724) in TNFα, and -592 (rs1800872), -819 (rs1800871), -1082 (rs1800896), and +1582 (rs1554286) in IL10 were determined by restriction fragment length polymorphism (RFLP) that consisted of water, 1 x buffer, enzymes and PCR products see Table 2.1. To prevent evaporation, 15 ul of sigma mineral oil was added to the mixture and incubated over night at 37^0C. Next day, a two colour gel dye was added to each sample and mixed.

2.3.4 Gel electrophoresis

Restricted products were resolved on either 10% polyacrylamide or 1.5% agarose gels by pipetting 10ul of restricted PCR products to the gel. Polyacrylamide gel electrophoresis were performed for TNFα (SNPs -308 and -857), and IL10 (SNPs 819, -1082, 1592) genotypes and agarose gel for IL10 (-592) genotype. A 10% polyacrylamide gel was constituted with water, 10X tris-borate EDTA (TBE) buffer, 30% acrylamide solution, ammonium per sulfate (APS) solution and tetramethylethylenediamine solution while the 1.5% agarose gel consisted of water, agarose, 10XTBE buffer and ethidium bromide (EtBr).

2.3.5 Gel imaging

Visualization of the restricted PCR products representing TNFα and IL10 genotypes performed using Gel Doc^{TM} EZ imager (Bio-Rad systems, USA).

2.4 Determination of serum IL10, IL18, TNFα, NGAL, cystatin C, and B2M

Concentrations of IL10; IL18; TNFα; NGAL and cystatin C were determined using Magnetic Luminex® Screening Assays (#LXSAHM-3, R&D Systems, Inc. Minneapolis, USA, in accordance with the manufacturer's instructions.

Serum was initially thawed and vortexed for 30 seconds. For Lipocalin-2/NGAL, KIM1 and cystatin C, sera were diluted 20-fold i.e. 10 ul sera: 190 ul Calibrator Diluent RD6-52 (supplied), and 2-fold i.e. 75 ul sera: 75 ul Calibrator Diluent (supplied) for IL18, TNFα and IL10 as per kit protocol. A set of 7 standards were prepared by addition of 100 ul of each cocktail to standard 1 and after mixing, a similar volume was removed and added to the next standard with an exception of the blank, see [Tables 2.2] and [Table 2.3].

Table 2.2 Standards preparation for NGAL and cystatin C

Standard	Volume of RD-52 diluent	Volume of standard
1	900 ul	100 ul Standard Cocktail J
2	200 ul	100 ul Standard 1
3	200 ul	100 ul Standard 2
4	200 ul	100 ul Standard 3
5	200 ul	100 ul Standard 4
6	200 ul	100 ul Standard 5
7	200 ul	100 ul Standard 6
Blank	200 ul	0

Table 2.3 Standard preparation for TNFα, IL10 and IL18

Standard	Volume of RD-52 diluent	Volume of standard
1	800 ul	100 ul Cocktail A and 100 ul Cocktail C
2	200 ul	100 ul Standard 1
3	200 ul	100 ul Standard 2
4	200 ul	100 ul Standard 3
5	200 ul	100 ul Standard 4
6	200 ul	100 ul Standard 5
7	200 ul	100 ul Standard 6
Blank	200 ul	0

The microparticle, biotinylated antibody and Streptavidin-Phycoerythrin (SA-PE) solutions were mixed in 15 ml aluminum foil covered tubes and vortexed for 30 seconds with additional 15 seconds ultra-sonication for microparticle cocktail.

Fifty microliters of microparticle cocktail solution and 50 ul standard/diluted samples were added to each microplate well, secured with a foil cover and left on a shaker for 2 hours. The plates were washed three times, followed by the addition of 50 ul of diluent Biotin Antibody Cocktail. After an hour duration on a shaker and another three washes, a 50 ul Streptavidin-PE was added to each well followed by 30 minutes on a shaker.

2.4.1 Biomarker concentration determination

The mean fluorescent intensities for TNFα, IL10, NGAL, KIM1, cystatin C, and IL18 were determined using BioPlex^{TM} 200 system (Bio-Rad, Texas, USA) in bead regions 12; 22; 53; 57; 75; and 78 respectively IL10, IL18, TNFα, NGAL, cystatin C were read in bead regions of 12, 22 and 78 respectively. Bio-Plex manager software, version 5 was used to generate a standard curve for concentrations determination.

2.4.2β-2-Microglobulin determination

Serum concentrations of β-2-microglobulin (β2M) were determined by enzyme linked immunosorbent assay (ELISA) (R&D systems, Inc.). Briefly, 20 ul of undiluted sera or standard were added to each well, followed by the application of 100 ul conjugate and 100 ul primary antibody. The plate was sealed and left to incubate on a shaker for 1 hour at room temperature. The wells were washed 6 times, followed by incubation with 100 ul substrate solution for 7 mins, and the reaction was stopped by the addition of 100 ul 0.5 M H_2SO_4. Optical density at 450 nm was measured on an ELx800 microplate reader (BioTek, Winooski, VT, USA). Protein concentrations were calculated using the 5 parameter logistic curve on MyAssays software (http://myassays.cloudapp.net/).

2.5 Determination of serum creatinine

Blood samples for serum creatinine were collected before and after contrast media. Serum creatinine was analyzed using the Jaffe method(130) (Siemens ADVIA 1200 chemistry system) and estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI)(131). The CKD-EPI equation belowhas demonstrated superiority to other equations (131).

$GFR=141 \text{ X } \min(SCr/\kappa, 1)^{\alpha} \text{ X } \max(SCr/\kappa,1)^{-1.209} \text{ X } 0.99^{Age} \text{ X } 1.018[\text{ if female}] \text{ X}1.159 [\text{if black}]$

$\kappa = 0.7$ if female and 0.0 if male

$\alpha=-0.329$ if female and -0.411 if male

Min = "The minimum of SCr/κ or 1 and max= the maximum of SCR/κ or 1"

2.6 Determination of the Microalbuminuria

Microalbuminuria was determined using the Chemistrip^{R} Micral 30 immune assay test (ROCHE 11544039172).

2.7 Determination of serum albumin

Determination of serum albumin was based on the principle of Doumns and Biggs in which serum albumin binds to bromocresol green solution (BCG) forming albumin-BCG compound. The latter is then measured at 596/694nm absorbance.

2.8Procedure for contrast media

Iopamidol (Jopamiron 370, Axim Pharmaceuticals in Italy); a low osmolar contrast agent was used in all contrastprocedures as intravenous or intra arteriolar in this study.

2.9 Statistical analysis

The statistics used are described in the respective chapters.

CHAPTER 3

3. RISK FACTORS AND OUTCOMES OF CONTRAST INDUCED NEPHROPATHY IN HOSPITALIZED SOUTH AFRICANS

Abstract

Background: Despite ranking third as a cause of hospital acquired acute kidney injury (AKI), iatrogenic contrast-induced nephropathy (CIN) impacts significantly on morbidity and mortality and is associated with high hospital costs. In Sub-Saharan Africa, the rates and risk factors for CIN and patient outcomes remain unexplored.

Methods: We conducted a prospective observational study at the Charlotte Maxeke Johannesburg Academic Hospital, in South Africa from July 1, 2014 to July 30, 2015. Hospitalized patients undergoing computed tomography scan contrast media administration and angiography were consecutively recruited to the study and followed up for development of AKI. CIN was defined as an increase in serum creatinine >25% or an absolute increase of >44 µmol/l from baseline at 48-72 hours post exposure to contrast media. Outcome variables were the occurrence of CIN, length of hospitalization and in-hospital mortality.

Results: We recruited 371 hospitalized patients with a mean age of 49.3±15.9. The rates of CIN assessed using an absolute or relative increase in serum creatinine from baseline was4.6% and 16.4% respectively. Anaemia was an independent predictor for the development of CIN (RR 1.71, 95% 1.01-2.87; p=0.04). The median serum albumin was 34 g/l (IQR: 29-39.5) and 38 g/l (IQR: 31-42) in the CIN and control groups respectively (p=0.01) and showed a significant trend for CIN development (RR 1.68 95% CI 0.96-2.92; p=0.06). Mortality was significantly increased in the CIN group (22.4% vs. 6.8%; p<0.001), and CIN together with anaemia increased mortality 2-fold (RR 2.39, 95% CI 1.20-4.75; p=0.01) and a 3-fold (RR 3.32, 95% CI 1.48-7.43; p=0.003) respectively.

Conclusion: CIN has a relatively high incidence in Sub-Saharan Africa and predicts poorer clinical outcomes. Presence of CIN and anaemia positively predicted mortality. Caution should be exercised in patients with hypoalbuminaemia and anaemia undergoing contrast media administration.

3.1 Introduction

Despite preventative guidelines, iatrogenic contrast-induced nephropathy (CIN) ranks third as a cause of hospital-acquired AKI and impacts significantly on morbidity and mortality and is associated with longer stays in hospitals with higher medical costs (1, 2, 5, 132).

The rates of CIN vary, depending on the study definitions employed and underlying risk factors, and ranges from 0.6% to 30%, or higher, among risk groups(9-11, 13). CIN is also associated with increased duration of hospitalization and early and late mortality. In-hospital mortality due to CIN ranges from 7-22% (41).McCullough *et al.*(41) reported early mortality of 1.1% in controls, 7.1% in CIN patients, and 35% in CIN patients that required dialysis. Due to increased comorbidities, hospitalized patients have increased risk for developing CIN compared to ambulatory patients(51).

Inflammation and endothelial dysfunction, together with reactive oxygen species (ROS), are implicated in the pathogenesis of CIN (13). Iodinated contrast media directly injures the renal tubular epithelium by producing ROS radicals that cause intra-renal vasoconstriction leading to ischaemia and death of tubular cells (3, 13, 51).Serum albumin is an important anti-oxidant that reduces the formation of oxygen free radicals and is important in expanding intravascular volume (133, 134).However, the role of serum albumin in reducing the incidence of CIN remains unexplored.

Sub-Saharan Africa has a dearth of data on rates of CIN. This study investigated the rates of CIN together with the influence of serum albumin, albuminuria, age, haemoglobin, and glomerular filtration (GFR) levels on CIN and patient outcomes.

3.2 Materials and methods

The procedures are described in chapter 2.

Statistical analysis

Statistical analyses were performed using Stata version 13 software (STATA, Inc, Texas).
Using conservative estimates for the prevalence of CIN, a minimum sample size of n=323 was required to sufficiently power this study. However based on the frequency of the rarest haplotypes for either the TNFα or IL-10 genes (at 5.2%) the samples size was be increased to 371. Continuous variables are presented as means and standard deviations when normally distributed, and as medians and interquartile ranges when non-normally distributed. Categorical variables are presented as counts and frequencies. Comparisons between CIN-positive (CIN+) and CIN-negative (CIN-) groups were assessed using a t-test for normally-distributed continuous variables, a Wilcoxon rank sum test for non-normally distributed continuous variables and a Pearson chi-squared test for categorical variables. P-values of less than 0.05 were considered significant. Multivariate analyses examined the associations of CIN and mortality with various risk factors and a generalized linear regression model built to adjust for confounding effects.

3.3 Results

3.3.1Baseline characteristics

After excluding 60 non-eligible patients [Figure 1], 371 participants remained in the study; 74.9% were black, 18.9% caucasian, 3.8% indian and 2.4% mixed race. Participants' baseline characteristics are reported in Table 1. Ages ranged from 18 to 92 years (mean 49.3±15.9). The rate of CIN was 16.4% (61 of 371) when based on 25% increase from baseline in serum creatinine and 4.6% when based on an absolute increase >44 umol/l. Baseline serum creatinine and GFR were not significantly different between the CIN and control groups; almost 97% had baseline GFR $\geq$ 60 ml/min/1.73m^2 while the remaining 3% had baseline GFR ranging from 42-59 ml/min/1.73m^2. Compared to the CIN-free group, CIN patients had significantly lower levels of baseline serum albumin and haemoglobin.

3.3.2 Independent predictors of CIN

After adjusting for all possible confounders, anaemia was found to be a predictor for CIN and additionally, low serum albumin showed a positive association for CIN development [Table 2].

