STUDY OF IRON PATHOPHYSIOLOGY FOR EARLY DIAGNOSIS OF ACUTE KIDNEY INJURY SECONDARY TO ISCHEMIA REPERFUSION INJURY FOLLOWING CARDIOPULMONARY BYPASS

By

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ABSTRACT

Currently, there are no successful therapies proven to ameliorate acute kidney injury (AKI). AKI secondary to ischemia reperfusion injury (IRI) leads to increased morbidity and mortality. The role of iron sequestration throughout cardiac surgery remains unclear, however it may be an important modifier of renal ischemia reperfusion injury. The primary goal was to characterize iron regulatory pathways in a prospective observational cohort of adult cardiac surgery patients in the context of clinical AKI predictors and in relation to clinical prediction alone. The secondary goal was to evaluate potential proteins that may act as non-invasive biomarkers for the early detection of AKI, including urine hepcidin-25, serum ferritin, serum transferrin saturation (TSAT) and urine lactotransferrin. We found that urine hepcidin-25 at postoperative day 1 and serum ferritin and TSAT at 1 hour into cardiopulmonary bypass were independent predictors of AKI avoidance on multivariate analysis, enhancing clinical prediction alone.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENT OF COPYRIGHT MATERIALS FOR WHICH PERMISSION WAS OBTAINED

Figure 1.1 Natural history of AKI. (Reproduced under the permission of American Society of Nephrology)

Figure 1.2 Proposed classification scheme, RIFLE criteria for acute kidney injury. (Open access article may be reproduced without formal permission)

Figure 1.3 Clinical and cellular phases of AKI in relation to temporal kidney function and glomerular filtration rate (GFR). (Reproduced from *Kidney International*, Vol 62, Issue 5, Sutton T, Fisher C and Molitoris B, Microvascular endothelial injury and dysfunction during ischemic acute renal failure, Pages No. 1539-1549, © 2002, with permission from Elsevier)

Figure 1.4 Tubular cell fate following renal injury. (Article may be reproduced without formal permission)

Table 1.1 The AKIN classification and staging system for AKI. (Reprinted from *Kidney International Supplements*, KDIGO, Chapter 2.1: Definition and classification of AKI, Pages No. 19-36, © 2012, with permission from Elsevier)

LIST OF ABBREVIATIONS

ABPP: Activity-based protein profiling

ACEI: Angiotensin-converting enzyme inhibitors

ADQI: Acute Dialysis Quality Initiative

AIC: Akaike information criterion

AKI: Acute kidney injury

AKIN: Acute Kidney Injury Network

ARB: Angiotensin receptor blockers

ATN: Acute tubular necrosis

ATP: Adenosine triphosphate

AUROC: Area under receiver operating characteristic

BSA: Bovine serum albumin

CABG: Coronary artery bypass grafting

CD: Cluster of differentiation

CKD: Chronic kidney disease

CPB: Cardiopulmonary bypass

Cr: Creatinine

CSA: Cardiac surgery associated

CV: Coefficient of variation

DAMPs: Damage-associated molecular patterns

DMSO: Dimethyl sulfoxide

DTT: Dithiothreitol

eGFR: Estimated glomerular filtration rate

ELISA: Enzyme-linked immunosorbent assay

EO: Ouabain

ESRD: End-stage renal disease

F-actin: Filamentous actin

FHB: Free hemoglobin

FP-TAMRA: Fluorophosphonate- tetramethyl-6-carboxyrhodamine

GFR: Glomerular filtration rate

HCl: Hydrochloric acid

HRP: Horseradish peroxidase

ICAM: Intercellular adhesion molecule

IDI: Integrated discrimination improvement

IL: Interleukin

INF: Interferon

IRI: Ischemic reperfusion injury

KDa: Kilodalton

KDIGO: Kidney Disease Improving Global Outcomes

KIM-1: Kidney injury marker-1

NAG: N-acetyl-β-d-glucosaminidase

NGAL: Neutrophil gelatinase-associated lipocalin

NRI: Net reclassification improvement

MOPS: 3-(N-morpholino) propanesulfonic acid

PBS: Phosphate-buffered saline

PDC: Potassium dichromate

POD: Post-operative day

RBC: Red blood cells

RIFLE: Risk, Injury, Failure, Loss and End-Stage Renal Disease

ROC: Receiver operating characteristic

ROS: Reactive oxygen species

RRT: Renal replacement therapy

SCr: Serum creatinine

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TGF: Transforming growth factor

Th: T-helper

TIBC: Total iron-binding capacity

TLR: Toll-like receptor

TMB: Tetramethylbenzidine

TNF: Tumor necrosis factor

TSAT: Transferrin saturation

UO: Urine output

Z-phe-arg-AMC: Z-phenylalanine-arginine-7-amido-4-methylcoumarin

1. INTRODUCTION

1.1.Chapter 1: Acute Kidney Injury (AKI)

Acute kidney injury (AKI) is an abrupt insult characterized by elevated serum creatinine from impaired kidney function and filtration (Kellum et al., 2015). Loss of kidney function impacts circulating fluid volume, electrolyte balance and metabolic homeostasis (Abdel-Kader & Palevsky, 2009), with the potential to lead to acute uremia, metabolic acidosis and hyperkalemia as well as progress to other chronic conditions such as chronic kidney disease (CKD) (Kellum et al., 2012). AKI remains a critical medical issue that is prevalent in patients of all ages at varying incidence rates across different countries (Susantitaphong et al., 2013).