3.3.3 CIN and mortality

Both duration of hospitalization and mortality were higher in the CIN group but only mortality was significantly increased; p <0.001(Figure 2). None of the patients who developed CIN required dialysis. Non-significant variables that included cardiac disease, malignancy, contrast volume and baseline eGFR and albumin were excluded in the final model. In multivariable analysis CIN and anaemia (but not comorbidities) were positive predictors for mortality (Table 3**)** and not comorbidities. Among the 13 deaths in the CIN group, 7 had malignancy and among the 20 deaths in the controls, 14 had malignancy. However, after adjusting for all confounders and comorbidities, malignancy was insignificantly associated with mortality

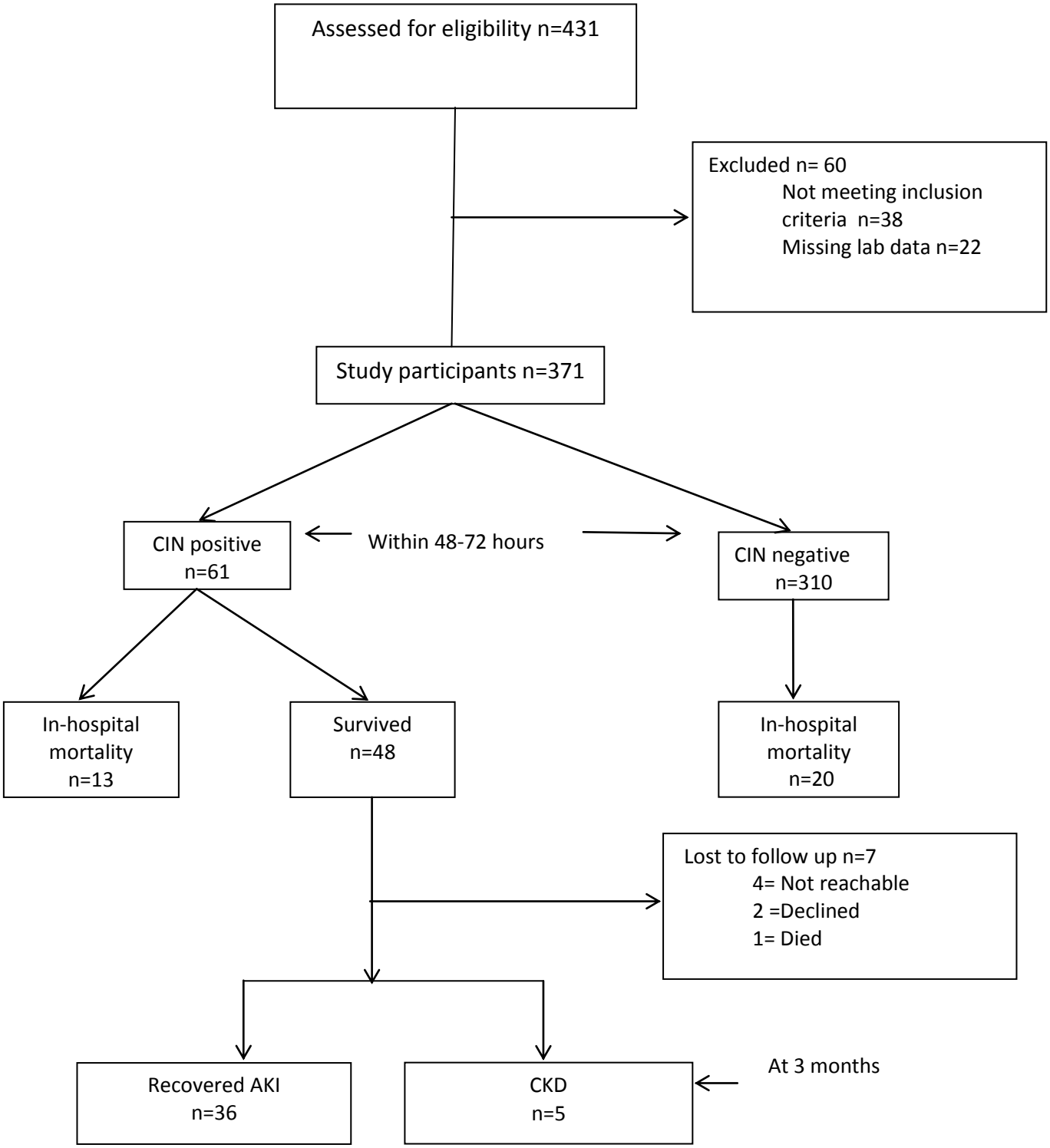

Figure 3.1 Results flow chart

Table 3.1 Baseline characteristics for study participants

Characteristic	ALL (n= 371)	CIN- (n= 310)	CIN+ (n= 310)	P
Age (years)	49.3±15.9	48.7±16.3	52.1 ±14.1	0.11
Gender, n (male, %)	210 (43.4)	174 (56.1)	36 (59.0)	0.86
Diabetes mellitus, n (%)	48 (13.2)	42 (13.8)	6 (10.3)	0.48
HTN, n (%)	93 (25.8)	78 (25.7)	15 (25.9	0.98
Malignancy (%)	159 (42.9)	136 (43.9)	23 (37.8)	0.34
Cardiac disease, n (%)	56 (15.1)	45 (14.5)	11 (18.0)	0.48
Stable CKD, n (%)	13 (3.6)	9 (2.9)	4 (6.9)	0.13
Systolic BP (mmHg)	119 (109-130)	119 (109.5-130	120 (106-132	0.43
Diastolic BP (mmHg)	73 (66-83)	73 (65-82.5)	74 (68-84)	0.18
Duration of hospitalization, n (%)	11 (6-20)	11 (5-18)	15(8-23)	0.52
Mortality, n (%)	**33 (9.3)**	**20 (6.8)**	**13 (22.4)**	**<0.001**
Pre-hydration, n (%)	38 (17.7)	28 (15.8)	10 (26.3)	0.12
N acetyl cysteine, n (%)	3 (0.8)	3 (1.0)	0 (0.0)	0.45
ACEI/ARBS, n (%)	69 (19.2)	59 (19.5)	10 (17.5)	0.73
Statins, n (%)	67 (18.6)	55 (18.2)	12 (20.7)	0.66
Diuretics, n (%)	70 (19.5)	55 (18.3)	15 (25.9)	0.18
Serum creatinine (µmol/l)	68 (53-85)	68.5 (54-84)	61 (52-89)	0.49
eGFR (ml/min per 1.73m^2	110.8 (89.5-134)	113.4 (89.9-134)	107 (89.5-133)	0.84
eGFR <60m//min/1.73m^2 , n (%)	13(3.5)	10 (76.9)	3 (23.1)	0.43
Urea (mmol/l)	4.8 (3.6-6.3)	4.9 (3.5-6.3)	4.7 (4-6.7)	0.08
Microalbuminuria, n (%)	58 (38.1)	46 (36.8)	12 (44.4)	0.46
Serum albumin g/l	37 (30-41)	38 (31-42)	34 (29-39.5)	0.01
Serum albumin <35g/l, n (%)	**113 (41.0)**	**82 (37.4)**	**31 (55.4)**	**0.02**
Haemoglobin (g/dl)	12.3 (9.8-14.3)	12.5 (10.1-14.4)	11 (9.3-13.4)	0.01
Anaemia, n (%) HB <11g/dl	117 (34.5)	89 (32.1)	28(50.9)	0.01
White cell count	8.1 (6.3 -11.7)	7.9 (6.3-11.7)	8.6 (5.9-13.9)	0.70
CRP (mg/L)	25.5 (10-70)	25.5 (10-70)	55 (12-115)	0.15
HIV positive status, n (%)	74(36.8)	61 (37.2)	13 (35.1)	0.58
Sodium (mmol/l)	139 (135-141)	139 (136-141)	138 (135-140)	0.17
Bicarbonate (mmol/l)	25 (22-27)	25 (22-27)	24 (20-26)	0.45
Phosphate (mmol/l)	1.1 (0.90-1.2)	1.10 (0.90-1.24)	1.00 (0.81-1.20)	0.10
Contrast volume (mls)	103.8 ±39.9	103.7 ±37.3	104.2 ±351.8	0.95
Contrast dose /CrCL ratio	0.88(0.72-1.16)	0.87 (0.72-1.17)	0.94 (0.71-1.1)	0.31
Ejection fraction %	63.1±14.0	63.1±14.0	59.1±13.3	0.31

CIN-, CIN absent; CIN+, CIN present; HTN, hypertension; ACEI/ARBs, angiotensin converting enzyme inhibitors/angiotensin receptor blockers, CRP, C-reactive protein; BP, Blood pressure; CKD, chronic kidney disease; Anaemia is defined as haemoglobin <11g/dL * Continuous variables expressed as mean ± SD when normally distributed and as medians (IQR) otherwise.

Table 3.2 Independent predictors forCIN

	Unadjusted			**Adjusted**		
	RR	95%CI	P value	RR	95%CI	P value
Age	1.01	0.99-1.02	0.14	1.00	0.99-1.03	0.89
Gender	1.04	0.66-1.66	0.86	1.37	0.82-2.29	0.22
Albumin level (<35g/dl)	1.60	1.00-2.56	0.05	**1.68**	**0.96-2.92**	**0.06**
Anaemia	0.89	0.81-0.98	0.025	**1.71**	**1.01-2.87**	**0.04**
Baseline eGFR	0.99	0.98-1.00	0.70			

*variables included in the final model were age, gender, anaemia, and albumin

Table 3.3 Independent predictors for mortality

	Unadjusted			**Adjusted**		
	RR	95% CI	P value	RR	95%CI	P value
CIN	**3.27**	**1.72-6.18**	**<0.0001**	**2.39**	**1.20-4.75**	**0.01***
Age	1.00	0.96-1.02	0.98	0.99	0.98-1.01	0.91
Anaemia	**3.61**	**1.75-7.45**	**0.001**	**3.32**	**1.48-7.43**	**0.003***
CM dose	0.99	0.98-0.99	0.02	0.99	0.99-1.00	0.55
Gender	1.06	0.55-2.05	0.18	0.96	0.49-1.89	0.91

*Included in final model were age, gender, anaemia, contrast media volume and CIN

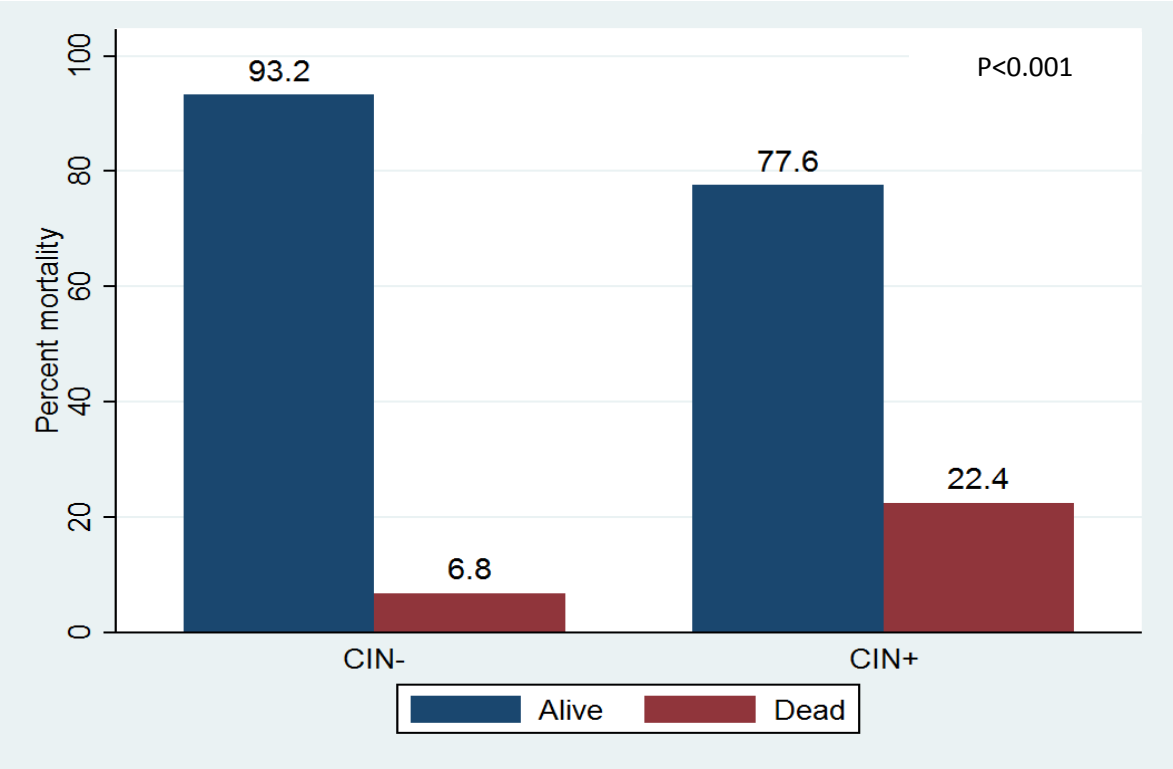

Figure 3.2 Contrast induced nephropathy and mortality

3.4 Discussion

The present study demonstrated a relatively high rate of CIN in hospitalized patients. Furthermore, the development of CIN resulted in increased mortality and highlighted the association of CIN with poorer clinical outcomes.