The global incidence rates and burden of AKI are poorly understood and difficult to grasp due to underreporting and inconsistent clinical diagnostic criteria's (Cerdá et al., 2008). Reports of increasing frequency, which is likely due to improved awareness and reporting, raises concern as to the real magnitude of this problem (Cerdá et al., 2008). Epidemiological studies are generally performed on data gathered at healthcare facilities in developed nations. A large scale study conducted in the United States using data reported by Medicare beneficiaries showed an average increase of 11% per year in AKI incidence between 1992 and 2001 with an average of 23.8 AKI cases per 1000 hospital discharges (Xue, Daniels, & Star, 2006). A more recent systematic review in North America, Northern Europe and Eastern Asia looked at AKI incidence in large cohort studies in hospital settings from 2004 to 2012 (Susantitaphong et al., 2013). Using 312 studies, the pooled incidence rates of AKI were 21.6% in adults and 33.7% in children. Furthermore, pooled mortality rates associated with AKI were 23.9% in adults and 13.8% in children (Susantitaphong et al., 2013). Adults and elderly populations (Waikar, Curhan, Wald,

McCarthy, & Chertow, 2006) are predominantly diagnosed with AKI in the developed world; whereas AKI is common in younger groups, including children in developing countries (Kohli et al., 2007; Vachvanichsanong, Dissaneewate, Lim, & McNeil, 2006).

On a smaller scale, AKI incidence rates have been studied in community and hospital settings. Patients admitted to the hospital with pre-existing AKI are categorized under 'community acquired AKI' and patients that develop AKI during their hospital stay are categorized under 'hospital acquired AKI' (Wonnacott, Meran, Amphlett, Talabani, & Phillips, 2014). An epidemiological study looking at patients admitted to two district hospitals between July 2011 and January 2012 in the United Kingdom found that community acquired AKI was more severe relative to hospital acquired cases. Incidence of community acquired AKI during this time was 4.3%, which was double that of hospital acquired AKI with a 2.1% rate. However, community acquired AKI had better short-term and long-term outcomes (Wonnacott et al., 2014). A more recent study looking at community acquired AKI in Malawi, East Africa between April to July of 2015 documented an incidence rate of 17.2% of medical admissions (Evans et al., 2017). A recent study looking at hospital acquired AKI noted variation in incidence dependent on ward location, with rates of 0.54%, 0.72% and 2.2% in medical, surgical and intensive care unit wards, respectively (Singh et al., 2013).

Hospital acquired AKI commonly occurs in surgical, pediatric, oncology and intensive care unit wards, where patients can experience both short-term and long-term adverse effects. Short-term adverse effects of AKI generally refer to issues associated with acute deterioration in renal function, commonly acid-base and electrolyte disturbances, uremia and volume overload (Doyle

& Forni, 2016). AKI has prognostic implications which include prolonged hospital stay duration, requirement of acute dialysis and even mortality (James & Wald, 2014). Mortality rates vary dependent on the etiology of AKI, with in-hospital rates reaching as high as 62% in critically ill patients (Chang et al., 2014). Importantly, a single stage 1 episode of AKI is independently associated with an increase in 10-year mortality (Doyle & Forni, 2016; Linder et al., 2014). Indeed, mortality was associated with increasing severity of AKI, stage 1 OR 1.70; stage 2 OR 2.95; stage 3 OR 6.88 (Hoste et al., 2015). In terms of AKI following cardiac intervention, mortality rates average around 15% to 30% within a 30-day period, dependent on the AKI definition (Rosner, 2005).

Patients that survive AKI may experience progressive loss of kidney function and have a higher risk of developing chronic kidney disease with subsequent end-stage renal disease (ESRD) and even mortality (Bonventre & Yang, 2011; Ishani et al., 2009). A prospective observational study looking at AKI patients that required dialysis observed that 14 % had developed CKD in a 5-year follow-up (Schiffl & Fischer, 2008). AKI in relation to patient outcome is demonstrated in a kidney transplant study that observed that long-term risk for graft loss was significantly higher in patients that received kidneys from donors with AKI versus those without AKI, $p = 0.02$ (Kolonko et al., 2011). The outcomes of these patients that experience AKI are dependent on the degree of renal injury followed by full or partial recovery with the possibility of further deterioration over time.