Our study showed a 16.4% rate of CIN, which was slightly higher than that reported in most recent studies. Overall, the reported incidence of CIN is variable, depending on the study definitions employed and underlying risk factors, and ranges from 5-30%(9-11, 13, 20, 37). Recent observational studies have reported low rates of CIN among hospitalized patients (33, 135). Shams-Edin Taher (33) observed a 11.5% rate of CIN in patients undergoing percutaneous intervention (PCI) while Salistre *et al*.(135)reported a 14% incidence of CIN in patients undergoing contrast CT administration in Brazil. A randomized controlled trial (RCT) in the USA found a 16.1% incidence of CIN in patients' undergoing PCI(35). The heterogeneous study population, variable comorbidities and non-standardized preventative prescriptions used by clinicians may explain the high rate of CIN in our study.

In this study, CIN patients had significantly lower levels of pre-contrast serum albumin compared to controls and showed a significant positive trend towards the development of CIN with serum albumin levels. Serum albumin is an important renoprotective, anti-inflammatory and anti-oxidant agent acting via several mechanisms; serum albumin binds non-ceruloplasmin copper and iron which are important in the formation of ROS and maintains renal perfusion by dilating renal vessels via its binding to nitrogen oxide forming S-nitro-albumin(29, 133, 136-138)However, during inflammation, albumin synthesis is reduced and activated neutrophils increase the levels of hypochlorous acid which inactivates albumin (133, 134, 136).Low serum albumin is probably a biomarker reflecting baseline susceptibility to acute or chronic inflammation or under nutrition. In two previous studies, Murat *et al*. and Song *et al.* found a positive association between low serum albumin and an increased risk for the development of CIN(136, 137).

A meta-analysis showed a positive association between decreased serum albumin and development of AKI as well as increased morbidity and mortality(139). The odds of developing AKI doubled with reduced serum albumin (29). Lee *et al.* reported 2-fold odds of developing post-operative AKI with decreased serum albumin (140).

Of interest in our study was the impact of baseline anaemia on patient outcomes. The rate of baseline anaemia was relatively high in our study population compared to controls and was associated with development of CIN and a 3-fold mortality risk. Limited studies have explored the association of anaemia with CIN and patient outcomes. Li *et al*. and Chong *et al*. in observational studies reported positive association between baseline anaemia and CIN (29, 141). In these studies the presence of anaemia was associated with an almost 2-fold risk of developing CIN (29, 141). In patients undergoing PCI, Mckechnie *etal*.similarly reported baseline anaemia as an independent predictor for mortality (142). The increased risk of CIN in patients with anaemia and poorer clinical outcomes is multifactorial in aetiology; in presence of anaemia, CIN induced renal ischaemia and hypoxia is significantly worsened in the outer renal medulla amidst already prevailing low oxygen tension and oxygen affinity and demand is increased (29, 143, 144).Additionally, anaemia also signifies underlying comorbidities and therefore it is a biomarker for severity of disease at time of contrast media administration and therefore increased mortality risk(29, 142).

Age, baseline renal function (determined by estimated glomerular filtration rate) and pre-hydration were not associated with the risk of CIN in our study. The positive association between CIN and age has been shown in the age group above 62 years (3).A RCT, found age above 75 years together with diabetes mellitus as independent predictors for CIN (35).In younger type two diabetic patients undergoing PCI, Zaki *et al*. reported age above 57 years to be a positive predictor for CIN (138). Our study population was relatively young, and fewer of our patients had multivessel diseases compared to previous studies. Baseline eGFR level was insignificantly associated with CIN in our study cohort. Previous studies have shown decreased risk of developing CIN with eGFR >60 ml/min per $1.73m^2$(135).

In an observational study, Weisbord *et al.* found <1% incidence of CIN in patients with eGFR >45-60 ml/min per 1.73m2 after contrast administration (145)In support of our study, Chong *et al*. found insignificant association between normal renal status and CIN development (141). In a cohort study of 80% study participants with eGFR >60 mls/min per 1.73m^2, Salistre *et al.* also reported no association between baseline eGFR and risk for CIN (135).

In our study cohort, 97% of study participants had normal baseline renal function based on eGFR with the remaining having eGFR ranging from 42-59 ml/min per 1.73$m^2$with nosignificant association with CIN.

Pre-hydration therapy was used in only 18% of patients in our study cohort compared to 70% seen in developed countries (146), and pre-hydration showed no impact on reducing the incidence of CIN. It is possible that only the patients at highest risk for CIN received pre-hydration and this possibly obscured any beneficial effects of pre-hydration. In previous studies, Salistre *et al*.observed no association between fluid administration and CIN occurrence (135).Diogo *et al*. reported a low rate of pre-hydration therapy in Brazil (19).

Similar to previous findings, our study demonstrated a higher mortality in the CIN group compared to controls and CIN together with anaemia were independent predictors of mortality (34).Rihal *et al*., in a retrospective registry review at the Mayo Clinic reported in-hospital mortality rates of 22.0% and 1.4% in the CIN and controls groups respectively and CIN was positively associated with a 10-fold increased odd of death(43). Furthermore, cardiac disease was associated with a 2-fold increased odd for death. Sadeghi *et al*. in a multicentre study found that 30 day mortality was significantly higher among patients with CIN compared to controls (16.2% vs. 1.2%; p <0.001) (34, 42).In-hospital mortality due to CIN in the National Cardiovascular Registry in USA was reported to be 9.1% (147).

Development of CIN also reflects underlying disease comorbidities at the time of contrast media administration. Despite increased mortality in the CIN group compared to controls, Sadeghi *et al.* found underlying cardiac disease as a positive predictor for mortality(42).

In other studies; age, cardiac disease and the requirement of dialysis were predictors for mortality in CIN participants (34). In our study, among the 13 deaths in the CIN group, 7 had a diagnosis of malignancy, however after adjusting for possible cofounders, only CIN and anaemia and not malignancy were predictors of mortality.

The strength of our study is that it was a prospective cohort with a large sample size. However, it is limited by being a single centre,non-interventional study conducted at a tertiary hospital. The study consisted of a heterogeneous population and prevention guidelines for CIN were not standardized or uniformly implemented.

3.5 Conclusion

Our study highlights the high rates of CIN and associated poorer clinical outcomes. Caution should be used when administering iodinated contrast media to hypoalbuminaemia and anaemic patients. To our knowledge, this is the first prospective study examining the risk and outcomes of CIN in Sub-Saharan Africa. This study advocates that preventative treatment guidelines for CIN should be standardized and uniformly implemented across various hospital departments.

CHAPTER 4

4. ACCURACY OF BIOMARKERS IN PREDICTING AND PROGNOSTICATING CONTRAST INDUCED NEPHROPATHY

Abstract

Background: Serum creatinine is suboptimal as a biomarker in the early diagnosis of contrast induced nephropathy (CIN) resulting in delayed interventions and leading to an increase in adverse clinical outcomes. This study investigated a panel of novel biomarkers in the early diagnosis of CIN and in predicting patient outcomes.

Methods: This was a single centre nested prospective case-controlled study that included 30 patients with CIN and 60 controls (those undergoing radio contrast studies) and were matched for race, gender and age in a case:control 1:2 ratio at all-time points. Sera for neutrophil gelatinase-associated lipocalin-2 (NGAL), cystatin C, beta-2 microglobulin (β2M), interleukin 18 (IL18), IL10, and tumor necrosis factor alpha (TNFα) were collected at four time points: baseline (pre-contrast), 24 hours, 48 hours and ≥5-7 days after contrast administration and their concentrations were determined using luminex assays and an enzyme linked immunosorbent assay for β2Mas per manufacturer's instructions. Novel biomarkers were compared to CIN and CIN mortality by generating the area under the receiver operating characteristics (AUROC) curves.

Results: The median cystatin C at 24 hour ($p<0.001$) and 48 hour ($p=0.01$) and β2M levels at all-time points were significantly higher in the CIN group compared to controls. The median 24 hour cystatin C and 48 hour β2M levels were 856.60 ng/ml (IQR 620.75-1002.96) vs. 617.42 ng/ml (IQR 533.11-805.20); $p<0.001$ and 5.3 µg/ml (IQR 3.8-6.9) vs. 3.3 µg/ml (IQR 2.7-4.5); $p<0.001$ with AUROC of 0.75 and 0.78 respectively for early diagnosis of CIN. Baseline IL18 ($p <=0.001$), β2M ($p=0.04$) and TNFα ($p=0.00$) levels were significantly higher in the non-surviving group and their AUROC were 0.83, 0.82 and 0.94 for CIN+ mortality respectively.

Baseline NGAL was a better marker at excluding patients at risk for CIN with positive predictive value (PPV) and negative predictive value (NPV) of 0.50 and 0.81 respectively. Cystatin C (P=0.003) and β2M (p=0.03) at 24 hours independently predicted CIN risk. β2M at baseline and 24 hours were associated with a 1.4-fold and 1.5-fold mortality odds respectively. **Conclusion:** Serumcystatin C showed the best diagnostic discrimination for CIN while IL18, β2M and TNFα showed the best discrimination performance for CIN mortality. NGAL is a dependable biomarker for excluding patients at higher risk for CIN.

4.1 Introduction

Despite increased morbidity and mortality linked with iodinated contrast media-induced nephropathy (CIN)(1, 2, 4),early interventions are delayed due to the suboptimal sensitivity and specificity of serum creatinine in the early diagnosis of CIN(79, 82-84, 86, 148). Almost 80% of subclinical kidney injury caused by contrast media should be identified within 24 hours using early diagnostic criteria(82).

Previous studies have demonstrated that neutrophil gelatinase lipocalin-2 (NGAL), cystatin C, interleukin 18 (L18) and beta-2 microglobulin (β2M) are more sensitive as earlier biomarkers of acute kidney injury (AKI) compared with serum creatinine(100, 115, 116, 122, 123, 148)and predicted adverse clinical outcomes (111, 125, 126). Biomarkers are needed in early identification of subclinical AKI characterised by small increases in levels of serum creatinine (148). In previous studies, even a small increase in serum creatinine exceeding 44.3 μmol/l was linked with a 7-fold mortality risk (148, 149).

Cystatin C and β2M, with molecular weights of 13 kDa and 11.8 kDa respectively, are non-glycosylated molecules that are increased in the circulation due to impaired glomerular filtration (85, 89). Cystatin C produced by all nucleated cells functions as an intracellular inhibitor of cysteine peptidase (88, 98) while β2M is found on major histocompatibility complex class-1 nucleated cells (117). NGAL, a 25 kDa glycoprotein is up regulated in 2-4 hours in patients undergoing radio contrast administration (79, 81, 83). In a multi-centre study, 24 hour serum cystatin C was a better predictor of CIN with an area under receiver-operative curve (AUROC) of 0.86 (115). Inflammatory cytokines (including IL18, TNFα and IL10) are upregulated in renal injury or damage (14, 90, 96) and released into plasma and urine (96); however, limited studies have explored their roles in AKI particularly CIN. Liu *et al*. in a recent meta-analysis of 23 studies, urine IL18 showed suboptimal sensitivity and specificity of 0.58 and 75% respectively (150).

Previous studies on novel biomarkers looked at homogenous populations characterised by non-heterogeneous AKI insults (surgery and non-mixed intensive care units), and a few were based on radio-contrast administration. Additionally, these studies were uncontrolled and evaluated only NGAL and cystatin C and none looked at other novel biomarkers.

Compared to urine, biomarker measurements using plasma/sera are unaffected by bacterial contamination and is also readily available, even in anuric patients (100). In a recent comparative observational study in Germany, Schley *et al.* demonstrated that plasma biomarkers had superior diagnostic accuracy for AKI after cardiopulmonary bypass (100). Our study investigated the diagnostic accuracy of serum NGAL, IL18, cystatin C, β2M, IL10, and TNFα in predicting CIN and their role in prognosticating patient outcomes.

4.2Materials and methods

This was a nested prospective case-controlled study that included 30 patients with CIN and 60 controls (not meeting the contrast induced kidney injury criteria) that were consecutively selected from patients undergoing contrast administration. The controls were matched for race, gender and age in a case: control ratio of 1:2 at each time interval. Sera were collected at 4 time point measurements: baseline (pre-contrast), 24 hours, 48 hours, and 5-7 days after contrast administration.

4.3.1 Study outcomes

The study outcomes were discrimination performance of the novel biomarkers for CIN at different time intervals (defined according to the European Society of Urogenital Radiology (ESUR) and in-hospital CIN associated mortality (50).

4.3.2 Statistical analysis

Biomarkers characteristics are described as medians and interquartile ranges (IQR) as values were not normally distributed. Comparisons of biomarkers with CIN and mortality were determined using the Wilcoxon-Mann-Whitney test.