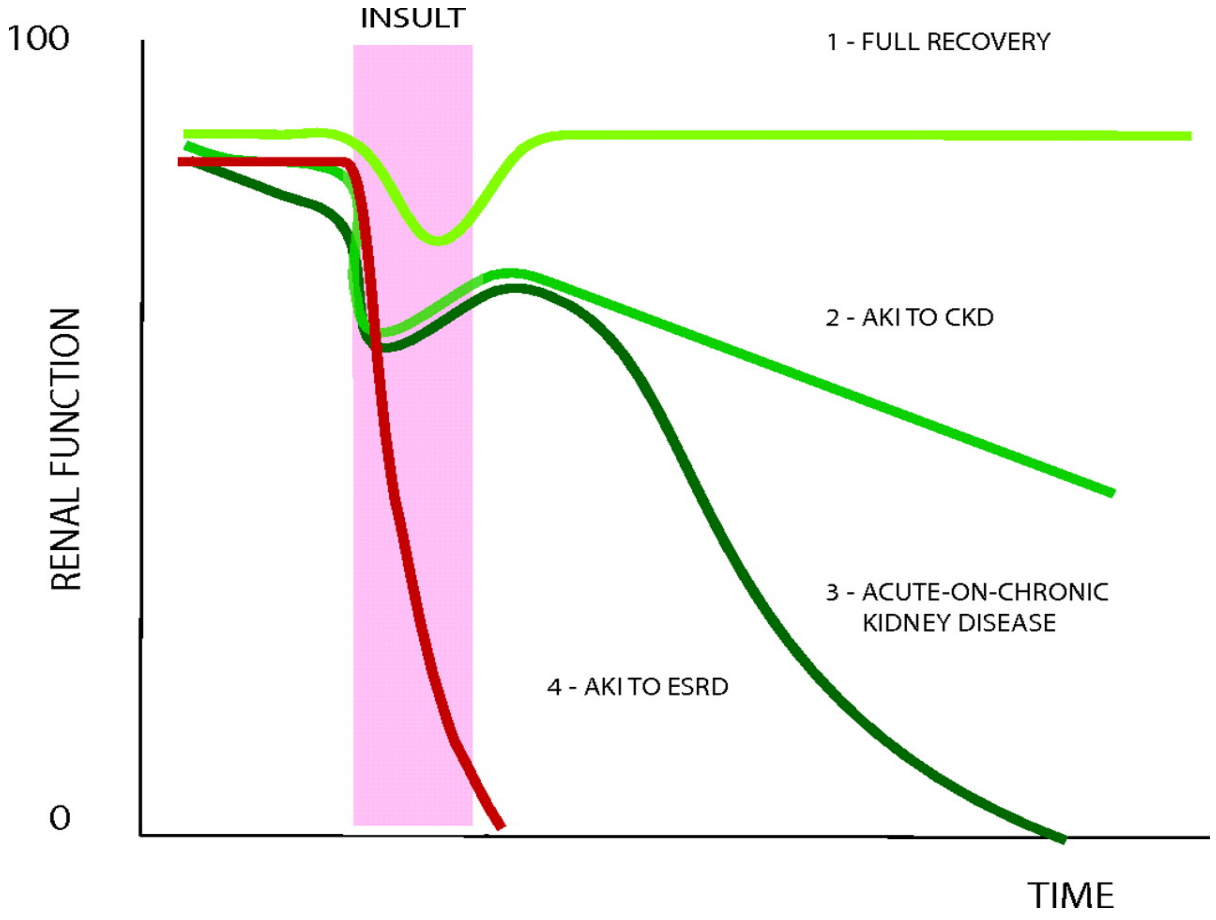

Figure 1.1 Natural history of AKI. Patients that survive AKI may experience a return to normal renal function, progress to chronic kidney disease (CKD), exacerbate progression of pre-existing CKD or evolve to end-stage renal disease (ESRD) with irreversible loss of kidney function. (Modified from Cerdá et al., 2008)

1.1.1. 2012 Kidney Disease: Improving Global Outcomes (KDIGO) Guidelines

The KDIGO clinical practice guidelines are an amalgamation of existing patient care guidelines into one comprehensive report to improve outcomes of common kidney conditions. These KDIGO guidelines were implemented globally in 2006 and the most recent version for AKI was updated in March 2012. Prior to this report, the diagnostic criteria of AKI and patient care were inconsistent among different healthcare facilities. Looking through the literature, over 35 different definitions have been used to describe AKI over the past few decades (Kellum, Levin,

Bouman, & Lameire, 2002). A standardized AKI diagnostic criteria is necessary to advance treatment procedures and medical research to ultimately improve patient survival and quality of life.

The KDIGO staging and definition of AKI is based on a combination of the Risk, Injury, Failure, Loss and End-Stage Renal Disease (ESRD) (RIFLE) criteria and the Acute Kidney Injury Network (AKIN) criteria. The RIFLE criteria was established by the Acute Dialysis Quality Initiative (ADQI) group in 2004 (Bellomo, Ronco, Kellum, Mehta, & Palevsky, 2004) to diagnose and classify AKI (Kellum et al., 2012). This criteria is subdivided into two groups: severity of AKI (risk, injury and failure) and outcome of AKI (loss and ESRD). AKI is defined using a combination of serum creatinine and glomerular filtration rate (GFR) in conjunction with urine output. However, only serum creatinine is used to diagnose clinical AKI because GFR is only validated in steady-state conditions, such as chronic kidney disease (Chiou & Hsu, 1975). Serum creatinine levels are impacted by many factors including patient age, gender, muscle composition and overall diet, but should remain in a homeostatic range (Bagshaw, George, & Bellomo, 2008).

Urine output is an important indicator of kidney function and tubular injury. However, changes to urine output may not be indicative of only AKI. Pre-renal azotemia can also cause a decline in urine output in patients without AKI (Abuelo, 2007). Pre-renal azotemia is a condition where excess nitrogen compounds accumulate in circulation due to reduced blood flow to the kidneys and is fluid responsive (R.M et al.,1976).

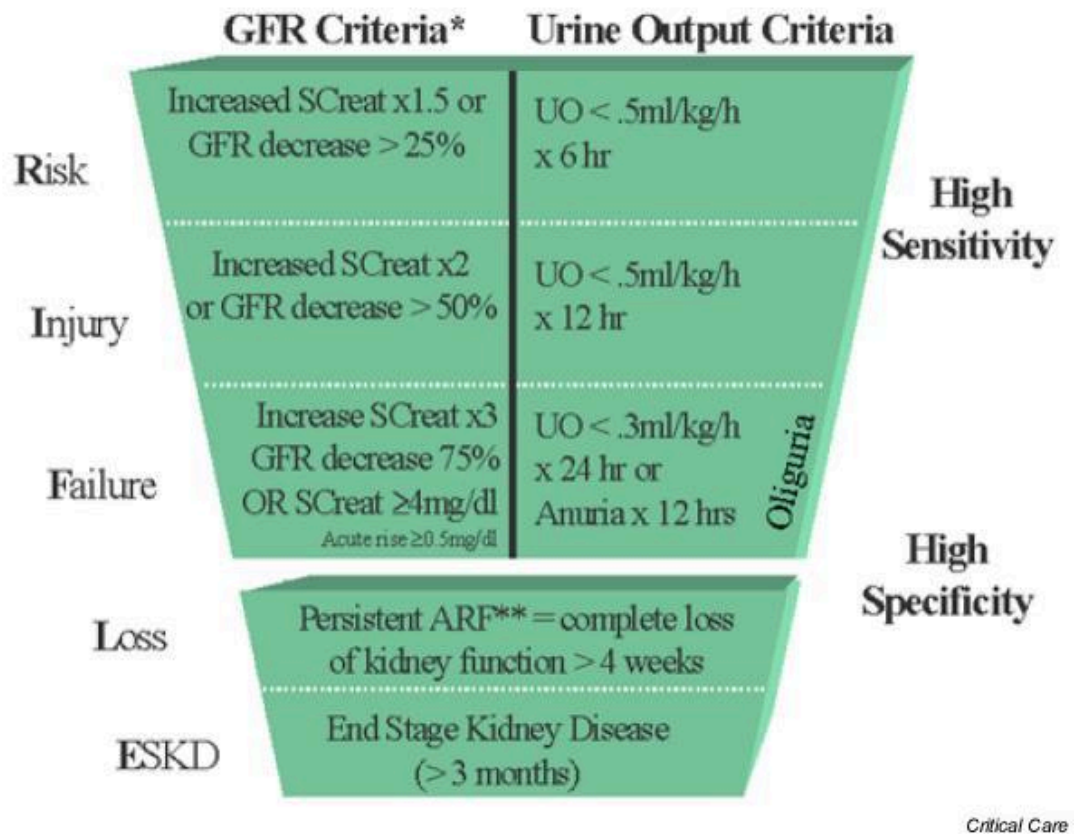

Figure 1.2 Proposed classification scheme, RIFLE criteria for acute kidney injury. A patient can fulfill the criteria through changes in serum creatinine or urine output or both. The Acute Dialysis Quality Initiative Group defines AKI severity (Risk, Injury, Failure) based on serum creatinine, GFR and urine output. AKI outcomes (Loss and End-stage kidney disease) are determined with continued loss of kidney function. ARF, acute renal failure; GFR, glomerular filtration rate; Screat, serum creatinine; UO, urine output. (Modified from Bellomo et al., 2004)

The RIFLE criteria was modified in 2005 by an international network of AKI researchers to develop the AKIN criteria (Mehta et al., 2007). The AKIN criteria focuses on serum creatinine and urine output. The severity of AKI was divided into three stages based on amount of serum creatinine increase and/or amount of urine output decrease. AKIN classification is particularly useful when the baseline serum creatinine (SCr) is unavailable, since SCr is monitored within a 48-hour period (eg: abrupt increase in SCr within 48 h).

Table 1.1 The AKIN classification and staging system for AKI. Serum creatinine increases and urine output decreases according to severity of AKI. (Modified from Kidney International Supplements, Section 2: AKI Definition)

Stage	Serum creatinine	Urine output
1	1.5–1.9 times baseline OR ⩾0.3 mg/dl (⩾26.5 μmol/l) increase	<0.5 ml/kg/h for 6–12 hours
2	2.0–2.9 times baseline	<0.5 ml/kg/h for ⩾12 hours
3	3.0 times baseline OR Increase in serum creatinine to ⩾4.0 mg/dl (⩾353.6 μmol/l) OR Initiation of renal replacement therapy OR, In patients <18 years, decrease in eGFR to <35 ml/min per 1.73 m^2	<0.3 ml/kg/h for ⩾24 hours OR Anuria for ⩾12 hours

1.1.2. Clinical AKI Etiology

The underlying causes of AKI are important to identify in order to improve patient treatment and recovery (Yang, Zhang, Wu, Zou, & Du, 2014). Clinical AKI etiology is divided into three main categories: pre-renal, intrinsic and post-renal dependent on the location of insult.

Pre-renal AKI is generally attributed to a decrease in intravascular volume and arterial pressure, which impairs perfusion to the kidneys (F. Yang et al., 2014; Rahman, Shad, & Smith. 2012). Cardiac surgery patients are commonly on medications that predispose them to AKI by potentially altering renal perfusion and autoregulation (maintenance of renal arterial pressure below 70mmHg) (Harty, 2014). For instance, angiotensin-converting enzyme inhibitors (ACEI) are anti-hypertensive agents that reduce the activity of the renin-angiotensin-aldosterone system

to promote secretion of sodium and water which can directly increase the effective circulating volume (Izzo & Weir, 2011). Furthermore, angiotensin receptor blockers (ARB) prevent angiotensin II from binding their corresponding receptor by competitive antagonism, which leads to efferent arteriole renovasodilation and alteration of renal perfusion (Barreras & Gurk-Turner, 2003). The most common medication used by cardiac surgery patients is aspirin, a non-steroidal anti-inflammatory drug (NSAID) that is involved in prostaglandin, cyclooxygenase-1 and cyclooxygenase-2 inhibition and subsequent afferent arteriole renovasoconstriction (Hörl, 2010). ACEIs, ARBs and NSAIDs are potential nephrotoxic agents that may indirectly cause AKI, thus cessation is appropriate in patients with deteriorating kidney function to correct renal perfusion and autoregulation (Harty, 2014). Correcting the underlying cause of AKI or discontinuing the problematic medication can often reverse pre-renal issues (R.M. et al., 1976; Rahman, Shad, 2012).

Intrinsic AKI occurs with injury to the vascular, tubular, glomerular or interstitial regions of the kidneys. A common presentation in hospitalized patients is acute tubular necrosis (ATN) (Rahman et al., 2012). Tubular damage generally occurs from decreased renal perfusion (eg: ischemia) or from endogenous (eg: hemoglobin hemolysis) and exogenous (eg: angiograph radio-contrast dye) nephrotoxic agents (Rahman, Shad, 2012). AKI secondary to ischemia reperfusion injury following cardiopulmonary bypass (CPB) surgery falls into this category. Patients that undergo cardiac surgery experience ischemic and hypoxic tubular insult beginning with extracorporeal circulation that continues on throughout cardiac surgery (D. Basile, Anderson, & Sutton, 2012; Bonventre & Yang, 2011). Reperfusion further contributes to injury

and amplifies tubular inflammation that continues into the postoperative period (Bonventre & Yang, 2011).

Post-renal AKI is associated with obstruction along the urinary tract network that may lead to waste buildup and hydronephrosis resulting in anuria or oliguria. Renal function generally improves if the obstruction is promptly relieved. (Rahman, Shad, 2012). Benign prostatic hyperplasia, bilateral ureteral stones, prostate cancer in men and gynecologic cancers in females have been associated with post-renal AKI (D. Basile et al., 2012; Choudhury & Ahmed, 2006).