To determine discrimination performance of biomarkers for CIN from non CIN and mortality (overall and CIN+ mortality), areas under receiver-operating curves (AUROC) were constructed. Sensitivity and specificity were calculated for each point on the curve and the optimal cut-point calculated by finding the point with the maximum Youden Index (Youden Index = sensitivity + specificity-1). Positive predictive values (PPVs) were calculated for the optimal cut-point using the formula: PPV=(number of true positives)/(number of true positives + number of false positives). Similarly, the negative predictive values (NPVs) were calculated for the optimal cut-point using the formula: NPV=(number of true negatives)/(number of true negatives + number of false negatives). Multivariable regression analysis of biomarkers, adjusted for age and gender, were performed to determine predictors of CIN and mortality.

4.3 Results

4.3.1 Biomarker characteristics in CIN patients

This nested case-controlled study included 30 CIN+ participants matched with 60 controls in a ratio of 1:2 and biomarker measurements were determined at four time points: baseline (pre-contrast), 24 hours, 48 hours and ≥5-7 days after radio contrast media administration. Overall mortality occurred in 15 individuals with 7 deaths restricted to the CIN+ patients. The biomarker characteristics are described in in Table 4.1. Compared to controls, the CIN+ group had increased median (IQR) levels of β2M at baseline ($p=0.04$) and 24 hours ($p=0.01$); and cystatin C at 24 hours ($p<0.001$). Pre-contrast medians (IQR) levels of IL18 ($p<0.001$), β2M ($p=0.04$), TNFα ($p<0.001$) including TNFα at 24 hours ($p<0.001$) were significantly higher in the group that demised [Table 4.3]. A non-significant association was also observed between rates of CIN in HIV infected individuals compared to controls and the characteristics of novel biomarkers in HIV infected patients compared to uninfected is described in Table 4.7.

4.3.2 Diagnostic accuracy ofbiomarkers in predicting CIN

The areas under the receiver operating curves (AUROC) were generated for determination of early CIN diagnosis and CIN mortality and only biomarkers that showed significance were considered. Table 4.1 and Figure 4.1 show biomarkers measurements in the CIN group vs. controls and their corresponding AUROC. Cystatin C at 24 hours and β2M at 48 hours demonstrated good early discrimination for CIN with AUROC of 0.75 and 0.78 respectively and together predicted CIN development after adjusting for priori-selected age and gender [Table 4.4].

Baseline β2M including the 48 hours independently predicted mortality [Table 4.2]. Table 4.2 describes the optimal cut-off values for bio-markers in predicting CIN. Serum NGAL (Table 4.2) at baseline and β2M showed superiority in excluding patients at risk of developing CIN.

The positive predictive values (PPV) and negative predictive values (NPV) for serum NGAL and β2M at baseline were 0.50 and 0.81, and 0.48 and 0.87, respectively. Cystatin C at 24 hours showed the best PPV and NPV of 0.75 and 0.77 respectively.

4.3.3 Diagnostic accuracy of biomarkers in prognosticating mortality

Table 4.2 and Figure 4.2 describe biomarker characteristics in the surviving and non-surviving groups after contrast administration. For prognosticating mortality, baseline pre-contrast measurements of IL18, β2Mand TNFα showed the best AUROCs of 0.83, 0.82 and 0.94 respectively. After adjusting for age, gender, as well as IL18 and TNFα, baseline β2M (including the 24 hours β_2M) independently predicted mortality.

Table 4.1 Biomarker characteristics in CIN+ and CIN- participants

	CIN+ (n=30)	**CIN- (n=60)**	**p-value**
variable	median (IQR)	median (IQR)	
NGAL_p (ng/ml)	100.31 (64.28-142.01)	74.33(43.97-127.99)	0.34
NGAL _24 (ng/ml)	99..61(72.26-135.98)	78.42 (51.12-107.00)	0.07
NGAL _48 (ng/ml)	83.81 (57.90-109.05)	60.91 (37.36-100.71)	0.13
NGAL _5 (ng/ml)	96.20 (74.48-156.66)	65.77 (51.94-72.44)	0.06
Cystatin C_ p (ng/ml)	711.45 (550.08-934.10)	687.41 (566.61-769.76)	0.25
Cystatin C_24 (ng/ml)	**856.59 (620.75-1002.96)**	**617.42 (533.11-805.20)**	**<0.001**
Cystatin C_48 (ng/ml)	764.32 (560.28-1010.71)	572.13 (461.67-708.11)	0.01
Cystatin C_5 (ng/ml)	811.52 (708.54-986.12)	596.14 (534.56-684.38)	0.01
ILl18_p (pg/ml)	170.41 (105.19-327.4)	123.73 (65.87-178.2)	0.13
IL18_24 (pg/ml)	152.32 (92.905-279.62)	122.36 (82.45-256.6)	0.40
IL18_48 (pg/ml)	137.62 (100.965-285.14)	95.75 (73-165.59)	0.06
IL18_5 (pg/ml)	133.385 (122.36-395.75)	131.93 (70.82-294.91)	0.64
B2M_p (µg/ml)	**4.4 (3.8-7.8)**	**3.8 (3.2-4.9)**	**0.04**
B2M _24(µg/ml)	**4.55 (3.9-7.55)**	**3.7 (2.9-4.8)**	**0.01**
B2M _48 (µg/ml)	5.1 (3.8-6.9)	3.3 (2.7-4.5)	<0.001
B2M _5(µg/ml)	12.1 (4.4-16.4)	3.7 (3.1-4.9)	0.01
TNFα_p (pg/ml)	4.87 (4.15-9.12)	4.6 (2.65-5.95)	0.12
TNFα_24 (pg/ml)	5.3 (4.15-6.7)	5.23 (3.43-7.39)	0.94
TNFα _48 (pg/ml)	5.9 (4.6-6.7)	4.29 (2.6-7.04)	0.06
TNFα _5 (pg/ml)	4.3 (3.43-5.23)	6.315 (4.26-8.26)	0.22
IL10_p (pg/ml)	4.94 (4.5-11.3)	4.1 (2.59-5.4)	0.19
IL10_24 (pg/ml)	4 (3.4-5.5)	3.9 (2.6-5)	0.45
IL10_48 (pg/ml)	4.6 (3.7-9.2)	3.4 (2-4.5)	0.10
IL10_5 (pg/ml)	9.5 (9.5-9.5)	4.2 (3.24-19.4)	0.51
Base creatinine (µmol/l)	69 (53-96)	67 (52-84.5)	0.69
Post creatinine (µmol/l)	104 (85-156)	63.5 (46.5-76.5)	<0.001

CIN-, CIN absent; CIN+, CIN present; NGAL, neutrophil gelatinase-associated lipocalin; cystatin C;IL, Interleukin; B2M, Beta 2 microglobulin; TNFα, Tumor necrosis factor alpha; p, pre-contrast baseline; 24, 24 hours; 48, 48 hours; 5, after 5 days after contrast studies.

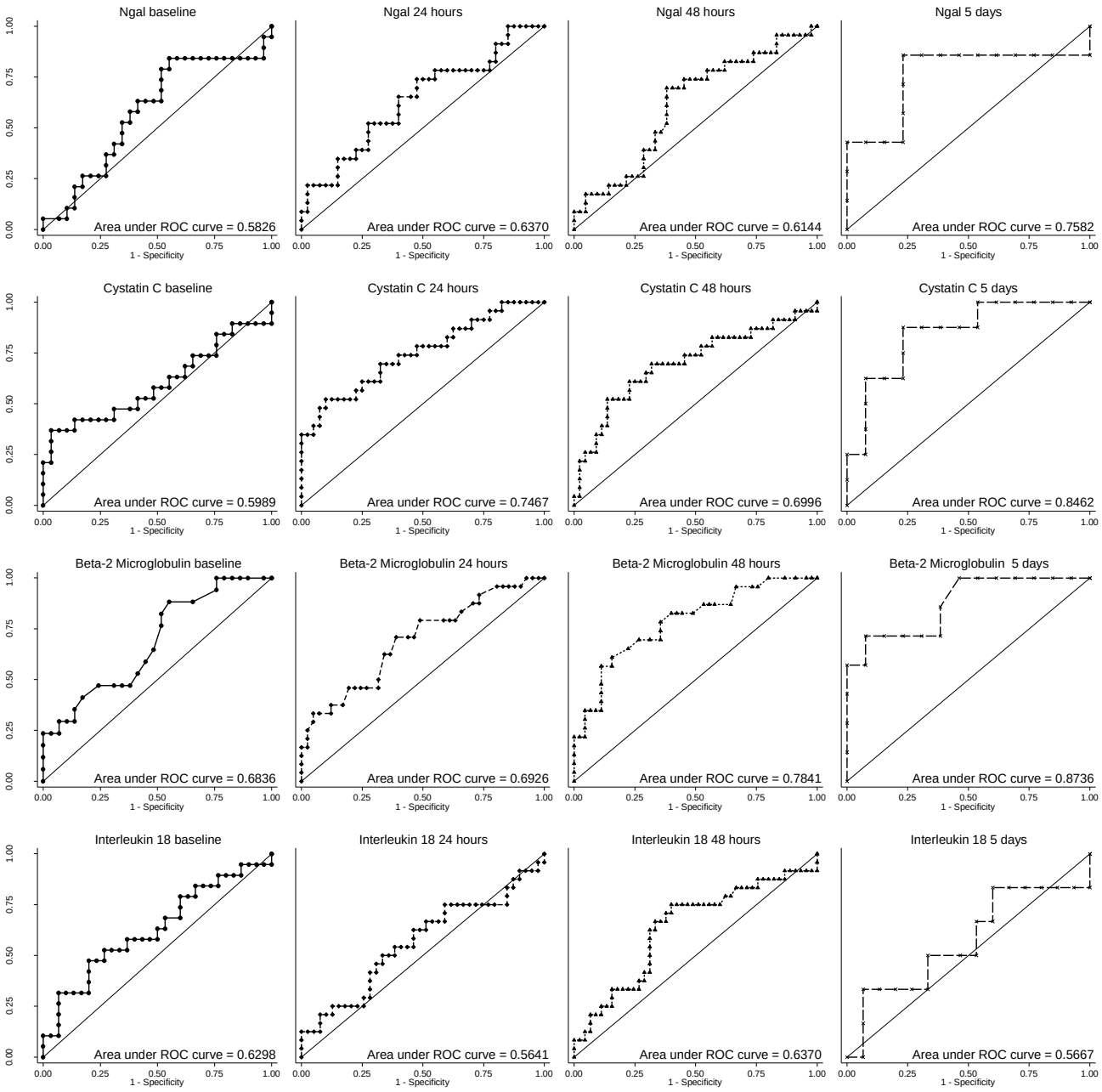

Figure 4.1 Diagnostic accuracy of biomarkers in predicting CIN.

Receiver operating characteristic curves (AUROC) demonstrating discrimination performance forCIN (at baseline, 24hours, 48 hours and ≥5-7 days after radio contrast administration).

Table 4.2 Positive and negative predictive values of biomarkers for patients with CIN

	Cut point	Sensitivity	Specificity	PPV	NPV
NGAL_p (ng/ml)	63.15	0.84	0.45	**0.50**	**0.81**
NGAL_24 (ng/ml)	80.81	0.74	0.52	0.47	0.78
NGAL_48 (ng/ml)	72.95	0.70	0.62	0.50	0.79
Cystatin C_p (ng/ml)	893.43	0.37	0.96	0.88	0.70
Cystatin C_24 (ng/ml)	856.59	0.52	0.90	**0.75**	**0.77**
Cystatin C_48 (ng/ml)	764.32	0.52	0.86	0.67	0.78
β2M_p (µg/ml)	3.6	0.88	0.45	**0.48**	**0.87**
β2M_24 (µg/ml)	4.3	0.71	0.61	0.51	0.78
β2M_48 (µg/ml)	5.1	0.57	0.88	0.67	0.80
IL18_p (pg/ml)	182.0	0.48	0.80	0.60	0.71
IL18_24 (pg/ml)	161.2	0.50	0.67	0.48	0.68
IL18_48 (pg/ml)	116.6	0.75	0.60	0.50	0.81

NGAL, neutrophil gelatinase-associated lipocalin; IL18, Interleukin 18; B2M, Beta 2 microglobulin;; p, pre contrast baseline; 24, 24 hours post contrast administration; 48, 48 hourspost contrast administration; PPV, positive predictive value; NPV, negative predictive value