1.2. Chapter 2: Cardiac Surgery-Associated Acute Kidney Injury (CSA-AKI)

Acute kidney injury secondary to ischemia reperfusion injury following cardiac surgery is a critical complication that leads to increased hospital stay, facility costs, morbidity and mortality (Chertow, Burdick, Honour, Bonventre, & Bates, 2005; Yamout, Levin, Rosa, Myrie, & Westergaard, 2015). On average, 31% of patients undergoing cardiac surgery are diagnosed with AKI, among which 2% of patients require dialysis (Chertow, Levy, Hammermeister, Grover, & Daley, 1998; Gailiunas et al., 1980; R.M. et al., 1976). A large prospective cohort study showed that severe AKI requiring dialysis developed in 1% to 5% of patients that underwent coronary artery bypass graft (CABG) or valve surgery between 1987 to 1994 (Chertow et al., 1997). Milder AKI, recognized as a serum creatinine >25% from baseline occurs in 17% of cardiac surgery patients (Mangano et al., 1998).

Regardless of whether patients develop mild or more severe forms of AKI following cardiac surgery, the potential short-term and long-term adverse effects are devastating. Severe cases of AKI following cardiac surgery are seen to produce 30-day mortality rates as high as 58.6% and 63% (Ostermann, Chang, & Riyadh, 2008; Chertow et al., 1997). Less severe cases are associated with increased short-term mortality by 19-fold (Mangano et al., 1998) and demonstrate a doubling in mortality up to 10 years post-cardiac surgery, regardless of whether renal function recovers to baseline (Lassnigg et al., 2004; Loef et al., 2005). Overall, cardiac surgery-associated acute kidney injury (CSA-AKI) is a risk factor of developing more serious kidney conditions such as chronic kidney disease, (Van Kuijk et al., 2010; L. Yang, Besschetnova, Brooks, Shah, & Bonventre, 2010) end-stage renal disease (Wald et al., 2009) and an independent risk factor of death (Chertow et al., 1998).

1.2.1. Cardiopulmonary Bypass (CPB)

The cardiopulmonary bypass circuit, commonly known as the heart-lung machine is standard protocol for cardiac surgery (Baehner et al., 2012). CPB is a form of artificial extracorporeal circulation that undertakes the function of the heart and lungs, circulating blood and oxygen throughout the operation. Systemic inflammation is a natural immunological response to CPB that affects all organs (Baehner et al., 2012). A combination of local inflammation and ischemia caused by the CPB machine can cause organ injury (Chai et al., 1999). Clinical issues that arise post-operation, range from cerebral, pulmonary, cardiac, hepatic and renal problems (Baehner et al., 2012). Cerebral injury can be presented as ischemic strokes, encephalopathy or neurocognitive dysfunction, with average incidence rates of 3.2%, 20.2% and 25%, respectively, within one month following CPB surgery (Hogue, Palin, & Arrowsmith, 2006; McKhann, Grega, Borowicz, Baumgartner, & Selnes, 2006; Newman et al., 2001). Pulmonary complications occur in 6.96% of patients that undergo CPB surgery (Ji et al., 2013). A more in-depth study on pulmonary complications observed incidence rates of 7.82% in patients that underwent coronary artery revascularization and 2.23% in patients that underwent valve replacement surgery (Al-qubati, Damag, & Noman, 2013). Hepatic complications are not well studied following cardiac surgery, however severe ischemic early liver injury post-cardiac surgery is strongly associated with mortality (Raman, Kochi, Morimatsu, Buxton, & Bellomo, 2002).

Our study looks at AKI secondary to ischemia reperfusion injury in adults that undergo CPB coronary artery bypass graft and/or valve repair/replacement surgery. The kidneys experience inadequate perfusion while on CPB. The kidneys are particularly susceptible to hypoxic injury due to their high metabolic requirements (Le Dorze, Legrand, Payen, & Ince, 2009). Tissue

perfusion of oxygen is influenced by the mean arterial pressure (MAP), hematocrit levels, temperature, pH fluctuations and bypass type (on-pump or off-pump) (Murphy, Hessel, & Groom, 2009). Renal ischemic insult is initiated with an impaired local supply of blood, oxygen and nutrients concurrent with insufficient removal of waste products (Bonventre & Yang, 2011). Upon discontinuation of CPB, patients experience reperfusion injury that can further exacerbate kidney damage. The cellular changes and immune responses that occur throughout this phenomenon can be conceptually described as initiation, extension, maintenance and repair of clinical AKI.

1.2.2. CSA-AKI Clinical Phases and Cellular Mechanisms

Cardiac surgery-associated acute kidney injury can be divided into 4 distinct clinical phases in relation to temporal glomerular filtration rate changes: initiation, extension, maintenance and repair. The initiation phase begins with reduced renal blood flow, causing vascular epithelial and tubular endothelial damage, which causes a decline in the glomerular filtration rate. The amplified innate immune and adaptive immune responses in the extension phase cause further decline in GFR. The maintenance phase is established at stable nadir GFR as repair mechanisms commence. Return to normal kidney function and improved GFR are observed in the repair phase (D. Basile et al., 2012).

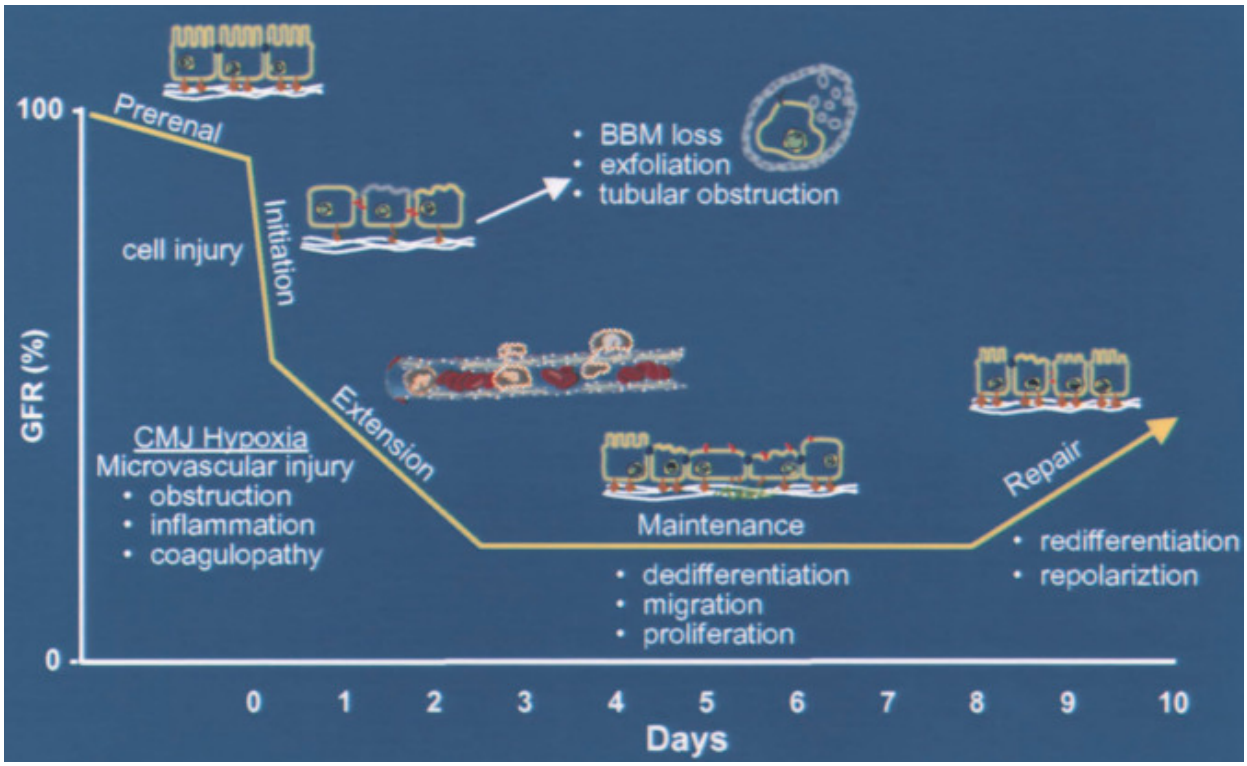

Figure 1.3 Clinical and cellular phases of AKI in relation to temporal kidney function and glomerular filtration rate (GFR). Initiation occurs with a reduction in renal blood flow, which in turn affects renal tubular epithelial cells and causes a subsequent decline in GFR. Further tubular cell injury occurs with ongoing inflammatory processes causing a further decline in GFR in the extension phase. The maintenance phase is represented by stable nadir GFR and the start of cellular repair mechanisms. The repair phase is marked by tubular repair and organ function, which leads to GFR improvement. (Modified from Sutton et al., 2002)

1.2.2.1. Phase 1: Initiation Phase

The initiation phase of AKI begins when the kidney microcirculation receives insufficient perfusion and oxygen for normal cellular processes (Bonventre & Yang, 2011). An accumulation of metabolic waste with an imbalance of oxygen supply and demand to the metabolically active renal tubules results in varying injury throughout ischemia. Tubular epithelial cells experience severe adenosine triphosphate (ATP) depletion that interrupts homeostatic cellular metabolism (Bonventre & Yang, 2011; D. Basile et al., 2012). ATP depletion is the starting point for failing cellular functions and mechanisms that may lead to tubular cell death and AKI. Another sequence of events that exacerbates tubular injury begins upon reperfusion following CPB discontinuation when the kidneys receive an influx of oxygen and inflammatory components. A combination of ATP depletion, vasoconstriction, inflammation and reactive oxygen species (ROS) contributes to renal tubular pathophysiological changes in the initiation phase.

1.2.2.1.1. Adenosine Triphosphate (ATP)

The architecture of the renal microvasculature network is extremely complex with high-energy demand for a steady oxygen supply, making it vulnerable to hypoxic injury (Le Dorze et al., 2009). Adequate oxygen at the cellular level is required by the mitochondria for oxidative phosphorylation (ATP production) to sustain organ function and performance. More specifically, animal models of AKI suggest that cells of the proximal tubule and thick ascending limb (Loop of Henle) are the most sensitive in terms of energy requirements to ischemic and hypoxic injury (Lieberthal & Nigam, 1998). However, some regions of the kidneys (eg: medulla collecting ducts) are more resistant to hypoxia and can maintain around 60% of baseline ATP levels while

increasing anaerobic glycolysis under stressful anoxic conditions (Bagnasco, Good, Balaban, & Burg, 1985).

The kidneys rely heavily on ATP to balance electrolytes (eg: tubules reabsorb 80 meq Na/g kidney/day) and regulate water balance throughout the tubular network (Manunta et al., 1998). The effects of ischemia on anesthetized dogs demonstrated that renal ATP concentrations fell 20% relative to control levels as early as 10 minutes into renal artery occlusion (Zito, 2010). Furthermore, renal ATP levels fell within minutes at the onset of ischemia, demonstrated with in vivo ^{31}P NMR spectroscopy (Stromski et al., 1986). Rapid restoration of ATP to hypoxic regions can reduce degree of injury, whereas impaired ATP recovery leads to kidney damage, as shown in a rat model of hypotension-induced AKI (Ratcliffe, Moonen, Holloway, Ledingham, & Radda, 1986).

1.2.2.1.2. Cellular Structure and Integrity

The inflammatory response begins with the shedding of the glycocalyx (Mulivor & Lipowsky, 2004) and disruption to the filamentous actin (f-actin) cytoskeleton framework, which alters tubular endothelial cell-to-cell contact (Ashworth & Molitoris, 1999). F-actin polymerization is highly sensitive to hypoxia and the decline in ATP (Kellerman & Bogusky, 1992). The disintegration of the cytoskeleton is characterized as one of the early morphological changes in AKI, resulting in the loss of the renal brush border microvilli in the proximal tubular region (S_1, S_2, S_3), seen within 15 min of reperfusion in a unilateral renal artery occlusion rat model (Venkatachalam, Bernard, Donohoe, & Levinsky, 1978). Another murine study looking at

reperfusion revealed loss of function at the endothelial barrier of the cortical tubule two hours post-reperfusion using two-photon microscopy (Sutton et al., 2003).