Table 4.3Biomarkers characteristics in surviving and non-surviving participants

	Death (n=15)	**Survivors (n=75)**	**p-value**
variable	median (IQR)	median (IQR)	
NGAL_p (ng/ml)	78.15 (37.01-105.11)	79.45 (57.28-131.51)	0.37
NGAL_24 (ng/ml)	71.38 (32.80-108.82)	82.72(58.28-119.27)	0.24
NGAL_48 (ng/ml)	88.99 (51.30-115.88	67.31 (41.00-100.71)	0.46
NGAl_5 (ng/ml)	116.37 (74.48-143.87)	66.11(43.57-96.20)	0.09
Cystatin C_p (ng/ml)	767.38 (633.02-893.43)	670.40 (548.52-789.58)	0.20
Cystatin C_24 (ng/ml)	723.70 (358.95-811.31)	694.25 (569.08-870.31)	0.34
Cystatin C_48 (ng/ml)	764.32 (240.03-843.25)	603.76 (505.55-760.62)	0.45
Cystatin C_5 (ng/ml)	740.40 (706.22-816.65)	668.11 (550.32-724.65)	0.15
IL18_p (pg/ml)	**301.54 (211.10-461.28)**	**109.00 (64.47-165.50)**	**<0.001**
IL18_24 (pg/ml)	203.30 (118.80-409.94)	125.18 (77.47-224.09)	0.09
IL18_48 (pg/ml)	181.67 (86.40-331.40)	110.20 (77.00-156.90)	0.19
IL18_5 (pg/ml)	356.50 (294.91-395.75)	122.36 (65.00-133.85)	<0.001
B2M_p(µg/ml)	**7.8 (3.7-9.7)**	**4.0 (3.2-5.4)**	**0.04**
B2M_24(µg/ml)	4.6 (3.8-7.4)	4.1 (3.3-5.2)	0.21
B2M_48(µg/ml)	4.6 (3.0-6.7)	3.7 (2.8-5.0)	0.09
B2M_5(µg/ml)	8.0 (5.5-16.4)	4.1 (3.1-5.0)	0.03
TNFα_p (pg/ml)	**8.26 (6.00-79.60)**	**4.15 (2.60-5.30)**	**<0.001**
TNFα _24 (pg/ml)	7.40 (5.45-14.69)	4.87 (3.23-5.60)	<0.001
TNFα _48 (pg/ml)	6.31 (3.08-11.37)	4.56 (2.70-6.70)	0.11
TNFα _5 (pg/ml)	8.98 (4.87-10.86)	4.60 (2.70-7.04)	0.02
IL10_p (pg/ml)	4.9 (3.6-17.8)	4.5 (3.0-5.5)	0.47
IL10_24 (pg/ml)	15.2 (4.2-33.4)	3.9 (2.9-5.0)	0.08
IL10_48 (pg/ml)	3.9 (3.0-14.8)	3.6 (2.5-5.0)	0.43
IL10_5 (pg/ml)	11.3 (3.2-19.4)	4.6 (3.9-9.5)	1.00
Base creatinine (µmol/l)	72 (44-96)	68 (55-85)	0.93
Post creatinine (µmol/l)	93 (45-126)	69 (54-91)	0.52

NGAL, neutrophil gelatinase-associated lipocalin; IL18, Interleukin 18;β2M, Beta 2 microglobulin; TNFα,Tumor necrosis factor alpha; IL10, interleukin 10; p, pre contrast; 24, 24 hours; 48, 48 hours; 5, after 5 days.

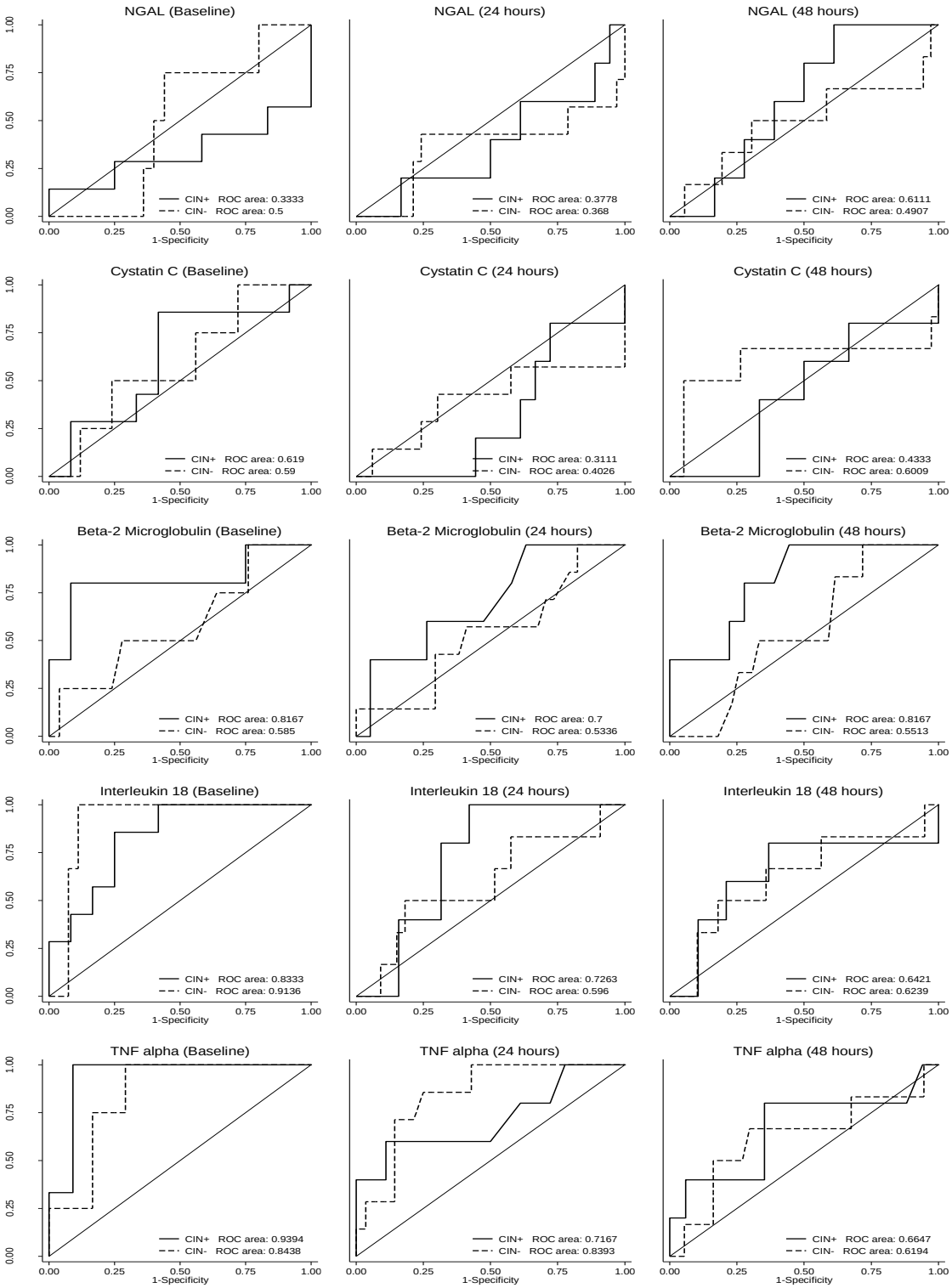

Figure 4.2 Diagnostic accuracy of biomarkers in predicting mortality. Receiver operating characteristic curves (AUROC) demonstrating discrimination for CIN diagnosis (at baseline, 24hours, 48 hours and ≥5-7 days pots radio contrast administration). CIN+ indicated by solid line and dashed line for CIN-.

Table 4.4 Biomarkers predicting CIN

	Unadjusted			Adjusted		
	Odds Ratio	95% Confidence interval	p-value	Odds Ratio	95% Confidence interval	p-value
Age	1.02	(0.99-1.06)	0.129	1.01	(0.97-1.06)	0.538
Gender (Female)	0.87	(0.36-2.12)	0.766	0.55	(0.15-1.98)	0.357
Cystatin C _24 hours	1.00	(1.00-1.00)	0.000	**1.00**	**(1.00-1.00)**	**0.003**
B2M _24 hours	1.46	(1.14-1.87)	0.003	**1.26**	**(1.02-1.56)**	**0.029**

Included in the model were age, gender B2M, Beta 2 microglobulin, and Cystatin C at 24 hours

Table 4.5 Pre-contrast biomarkers predicting mortality

	Unadjusted			Adjusted		
	Odds Ratio	95% Confidence interval	p-value	Odds Ratio	95% Confidence interval	p-value
age	1.01	(0.97-1.05)	0.611	1.02	(0.98-1.07)	0.331
Gender (Female)	0.54	(0.17-1.75)	0.305	1.25	(0.29-5.40)	0.763
B2M_p	1.49	(1.12-1.99)	0.006	**1.41**	**(1.09-1.84)**	**0.010**
IL18p	1.01	(1.00-1.01)	0.017	1.00	(1.00-1.01)	0.582

Included in the model were age, gender, B2M, Beta 2 microglobulin and IL18, interleukin 18 at baseline; p, baseline

Table 4.6 Biomarkers predicting mortality at 24 hours post radio contrast administration

	Unadjusted			Adjusted		
	Odds Ratio	95% Confidence interval	p-value	Odds Ratio	95% Confidence interval	p-value
age	1.01	(0.97-1.05)	0.611	1.01	(0.97-1.06)	0.588
Female gender	0.54	(0.17-1.75)	0.305	0.90	(0.27-3.05)	0.868
b2m_24 (µg/ml)	1.19	(0.98-1.45)	0.085	**1.51**	**(1.15-1.98)**	**0.003**
tnfa_24 pg/ml	1.23	(1.03-1.47)	0.020	0.96	(0.89-1.02)	0.196

Included in the model were age, gender, B2M, Beta 2 microglobulin; TNFα, Tumor necrosis factor alpha at 24 hours after contrast administration

Table 4.7Comparison of levels biomarkers in HIV infected patients

Characteristic	HIV+ (n=22)	HIV- (n=37)	p-value
NGAL-Pre (ng/ml)	78.67 (37.86-160.97)	78.15 (43.92-115.46)	0.811
NGAL-48 hours (ng/ml)	54.41(36.57-121.13)	88.85 (60.31-115.88)	0.115
Cystatin C Pre (ng/ml)	616.90 (547.71-872.31)	705.73 (566.61-769.76)	0.727
Cystatin C 48 hours (ng/ml)	564.53(382.54-761.88)	693.16 (483.97-764.32)	0.391
IL-18 Pre (pg/ml)	109.00 (87.47-165.50)	126.80 (61.73-217.60)	0.797
IL-18 48 hours (pg/ml)	128.31 (85.30-417.51)	101.73 (61.00-173.63)	0.166
TNFα Pre (pg/ml)	4.60 (3.43-5.59)	5.09 (3.10-7.40)	0.745
TNFα 48 hours (pg/ml)	5.65 (3.43-7.93)	4.62 (2.53-6.31)	0.175
IL-10 Pre (pg/ml)	3.9 (3.1-4.5)	4.7 (3.6-8.3)	0.522
IL-10 48 hours (pg/ml)	3.6 (1.9-5.0)	2.6 (1.8-3.7)	0.263
Base creatinine µmol/l	61 (54-89)	71 (58-90)	0.551
Post creatinine µmol/	63 (46-91)	75 (57-95)	0.221
Microalbuminuria (+)	5 (45.5%)	9 (47.37%)	0.919
Proteinuria (+)	1 (10%)	3 (15.8%)	0.667
B2M-Pre µg/ml	4.0 (3.2-4.9)	4.0 (3.4-7.8)	0.410
B2M 48 hours µg/ml	3.5 (2.8-4.6)	4.4 (2.8-5.5)	0.566

CIN-, CIN absent; CIN+, CIN present; NGAL, neutrophil gelatinase-associated lipocalin; IL10, Interleukin 10; IL18, Interleukin 18; B2M, Beta 2 microglobulin; p, pre contrast baseline; 24, 24 hours post contrast administration; 48, 48 hourspost contrast administration.

4.4 Discussion

This prospective case-controlled study confirmed that increased levels of novel biomarkersdemonstrated better early diagnostic accuracy for CIN compared to serum creatinine and they are dependable in excluding patients at risk of developing CIN. Additionally the increased levels of novel biomarkers demonstrated prognostic significance compared to serum creatinine. To our knowledge, this the first prospective and case-controlled study that compared and associated a panel of novel structural, filtration and inflammatory biomarkers in diagnosis and prognostication in patients undergoing contrast studies.

Our study showed that cystatin C at 24 hours after contrast administration showed best discrimination for CIN, which is consistent with previous observational and meta-analysis studies (100, 115, 151, 152). In a recent study of patients undergoing cardiopulmonary bypass surgery with cystatin C measurement at 2, 4, 24 and 72 hours after surgery, serum cystatin C levels significantly peaked at 24 hours in the acute kidney injury (AKI) group compared to controls (100). The cystatin C AUROC at 24 hours in discriminating presence of AKI after surgery in patients with normal baseline renal function was 0.75, a finding very similar to our study (100).