Alterations to tubular epithelial cells influence vascular endothelial cell structure and integrity (Basile, 2007). Endothelial cell tight junctions quickly lose integrity with disruption to the cytoskeleton, contributing to the loss of cell polarity (Molitoris, Leiser, & Wagner, 1997). Cell polarity is regularly maintained by tight junctions and tight adherens that are linked to the actin network by transmembrane bridging and cytoplasmic proteins (Lee, Huang, & Ward, 2006). The vascular endothelial cadherins breakdown and loosen the tight junctions, allowing inflammatory cells to pass into the interstitium (Sutton et al., 2003). This sequence of events culminates in an increase in microvascular permeability, allowing fluid to leak into the interstitial region and cause local edema (Basile, 2007). Both viable tubular cells with weakened structural integrity and irreversibly damaged cells slough off into the tubular lumen and hamper filtration and cause backleak of the filtrate (Sancho-Martinez et al; D. Basile et al., 2012). AKI severity is dependent on the extent of these cellular alterations and duration of ischemic insult that continues on into the extension phase (D. Basile et al., 2012).

1.2.2.1.3. Adhesion Molecules

The damaged tubular epithelial cells actively generate pro-inflammatory and chemotactic cytokines (eg: TNF-α, IL-8, IL-6, IL-1β, TGF-β and RANTES) that traffic inflammatory cells to local regions of injury (Bonventre & Zuk, 2004). Innate inflammatory cells that circulate through the renal vascular network infiltrate damaged areas and adhere to upregulated adhesion molecules. Surface expression of intercellular adhesion molecule 1 (ICAM-1), P-selectin and E-

selectin are upregulated with progression of vascular endothelial damage which in turn initiates endothelium-leukocyte interaction (Kelly et al., 1996). Increased ICAM-1 expression on endothelial cells allow for tight binding of leukocytes to their counter β-integrin receptor (Smith et al., 1988). ICAM-1 deficient rats demonstrated markedly less leukocyte infiltration relative to the control group in a ischemia reperfusion injury (IRI) model (Kelly et al., 1996). Similarly, interfering with P-selectin in a separate rat IRI model has been shown to mitigate the degree of tubular necrosis and reduce overall mortality (Nemoto et al., 2001). Fractalkine (CX3CL1) is another adhesion molecule that is expressed by damaged endothelial cells which behave as a potent chemoattractant and adhere to monocytes and macrophages that contain CX3CL1 receptors: CX3CR1 and CCR2. In a murine model, blocking the fractalkine pathway reduced the severity of AKI by significantly reducing macrophage infiltration (D. Basile et al., 2012; Oh et al., 2008).

1.2.2.1.4. Inflammatory Cell Types

Neutrophils are the main inflammatory cells that contribute to inflammation in the early stages of AKI, predominantly observed in the outer medullary region. Neutrophils are increased by a factor of 12 in the outer medulla and by a factor of 14 in the inner medulla relative to controls as early as 2 hours following reperfusion in rats that sustained 45 minutes of renal artery clamping (Willinger, Schramek, & Pfaller, 1992). IL-8 is a crucial chemokine responsible for neutrophil recruitment, as seen in kidneys following reperfusion (Araki et al., 2006). Neutrophils attach to the activated endothelium using upregulated adhesion molecules such as ICAM-1 and transmigrate into the interstitial regions (D. Basile et al., 2012). Mice deficient in ICAM-1 were better protected against neutrophil migration into regions of renal injury (Kelly et al., 1996).

Neutrophils congregate in injured regions early on in the inflammatory process and cause local obstruction of capillaries that alter renal hemodynamics and exacerbate local hypoxia (D. Basile et al., 2012). Additionally, neutrophils indirectly amplify vasoconstriction and worsen hemodynamics by generating ROS and vasoactive lipids (Bonventre & Yang, 2011). Neutrophils leak into the interstitial region and produce cytokines that cause edema, which increases the interstitial pressure and slows down regional blood flow. Specifically, neutrophils produce large amounts of IL-17, that enhanced INF-ϒ mediated neutrophil migration into damages regions in a mouse model (Li et al., 2008).

Macrophages are another important early contributor of inflammation in AKI with infiltration into tubular and interstitial regions facilitated by the fractalkine pathway (Oh et al., 2008). A mouse study looked at the kinetics of macrophage infiltration in damaged regions and found a significant increase at 1 hour post-reperfusion, that peaks by 24 hours and persists locally for 1 week (Li et al., 2008). Macrophages excrete high levels of cytokines (eg: IL-1a, IL-6, IL 12, TNF-α) in the interstitial regions which further activates other inflammatory components that contribute to inflammation (Li et al., 2008; Kinsey, Li, & Okusa, 2008). Macrophages are presented in the M1 phenotype in an environment high in pro-inflammatory cytokines. M1 macrophages are responsible for producing cytokines (eg: IL-1β, TNF-α) that are involved in T-helper 1 (T_h) polarization and responses (Bonventre & Yang, 2011).

The role of dendritic cells in the kidneys during IRI is unclear, however they are the most abundant leukocytes in mouse kidneys, which suggests a potential role in inflammation (Bonventre & Yang, 2011). Dendritic cells were observed as the prominent secretor of pro-

inflammatory cytokine TNF-α in a model of AKI following IRI (Dong, Bachman, Miller, Nath, & Griffin, 2008).

Both $CD8^+$ and $CD4^+$ T-lymphocytes have been implicated in AKI and identified in rat renal tissue of an IRI model (Takada, Nadeau, Shaw, Marquette, & Tilney, 1997). The role of $CD8^+$ T-lymphocytes is unknown, however, mice deficient in $CD4^+$ T-lymphocytes were protected from structural and functional damage in the post-ischemic kidney, with injury upon reconstitution (Burne et al., 2001; Day, 2004).