In another study limited to chronic kidney disease (CKD) patients undergoing angiography, Briguori *et al.* observed a non-significant differences between levels of cystatin C in the CIN group compared to controls at baseline; however at 24 hours after contrast media administration, cystatin C was significantly higher in the CIN group (151). In this study, the AUROC at 24 hours was 0.92 and higher, compared to our study (151). This study cohort was composed of patients with CKD (151)whereas our study patients had almost normal baseline renal function. Previous studies have shown high AUROC in CKD patients compared to patients with normal renal function (100).

In a meta-analysis that included 19 studies, Zhang *et al.* reported best discrimination for AKI with cystatin C measured within 24 hours in patients with homogenous insults to the kidney and after 24 hours in patients with non-homogenous insult to the kidney (115).

In this meta-analysis, the best AUROC for AKI were 0.81 at 12 hours after cardiac surgery and 0.92 after contrast media administration (115); however this meta-analysis majorly comprised studies of patients with homogenous insults to the kidney and only one study on CIN in CKD patients was included in this study (115).

There are several reasons why cystatin C demonstrates superiority over serum creatinine in detecting early AKI, including CIN (152). Cystatin C belonging to the cystatin is a small molecule of 13 kDa that intracellularly inhibits cystatin peptidases (109). Secretion of cystatin C into the circulation is linear (109, 152) and unaffected by muscle mass and volume as compared to serum creatinine.(152).

In our study cohort, increased levels of beta 2 microglobulin (β2M) demonstrated early prediction for CIN and mortality. Several studies on β2M and outcomes have looked at populations with underlying chronic kidney disease (116). In a study limited to a paediatric population, El-Frargy *et al.* found significantly increased levels of baseline β2M in AKI patients compared to controls; however the levels of serum creatinine remained unchanged (153). After 72 hours, β2M demonstrated superiority in detection of AKI with sensitivity and specificity of 98% and 80% vs. 46% and 53% for serum creatinine (153). In another paediatric study (154), Herrero-Morrin *et al.* also demonstrated higher levels of β2M in the AKI group compared to controls despite insignificant differences in levels of serum creatinine (154). β2M also demonstrated superiority early detection of AKI with AUROC of 0.80 vs.0.63 for serum creatinine (154). This study defined renal disease as glomerular filtration rate (eGFR) <80 mls/min per 1.73m^2(154).

Increased serum β2M levels were linked with mortality in our study, a finding previously reported (155, 156), In the CKD patients who died, increased β2M was an independent predictor of mortality (155). Two reasons could explain the association between increased levels of β2M and mortality in our study cohort. The underlying comorbidity of our study cohort was predominantly malignancy and in previous studies, malignancy together with micro inflammation is associated with increased secretion for β2M(156). In the presence of renal disease including CIN, the impact of high β2M is heightened (156).

Compared to serum creatinine, β2M performed superiorly in the diagnosis of CIN, similar to a few limited studies. β2M is a filtration biomarker produced by nucleated cells (116) and undergoes almost complete metabolism in the kidney and is completely absorbed by megalin mediated endocytosis (116, 117) and is also least affected by extra renal factors (157).

In our study, NGAL showed dependable accuracy in excluding patients at risk of development of CIN. This finding is supported by two recent studies limited to CIN; one conducted in Italy among patients with underlying renal disease who underwent angiography (158) and another in Iran that was conducted in patients with normal renal function (159). In the Italian study, Quintaville *et al*. reported a NGAL positive predictive value (PPV) and negative predictive value (NPV) of 20% and 93% respectively (158), thus demonstrating superiority of NGAL in excluding patients at risk for CIN and suboptimal accuracy for diagnosing CIN. In patients with normal renal function undergoing angiography, Khatami *et al.* reported NGAL positive and negative predictive values of 9.4% and 97.1 % respectively, with suboptimal area under the receiver operative curves (159). Several studies correlating NGAL in the diagnosis of AKI were limited to patients with homogenous insults to the kidney such as surgical patients (101, 107). In these studies, early NGAL discrimination for AKI was observed within 2-4 hours after an insult (100, 101). In non CKD population, Schley *et al.* recently reported a high AUC-ROC of 0.85 in the non CKD group at 4 hours after surgery (100). However, in patients with CIN, the efficacy of NGAL in discriminating performance was conflicting. A recent meta-analysis of 10 studies and limited to NGAL discriminating for CIN showed variable AUROC (83). In this meta-analysis, 4 studies looked at patients with CKD (83).

Patients undergoing contrast administration are characterised by various heterogeneous insults to the kidney (160) compared to cardiac surgery and additionally the underlying comorbidities influence NGAL productions including baseline kidney function.The low diagnostic accuracy for NGAL in CIN could be due to the presence of underlying heterogeneity in kidney function at baseline in these patients and the influence of varying comorbidities (158).

In our study serum IL18 together with TNFα demonstrated a prognostic significance with CIN+ mortality despite the poor the discrimination performance for CIN.

In a recent meta-analysis correlating urine IL18 with AKI, the discrimination performance of IL18 was modest with an AUROC of 0.66 in adults (161) and was lower compared to other serum biomarkers. Increased IL18 levels are shown with acute tubular necrosis, urine tract infections, pre-renal failure and therefore may not purely reflect an injury to the kidney (161). Therefore our study was limited to serum IL18.

Similar to previous studies (123, 162), our study showed increased levels of serum IL18 in the non-surviving group of our study cohort. In previous study, an increase from 25-500 pg/ml in urine IL18 at baseline predicted an up to 5-fold mortality risk (123). Increased levels of serum IL18 are linked with dysfunction of cardiac myocytes, vascular injury and apoptosis (163).

Our study found no association between HIV status and CIN or HIV positivity with levels of novel biomarkers. In previous studies, Landro *et al.*(129) reported decreased levels of plasma NGAL in HIV infected patients compared to controls. However after initiation of antiretroviral therapy, levels of NGAL increased. The insignificant difference in levels of biomarkers is a reflection of good immune competence of patients in this cohort and the increased accessibility to anti-retroviral therapy.

The strength of this investigation was that it was a prospective case-controlled study and evaluated and compared several biomarkers in discriminating CIN and patient outcomes. Our study population was characterised as heterogeneous unlike previous studies. Despite this, our study was a single centre conducted at a tertiary hospital and the study analysed only serum measurements.

4.5 Conclusions

Compared to serum creatinine novel biomarkers have better diagnostic discrimination for CIN and prognosticating patient outcomes in patients with heterogeneous insults to the kidney.

CHAPTER 5

5.0 Influence of Genetic Polymorphisms of TNFα and IL10 on Contrast Induced Nephropathy and Outcomes in Black South Africans

Abstract

Despite positive associations of genetic polymorphisms in TNFα and IL10 with contrast induced nephropathy (CIN) among Chinese populations, its role in other populations remains unexplored.

Methods

In this prospective observational study, 208 black Africans patients undergoing contrast administration were genotyped for TNFα and IL10 genes cytokine polymorphisms. Genomic DNA was extracted from peripheral blood samples using the Maxwell DNA purification kit (Promega AS1010, USA). The TNFα genotypes -308 (rs1800629) and -857(rs1799724), and IL10 genotypes -592(rs1800872), -819(rs1800871), -1082 (rs1800896) and +1582(rs1554286) were determined by restriction fragment length polymorphism (RFLP). The study outcomes measures were genetic susceptibility to development of CIN (defined as serum creatinine > 25% from baseline) and mortality risk in patients with CIN.

Results

This study showed significant increase in the frequency of the TNFα -308 AA genotype in CIN patients compared to controls (13.3% vs.1.82%, p=0.016), In addition, the presence of the TNFα-308 AA (high producer) vs. GG+GA genotypes was associated with a 9-fold CIN risk (9.24, 95% CI, 1.88-45, p=0.006). The IL10-1082 AA genotype (low producer) was significantly high in the non-surviving CIN+ patients compared to controls (p=0.011) and also showed a significant trend in the non-surviving patients without CIN (p=0.05).

Conclusion

The presence of the TNFα-308 AA genotype is a predisposing factor for CIN development while the IL10-1082 AA (the low producer) genotype decreases survival in CIN patients.

5.1 Introduction

In recent studies, inflammatory cytokines including tumor necrosis factor alpha (TNFα) and interleukin (IL10) are implicated in the pathogenesis of ischaemic acute kidney injury (AKI) including contrast induced nephropathy (CIN) (14, 67-69). Treatment of CIN remains supportive (9) and knowledge of existing risk factors and methods for CIN identification are suboptimal in diagnosis, determining CIN risk and patient outcomes. Therefore, inflammatory cytokine genetic susceptibility to CIN is an important marker in CIN identification and in guiding directed therapy.

Genetic polymorphisms in TNFα and IL10 influence upregulation of inflammatory cytokines and consequently increase susceptibility to inflammatory diseases (75) including CIN (14) and result in poor clinical patient outcomes (76). Among the Han Chinese populations with CIN, Chang *et al*. found a 2-fold increased frequency in the A-allele (rs1800629) compared to controls (OR 2.01, 95% 1.13-3.35, p=0.02) (14). The TNFα gene has several single nucleotide polymorphism (SNP) within its promoter region, however TNFα-308 remains the mostly associated with disease (75). The presence of the lesser adenine (A-allele) at position -308 of the TNFα gene is positively associated with a 6-fold increased transcription rate (75). IL10 a 35 kDa anti-inflammatory cytokine localised to chromosome 10 has several SNPs in its promoter region including the IL10 -1082 genotype (14) and almost 75% secretion of the IL10 cytokine is genetically determined (76). Recently, Chang *et al.* reported positive association between the IL10-1082 G-allele and CIN risk (14). There are limited studies and only three meta-analyses (164-166) that have explored susceptibility genes (including inflammatory cytokines genes) linked with AKI and these studies demonstrated conflicting results.

Only one study examined the association of genetic polymorphisms in inflammatory cytokine genes with CIN and reported positive association (14) thereby providing potential targets for future anti-inflammatory therapies.

TNFα is an inflammatory cytokine that accelerated acute kidney injury while IL10 accelerates recovery (14). Identification of protective genetic polymorphisms in these inflammatory cytokines will help in the implementation of targeted modalities that will lessen the impact of CIN. Our study investigated the impact of TNFα and IL10 SNPs on CIN and patient outcomes in black Africans.

5.2 Materials and methods

Study methods are described in chapter 2.This prospective observational study included 208 black South Africans undergoing contrast media administration at Charlotte Maxeke Johannesburg Academic Hospital and was approved by Human Research Ethics Committee of the University of the Witwatersrand. All study participants gave a written informed consent.

Statistical analysis

Categorical data are presented as frequencies and percentages. A Fisher's exact test was employed to analyze differences between genotype frequencies among the CIN group and controls and also among the surviving and non- surviving group. Genotype susceptibility to CIN was determined by calculating odd ratios (ORs) and 95% CI with p values <0.05 regarded as significant.

5.3 Results

In this prospective cohort study, 208 black Africans South Africans were genotyped for TNFα and IL10 genes. Among these genotyped participants, 32 (15%) developed CIN. Controls were those not meeting CIN diagnostic criteria. The overall mortality in this study cohort was 21 with 12 occurring in the CIN+ black South Africans.

5.1 Associations of TNFα and IL10 polymorphism with CIN

The comparison of the frequency allele distribution between the CIN group and controls is described in Table 5.1. TNFα -308 AA (high producer) was significantly higher in the CIN group compared to controls (p=0.016) and was associated with a 9-fold CIN risk. The other TNFα and IL10 genotypes showed non-significant frequency differences between the CIN group and CIN negatives.

5.2 Associations of TNFα and IL10 polymorphisms with overall mortality

Table 5.2 describes an association of the genotypes with cause mortality. The IL10-1082 low producer (AA-allele) showed a trend for significance between the surviving groups and non-surviving group (p=0.05). When mortality was restricted to the patients who developed CIN, there was increased frequency of the IL10-1082 AA-allele in the CIN+ patients who died vs. surviving CIN+ patients (p=0.011). The other genotypes showed a non-significant association with overall mortality as well as mortality restricted to the CIN+ groups only.

Table5.1 Associations of TNFα and IL10 polymorphism with CIN

SNP ID	Genotype	CIN+ (n=32)	CIN- (n=176)	p value	OR 95% CI	p value
TNFα 308 Low producer	GG	15 (50%)	104(63.0%)	0.02	1(Ref)	
High producer	GA	11 (36.7%)	58 (35.2%)		1.31(0.56-3.05)	0.52
High producer	AA	4 (13.3%)	3 (1.8%)		9.24(1.88-45.4)	0.006*
TNFα-857	CC	5 (18.5%)	38 (24.7%)	0.77	1 (Ref)	
	CT	20 (74.1%)	106 (68.8%)		1.43(0.50-4.08)	0.50
	TT	2 (7.4%)	10 (6.5%)		1.52 (0.26-9.02)	0.65
IL10-592	CC	14 (46.7%)	76 (43.9%)	0.32	1 (Ref)	
	CA	13 (43.3%)	89 (51.5%)		0.79 (0.35-1.79)	0.57
	AA	3 (10.3%)	8 (4.6%)		2.03(0.48-8.63)	0.35
IL10-819	TT	3 (9.4%)	11 (6.3%)	0.75	1 (Ref)	
	TC	15 (46.9%)	88 (50.0%)			
	CC	14 (43.7%)	77 (43.8%)		0.67(0.16-2.69)	0.57
IL10-1082 Low producer	AA	3 (9.1%)	10 (5.6%)	0.60	1 (Ref)	
Intermediate	AG	25 (78.1%)	143 (82.7%)		0.58 (0.14-2.25)	0.43
High producer	GG	4 (12.1%)	21 (11.7%)		0.63(0.11-3.39)	0.59
IL10+1582	TT	6 (19.4%)	22 (14.4%)	0.77	0.72 (0.26-1.97)	0.52
	TC	23 (74.2%)	117 (76.8%)			
	CC	2 (6.5%)	14 (9.2%)		0.52(0.09-2.96)	0.46

*OR for TNFα -308 (rs1800896) AA vs. AG+GG

Table5.2 Associations of TNFα and IL10 polymorphisms with overall mortality

SNP ID	Genotype	Mortality + (n=21)	Mortality – (n=187)	p value	OR 95% CI	p value
TNFα 308	GG	12 (70.6%)	102 (60%)	0.79	Ref (1)	
Low producer						
High producer	GA	5 (29.4%)	61 (35.9%)		0.52 (0.23-2.07)	0.51
High producer	AA	0 (0.0%)	7 (4%)			
TNFα-857	CC	6 (35.3%)	35 (22.3%)	0.19	1 (Ref)	
	CT	9 (52.9%)	113 (71.9%)		0.46(0.15-1.39)	0.17
	TT	2 (11.8%)	9 (5.7%)		1.29 (0.22-7.5)	0.77
IL10-592	CC	11 (52.4%)	73 (41.9%)	0.29	1 (Ref)	
	CA	8 (38.0%)	92 (52.9%)		0.57(0.22-1.50)	0.26
	AA	2(9.5%)	9 (5.2%)		1.47(0.28-7.74)	0.65
IL10-819	TT	2 (9.5%)	12 (6.7%)	0.75	1 (Ref)	
	TC	9(42.9%)	92 (51.4%)		0.58(011-3.04)	0.52
	CC	10 (47.6%)	75 (41.9%)		0.80 (0.15-4.10)	0.79
IL10-1082	AA	4(18.2%)	9 (4.9%)	0.05	1(Ref)	
Low producer						
Intermediate	AG	14 (66.1%)	153 (83.6%)		0.22(0.14-2.25)	0.02*
High producer	GG	3 (13.6%)	21 (11.5%)		0.32(0.05-1.73)	0.19
IL10+1582	TT	2 (12.5%)	25 (15.5%)	0.77	1 (ref)	
	TC	13 (81.3%)	122 (75.8%)		1.33(0.28-6.27)	0.72
	CC	1(6.3%)	14 (8.7%)		0.89(0.07-10.7)	0.92

5.4 Discussion

This prospective observational study and the first in Africans confirmed the positive association of inflammatory TNFα and IL10 genes polymorphisms with CIN including CIN mortality. In our study, the presence of the TNFα high producer AA genotype was associated with an almost 10-fold CIN risk and presence of IL10 AA (low producer) genotype was significantly high in the non-surviving CIN+ patients.

In this study, the presence of TNFα-308 high producer allele increased the risk for developing CIN. Our finding is similar to a previous study conducted among the Han Chinese population (14). In this study Chang *et al.* demonstrated positive association between high producer TNFα 308 alleles and development of CIN. In patients with autoimmune disease, Ivanova *et al*. also reported a 3.4-fold increase risk for systemic lupus erythromatosis in patients carrying the TNFα -308 A-alleles(167). In a recent study in the USA, Susantitaphong *et al.* observed increased frequency of the TNFα-308 AA-alleles in patients with high peak serum creatinine and high levels of novel biomarkers (168).

Inflammatory cytokines including TNFα play significant roles in the pathogenesis of CIN as well as in other forms of AKI (14, 63) and genetics determines individual variation in secretion of TNFα and other cytokines (167). Jaber *et al* observed increased levels of TNFα and IL10 cytokines in patients carrying the TNFα -308 and IL10-1082 high producer alleles (76). TNFα, mapped to chromosome 6, is upregulated during renal injury (14, 63) and performs the following functions; recruitment of inflammatory cells and other inflammatory cytokines; increases upregulation of various adhesion molecules (ICAM, VCAM) (72). Upon activation, TNFα binds to its receptors (TNFR1 and TNFR2) and this leads to activation of the TNF receptor associated factor-2 and NF-kβ, ultimately leading to inflammation and cell death (72).

Few studies have investigated genetic susceptibility of inflammatory cytokines with AKI, and including CIN (164). Additionally these limited studies (including the only three meta-analyses) have demonstrated conflicting results (164, 166, 169).

In a meta-analysis that included 11 studies, the presence of the high producer TNFα was associated with a decrease in patient survival (169). However, only one study in these meta-analyses was limited to inflammatory cytokine genes. Other observational studies showed non-significant association between the TNFα-308 and other genotypes with AKI or patient outcomes (164).

In a recent prospective and observational study of patients hospitalised to an intensive care unit (ICU) in Spain, Cardinal-Fernandez *et al.* reported non-significant differences in the frequency of the TNFα -308 A-allele between the AKI group and controls (169).

Our study showed increased frequency in the IL10-1082 low producer allele in the CIN+ non-surviving patients. Additionally, there was a trend for increased frequency in the IL10-1082 low producer genotypein patients without CIN who died. In a prospective nested control ICU based study in Brazil, the IL10 -1082 low producer allele in combination with TNFα positively predicted mortality (170). In this study, the IL10 -1082 genotype showed a non-significant frequency increase between the AKI and controls groups (170), a similar finding in our study. However, Jaber *et al.* reported decreased AKI mortality in patients with IL10 intermediate or high producer genotypes (76).

There are several reasons for increased CIN mortality in patients with the IL10-1082 low producer allele. The IL10 gene mapped to chromosome 1, is an important inflammatory cytokine that inhibits transcription of pro-inflammatory cytokines (TNF, IL-1 and IL6) and also down regulated production of nitric oxide (171)(78). In animal studies, IL10 is also important in induction of heme oxygenase, an enzyme that metabolizes heme to carbon monoxide, biliverdin and iron. Heme oxide functions as an oxidative stress responding molecule in various inflammatory states (171). The metabolites of heme functions as anti-oxidants and also inhibits inflammatory cytokines (171).

Similar to other previous findings in AKI (172), the IL10 gene polymorphisms were not associated with CIN in our study. In a paediatric cohort study, Treszl *et al*. observed insignificant differences in the frequency of the IL10 genotypes (including TNFα -308) between the AKI groups and controls (172). However few studies have explored the association of IL10 gene polymorphisms with AKI

The strength of this study is that it was a prospective observational study and the first to explore association of cytokine genetic susceptibility with CIN.

Our study also confirms Chang *et al.* findings which demonstrated a positive association of these genes in the Han Chinese population. However due to conflicting results on the association of ischaemic AKI and cytokine genes, there is need to replicate the studies in large populations and other races.

5.5 Conclusion

The TNFα 308 AA (high producer) genotypeincreases susceptibility to development of CIN while the IL10 -1082 AA (low producer) decreases survival in CIN patients.

CHAPTER 6

6. DISCUSSION

The hypothesis tested in this study were three-fold; that the rates of CIN were higher among hospitalised South Africans compared to developed countries, that the cytokine gene polymorphisms (TNFα and IL10 genes) were positively linked with CIN development and patient outcomes and lastly that novel biomarkers were accurate in the early prediction of CIN and prognosticating.

6.1 Summary of study findings

This study revealed the following;

1.6.1 There is an increased rate of CIN in hospitalised South Africans compared to developed countries with anaemia acting as an independent risk factor.

1.6.2 The novel biomarker cystatin C showed the best discrimination for early diagnosis of CIN at 24 hours and together with together with β2M at 48 hours after contrast media administration. Baseline NGAL (pre-contrast) demonstrated early accuracy for excluding patients at risk of developing CIN.

1.6.3 The novel biomarkers at baseline (IL18, β2M and TNFα) demonstrated best prognostic significance for CIN mortality while IL18 and TNFα at baseline showed prognostic significance for overall mortality.

1.6.4 Genetic polymorphisms in the cytokine genes (TNFα and IL10) showed positive association with CIN development including CIN mortality. The TNFα -308 AA-allele (high producer) showed increased frequency in the CIN group compared to controls and was associated with an almost 10-fold CIN risk. The IL10-1082 (low producer) showed increased frequency with CIN mortality risk. Table 6.1 summarizes the study findings

Table 6.1 A summary of the study findings

	Objective	**Chapter**	**Findings**
1	Determination of rates of CIN	3	The rates of CIN were 16.4% and relatively high compared to developed countries. CIN was defined using the ESUR criteria: an increase in serum creatinine > of 25% or >44 µmol/l from baseline. The >25% relative increase criterion is also supported by the Contrast Induced Nephropathy Consensus Working Group.
2	Risk factors for CIN	3	In an adjusted model, presence of anaemia was associated with a 2-fold odds for developing CIN (p=0.04). Levels of albumin were significantly lower in the CIN group (p=0.01) in our study, and showed a significant trend for CIN risk in adjusted model (p=0.06)
3	Outcomes of CIN	3	The rates of in-hospital mortality was significantly high in CIN group, 22.4% vs.6.8%, p<0.001
4	Predictors of mortality		CIN together with anaemia were associated with 2-fold (p=0.01) and 3-fold (p=0.03) mortality risks. Of the 13 deaths in the CIN group, 7 had malignancy. After adjusting for

			comorbidities, CIN remained an independent predictors
5	Accuracy of biomarkers in predicting CIN	4	Cystatin C at 24 hours and B2M at 48 hours showed best discrimination for CIN with AUROC of 0.75 ($p<0.001$) and 0.78 ($p<0.01$) respectively. Baseline NGAL demonstrated accuracy in excluding patients at risk of developing CIN
6	Accuracy of biomarkers in prognosticating CIN patient outcomes	4	Baseline IL18, β2M and TNFα showed best discrimination for CIN+ mortality. The AUROC were 0.83 ($p<0.001$), 0.82($p<0.04$) and 0.94 ($p<0.001$) respectively. In the controls, IL18 and TNFα showed best discrimination of mortality with AUROC of 0.91 and 0.84.

7	Influence of genetic (TNFα and IL10 genes) susceptibility to CIN	5	The CIN group showed a significant increased frequency in the TNF-308 A-allele (high producer) compared to G-allele (p=0.016). Presence of the A-allele was associated with a 10-fold CIN risk (p=0.006)
8	Influence of genetic (TNFα and IL10 genes) susceptibility to CIN mortality	5	The IL10-1082 AA genotype showed a significant frequent increase in the non-surviving participants in the study cohort (p=0.046). When restricted to CIN+ group, the IL10 low producer was higher in the non-surviving group (p=0.011)

6.2 Rates and risk factors for CIN

In South Africa including Sub-Saharan Africa, this is the first prospective study that has examined the risk and outcomes of CIN among hospitalised patients. The rate of CIN in our study was 16.4% (defined according to the updated guidelines of European Society of urogenital Radiation (ESUR) (50, 53).

The rate of CIN in this study was relatively high compared to previous findings in developed countries. Sherma *et al*. in Israel (1), Chang *et al*. in Taiwan (14) and Mitchel *et al.* in USA (132) reported 4.5%, 10% and 14% rates of CIN respectively.

In a recent observational study and randomized controlled trial, Shams-Eddin Taher *et al.*(33) and Narula *et al*. (35) reported rates of CIN of 11.5% and 16.1% rates of CIN respectively. The relatively high rates of CIN in this study could be explained by two reasons; the non-standardised prescription practices by clinicians, including pre-hydration, and the heterogeneous comorbidities of our study population at the time of contrast administration.

The presence of anaemia at baseline was associated with almost 2-fold and 3-fold increased rates of CIN and mortality risks respectively. In previous studies, anaemia was positively associated with CIN development (53); however; there are limited studies on the association of anaemia with CIN mortality. In an observational study, Lin *et al*. (29) observed a 2-fold CIN risk in patients with underlying anaemia while McKechnie *et al.* reported positive association of baseline anaemia with mortality (142). There are several reasons why baseline anaemia is associated with CIN risk including mortality. Firstly only 10% of renal blood flow is directed to the renal medulla despite the increased metabolic activities occurring in the medulla (65).

In the presence of contrast media, significant vasoconstriction (mediated by adenosine and endothelins) occurs in the medulla that further reduces the oxygen partial pressures to as low as 10 mmHg (25). Presence of anaemia is a reflection of severity of the comorbidities (142) at baseline and therefore a traditional biomarker signifying CIN prognosis.

We observed significantly low serum albumin levels in the CIN cohort compared to controls and the decreased albumin also showed a significant trend for almost 2-fold CIN risk. Serum albumin is an important anti-oxidant that decreases formation of ROS through its binding of non-ceruloplasmin copper and iron (133). Endothelial dysfunction and inflammation leads to production of reactive oxygen species ROS) implicated in CIN (13).

Additionally, albumin is a biomarker reflecting underlying inflammation or under nutrition in patients.

6.2.1 Mortality and CIN

We observed significantly high mortality in the CIN group compared to controls (22.4% vs. 6.8%, $p<0.001$). After adjusting for underlying confounders including patient comorbidies, (malignancy and cardiac disease), CIN predicted 2 fold mortality risk. Our findings are similar to previous observational studies in developed countries that reported increased mortalities in patients with CIN(34). Rihal *et al*.(43) and Sadeghi *et al*.(42) found mortality of 22.0% vs.1.4% and 16.2% vs. 1.2% mortalities in the CIN group compared to controls respectively.

6.3 Accuracy of biomarkers in the diagnosis of CIN and prognostication

The novel biomarkers demonstrated diagnostic accuracy (Cystatin C at 24 hours and β2Mat 48 hours) for CIN and CIN prognosis (baseline β2M, Interleukin18 and TNFα). Additionally, the novel biomarkers showed accuracy in excluding patients at risk of developing CIN (baseline NGAL). To our knowledge, this is the first prospective case-controlled study to compare a panel of novel biomarker (filtration, structural and inflammatory) on the development of CIN and patient outcomes.

Similar to previous findings, Cystatin C at 24 hours demonstrated best accuracy for CIN diagnosis compared to serum creatinine (100, 151). In patients undergoing cardiopulmonary bypass surgery, Schley *et al.* showed that the best cystatin discrimination for acute kidney injury (AKI) was at 24 hours after an insult. In this study, the area under receiver operating curves (AUROC) at 24 hours after surgery was superior compared to the AUROC at baseline, 2 hours or 4 hours (100). A similar finding was observed by Briguori *et al* who reported best cystatin C AUROC of 0.92 at 24 hours in patients undergoing contrast media administration (151).

Consistent with two recent finding, NGAL demonstrated best accuracy for excluding patients at higher risk for developing CIN. In these studies conducted in Italy and Iran, Quintaville et al.(158) and Khatami *et al.*(159) reported 20% vs. 93% and 9.4% vs. 97.1% positive predictive value and negative predictive values respectively.

Despite limited studies associating β2M and CIN, β2M showed best diagnosis accuracy for CIN and CIN mortality in our study. In few studies limited to AKI, β2M showed best discrimination of AKI with an AUROC of 0.80 (154).In a study limited to CKD patients, increased levels of β2M were positively associated with mortality (155, 156), a similar finding to our study.

Damaging renal injury biomarkers (IL18 and TNFα) at baseline showed prognostic significance for CIN mortality. In previous studies, Parikh *et al.*reported a5-fold mortality risk in patients with increased IL18 levels (123). Interleukin l8 and TNFα are pro inflammatory cytokines secreted by both injured renal tissue and infiltrating inflammatory cells (67, 68) and therefore, the positive association of damaging biomarkers with mortality probably reflects severity of the underlying inflammatory in patients at risk.

6.4 The influence of TNFα and IL10 cytokine gene polymorphisms with CIN and patient outcomes

This is the first study in black South Africans and all black populations to demonstrate cytokine gene polymorphisms and susceptibility to CIN and mortality. We observed increased frequency of the TNFα-308 high producer A-allele in the CIN group and this was associated with an almost 10-fold CIN risk. Our findings are similar to Chang *et al.* who found positive association of the TNF high producer alleles with CIN among the Han Chinese population (14). Similarly, Ivanova *et al.* found a positive association between the TNFα-308 A-allele with autoimmune diseases (167). Few studies have been conducted on the TNFα gene polymorphisms and IL10 genes polymorphisms and susceptibility to AKI. Only one study by Chang *et al.* has been conducted that explored genetic susceptibility to CIN in Asian populations.

We observed an increased frequency increase in the IL10 -1082 A-allele (low producer) in the non-surviving CIN+ patients compared to controls despite the low sample size in this nested study of black Africans. Our findings are similar to Jaber *et al.* who reported decreased AKI mortality in the IL10-1082 high producer compared to the low producer (A-allele) (76).

6.5 National and global importance of this study

This is the first study describing the impact and prognostic implications of CIN in hospitalised patients (including Sub-Saharan Africa) and the first to demonstrate the positive genetic susceptibility to CIN in blacks carrying polymorphisms in the TNFα and IL10 genes.

This study has highlighted the increased prevalence of contrast induced nephropathy and mortality. Despite being conducted at a tertiary hospital, this study calls for unified and standardised guidelines for prevention of contrast induced nephropathy. Additionally, extra caution should be taken in patients with underlying anaemia and low albumin who demonstrated an increased risk for contrast induced nephropathy. With the increasing rates of non-communicable diseases (such as diabetes and cardiac diseases) in Sub-Saharan Africa which are known risk factors, the rates of CIN will also increase.

Our study and the recent study in Asians (Chang *et al*) have shown the positive influence of inflammatory cytokine genes on CIN. In our study, the presence of high producer TNFα-308 increased susceptibility to CIN among black South Africans. Additionally non-surviving CIN+ patients showed increased frequency of the IL10-1082 low producer. TNFα is linked with positive acceleration of renal injury while IL10 increases repair. These findings highlight the need to replicate these studies in large cohorts and other races. Randomized controlled studies should address replacement of IL-10 or anti-TNFα (including other anti-inflammatory therapies) in genetically susceptible individuals.

The novel biomarkers showed early diagnosis accuracy and prognostic accuracy. Additionally, novel biomarkers also demonstrated accuracy for excluding patients at risk for CIN. This is important nationally and globally in early identification of patients at risk; however, few studies have explored these biomarkers in CIN.

Therefore optimal cut-off points remain variable in different studies including what is the best timing in patients with heterogeneous (CIN group) insults to the kidney.

6.6 Future research and recommendations

Future research should address the following;

1. Multi-institutional studies should be conducted on rates of CIN in South Africa and other Sub-Saharan countries. Workable and standardised recommendations on fluid pre-hydration should be implemented in patients undergoing contrast administration procedures especially in high risk groups.
2. Studies on genetic susceptibility of TNFα and IL10 genes including other genes inflammatory cytokines (IL18, FGF) should be conducted in other races. Additionally, these studies should also explore the impact of IL10 or ant-TNFα replacement therapy (including other anti-inflammatory therapies) in genetically susceptible individuals
3. Our study addressed a panel of biomarkers assessing the diagnostic accuracy and prognosis in CIN patients. Future studies should address the best optimal cut-off limits and timings for specimen collection in such CIN groups. NGAL showed accuracy in excluding patients at risk for CIN and therefore future studies should address the impact on decreased CIN risk by measuring NGAL at baseline in patients undergoing contrast administration.

6.7Study limitations

The positive aspect of this study is that it was a prospective observational study (with a nested case-controlled component) investigating novel biomarkers with a large sample size.

Despite this, there are limitations of study; it was a non- interventional study and conducted a single tertiary hospital. The study was also limited to few single nucleotide polymorphisms (SNPs) that were selected based on previous findings in which they had shown positive association with acute kidney injury in other populations. Despite blood and urine demonstrating similar findings for novel biomarkers, this study should have compared the blood and urine performance for biomarkers. However, timing, storage and contaminants in urine remain a challenge.

6.8 Conclusion

The objectives of this thesis were to determine the rates and risk factors of CIN in hospitalised South Africans, determine the influence of genetic susceptibility (TNFα and IL10 genes) in CIN patients and the accuracy of novel biomarkers in early CIN diagnosis and prognostication. This thesis has confirmed the relatively high rates of CIN and underlying risk factors and patient outcomes. It has also confirmed the positive genetic susceptibility to CIN in black South Africans. Lastly, biomarkers for early identification of CIN and prognostication have been identified including those for identifying individuals at risk for CIN.

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APPENDIX A

M140467

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140467

NAME: (Principal Investigator)	Dr Justor Banda
DEPARTMENT:	Internal Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Biomarkers in Acute Kidney Injury Due to Contrast Induced Nephropathy
DATE CONSIDERED:	25/04/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Saraledevi Naicker and Dr Raquel Duarte
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 26/05/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

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APPENDIX C

Participants information sheet/ informed consent forms (general and genetics)

Introduction

Good day, my name is Justor Banda, a medical doctor in the Division of Nephrology at CharlotteMaxekeJohannesburgAcademicHospital. I am doing a PhD research study at the University of the Witwatersrand. You are invited to consider participating in this research study. Your participation is voluntary and you can withdraw from the study without any prejudice and you will continue to receive standard of care treatment. It is important that you read and fully understand information on this leaflet as it will help you in making the decision. If unable to read or understand English, an interpreter will be provided for you.

Purpose of the study

I am conducting a research study to look at reasons why some people exposed to x-rays with substance suffer kidney injury and see how we can possibly identify higher risk individuals to prevent kidney injury. The human body has two kidneys that perform different functions; for example the removal of waste products and forming urine. In order to determine why some people suffer kidney injury following exposure to contrast and others do not, I will look for inheritable factors called genes that identify those with increased risk to kidney injury after x-ray procedures.

Genes are basically a collection of information or instructions that form the makeup of a human being and decide how he/she behaves.

I will look for the TNFα and IL10 genetic differences or changes which affect the kidney when exposed to contrast media. The TNFα is involved in inflammation while the IL10 is protective.

Individuals with some differences or mutations in their genes have increased risks of kidney injury compared to those without changes when exposed to x-rays with contrast. Mutations are permanent changes in the genes and affect how information is interpreted by the body. From this study, I will be able to find out if there are differences in the genes among those who develop kidney disease after x-rays studies and those who do not.

Procedures of the study

If you agree to participate in the study, your medical records will be reviewed. A medical doctor will examine you and collect information assessing risk factors associated with development of kidney disease from contrast media studies. Blood and urine samples will be collected and an examination of your heart will be done in order to determine renal and heart status. An initial total of 10mls (2 teaspoons of blood) will be drawn prior to x-ray exposure and half of this sample will be stored for determination of the inheritable factors (genes) predisposing to kidney injury following contrast administration (genetic analysis). Subsequent blood samples of 5mls (half spoon) will be drawn 4hours after the procedure and at days 1, 2, 5 and at 3 months in order to look at kidney recovery.

Benefits of the study

The benefits of participating in this study are that we will be able to identify earlier those at risk of developing kidney injury post exposure to contrast media administration. Participants in this study will be screened for kidney dysfunction and those with kidney diseases found during screening procedures will be referred to the Division of Nephrology for further management. The findings from the study will help in formulating intervention strategies for kidney disease following radio contrast administration.

Possible risks

Blood collection may cause pain at site of puncture. Air emboli and infection are very rare complications that can occur after vein puncture. Qualified personnel will collect the blood samples to prevent such complications.

Financial arrangements

You will not be paid for participating in the study. However, there will be no costs to youfor any related study visits and procedures, as any costs incurred will be compensated by research funds.

Confidentiality

All information obtained during the course of this study will be kept strictly confidential. Your records will be given unique identification numbers and the initial identification details won't be used. All physical records will be kept in a locked locker with access limited to the research team. Electronic data will be password protected.

Source of information

If you have any questions, concerns and clarifications, please contact the following; Dr Justor Banda will be reachable 24 hours every day on the number, 073-409-3680.

Additional information can be obtained from the chairperson of Witwatersrand University Human Research Ethics Committee, Professor Cleaton Jones on 011-717-2301

Informed Consent Form: General

I confirm that I have been informed about the study by Dr Justor Banda. I understand that my personal details will be kept strictly confidential and that I may at any stage withdraw my consent and participation in the study and continue to receive the appropriate treatment. I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

PARTICIPANT (printed name)……

Signature or thumb print …… **Date…….**

Witness (printed name)……

Signature……...Date…….

I, Dr Justor Banda confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

…….. Signature……Date……

Printed Name

Informed consent form: DNA

I hereby confirm that I have been informed about the study by Dr. Justor Banda about the nature, benefits and risks of the genes study. I understand that my personal details (any identifying data) will kept strictly confidential. I have had the opportunity to ask questions and I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

PARTICIPANT (printed name)……

Signature or thumb print …… **Date** …**……**

Witness (printed name)…….

Signature……...Date……

I, Dr Justor Banda confirm that the participant has been fully informed about the nature of the above study.

STUDY INVESTIGATOR

Name …….. Signature……Date…….