1.2.2.1.5. Vasoconstriction

Concurrent with inflammation, the damaged vascular endothelium promotes an increase in endothelin-1, angiotensin II, thromboxane A2, and adenosine, which in turn stimulates the sympathetic nervous system and contributes to vasoconstriction of the small arterioles in the kidney (Da Silveira et al., 2010; Kurata et al., 2005). Arteriolar vasoconstriction reduces local blood flow and delivery of oxygen. Vasoconstriction is indirectly amplified as damaged endothelial cells reduce normal production of vasodilatory substances such as nitric oxide (Kwon, Hong, & Ramesh, 2009). Decreased nitric oxide levels impact the regulation of renal plasma flow, renin secretion and the glomerular filtration rate, which influences oxygen delivery (Blantz et al., 2002). Furthermore, as leukocyte-endothelial adhesion and activation occurs, vasoactive cytokines (eg: TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-18, IL-32) are produced by damaged vascular endothelial cells and promote a vigorous pro-inflammatory response (Bonventre & Zuk, 2004). Vasoconstriction alongside endothelium-leukocyte adhesion induced small vessel

occlusion compromises microcirculation which manifests hemodynamic dysregulation (Pettilä & Bellomo, 2014).

1.2.2.1.6. Reactive Oxygen Species (ROS)

Reactive oxygen species are a group of chemically reactive molecules that are derived from oxygen (Nath & Norby, 2000; Sedeek, Nasrallah, Touyz, & Hébert, 2013). In physiological conditions, the kidneys produce a basal level of ROS that play an important biological role in the regulation of cell defense, hormone production, ion channels, transcription factors and gene expression (Sedeek et al., 2013). In pathophysiological conditions, an excess of ROS production can cause oxidative stress, inflammation, DNA damage and cellular injury/death.

Increased ROS activity is a major factor of tubular injury in AKI (Dobashi, Ghosh, Orak, Singh, & Singh, 2000); ROS systems are associated with cisplatin-induced AKI (Bae et al., 2009) and gentamicin nephrotoxicity in murine models (Lopez-Novoa, Quiros, Vicente, Morales, & Lopez-Hernandez, 2011). The energetics of the mitochondrion is impaired in hypoxic conditions throughout IRI and intracellular ROS production is elevated which causes tubular lipid peroxidation (Lemasters et al., 1998). ROS varieties in renal injury includes hydroxyl radicals, which generate the greatest cytotoxic effects, superoxide anions and hydrogen peroxide (Bolisetty & Jaimes, 2013). Dismutation of superoxide ions can yield hydrogen peroxide that can further react with catalytic iron (free unbound iron) to generate highly reactive hydroxyl radicals (Basile, Fredrich, Weihrauch, Hattan, & Chilian, 2004). ROS can also indirectly impact the degree of vasoconstriction and renal vascular resistance. Particularly, superoxide production can augment renal vasoconstriction of the renal medullary region. The superoxide anion is generated

when oxygen accepts a single electron and increased production is correlated with increased reactivity of Angiotensin II in both the renal cortex and medulla (Zou, Li, & Cowley, 2001). Tubular cells contain defense mechanisms, including superoxide dismutase, catalase and glutathione S transferase that partially mitigates damage (Martin-Sanchez et al., 2016).

1.2.2.2. Phase 2: Extension Phase

The initiation phase transitions into the extension phase as the GFR starts to further decline. During this phase, the pro-inflammatory response is amplified as innate immune cells (eg: neutrophils and macrophages) continue to influx to the damaged vascular endothelium and transmigrate to the interstitial region and destroy tubular cells. Significant inflammation is seen generally seen 24 hours post ischemia, especially in the outer medulla in animal models of ischemic injury (Kelly et al., 1996).

Immune cells and damaged renal parenchymal cells release cytokines (eg: IL-6, IL-12, TNF-α) that further amplify the inflammatory cascade. Tubular cells upregulate toll-like receptors (TLR) and complement receptors that propagate inflammation. In AKI, tubular cells have been seen to specifically upregulate TLR2 and TLR4 that sense endogenous damage-associated molecular patterns (DAMPs) and contribute to antigen-independent inflammation in AKI (Jang, Kim, Park, & Park, 2008; Vallés, Lorenzo, Bocanegra, & Vallés, 2014). Binding of TLR's induce intracellular signal transduction, which activates effector cells via kinase and NF-κB (Jang et al., 2008). The complement system simultaneously plays a crucial injurious role in AKI (Donnahoo et al., 1999). The complement system induces upregulation of cell adhesion molecules and chemokine production (Bonventre & Zuk, 2004). Specifically, the alternative complement

pathway has been implicated in AKI with selective inhibition providing renoprotection (Thurman, Ljubanovic, Edelstein, Gilkeson, & Holers, 2003). Another study looked at the innate response in an ischemic AKI model and noted a decrease of CXC chemokine production and neutrophil infiltration by half when the C5a receptor was blocked (de Vries et al., 2003). With extended inflammation, tubular cells can undergo cell death in the form on apoptosis, necrosis and ferroptosis.

1.2.2.3. Phase 3: Maintenance Phase

The extension phase transitions into the maintenance phase several days after initial ischemic insult when nadir GFR levels are stabilized. Tubular cells undergo repair by dedifferentiation, proliferation, redifferentiation, and migration to re-establish cellular composition and integrity (Bonventre & Zuk, 2004). Tubular epithelial cells re-establish intercellular homeostasis and polarity (Harris, 1997). Local blood flow returns to normal levels, improving cellular function and subsequent organ function. Tubular structure and function alteration is most pronounced at the outer medullary region (eg: straight proximal tubule and thick ascending limb) where a complex metabolically active microvasculature network exists.

1.2.2.4. Phase 4: Repair Phase

The repair phase begins as cellular functions normalize and kidney function returns, seen by improvement in GFR. Dependent on the duration and continuum of cell death in the tubular region, select areas of sub-lethal injury can repair while others remain irreversibly damaged (Bonventre & Yang, 2011). In terms of immune cells, M2 macrophages are abundant in the repair phase of AKI, implicating a beneficial role in recovery. The M2 phenotype is induced by

complement factors, cell death and certain cytokines (eg: macrophage colony stimulating factor & IL-4) (N. Wang, Liang, & Zen, 2014). The M2 macrophage subset secretes anti-inflammatory cytokines (eg: IL-10 & TGF-β) involved in tissue repair and angiogenesis and drives T-helper 2 repair responses (Bonventre & Yang, 2011; Duffield, 2010; Yiping Wang & Harris, 2011). Programming macrophages into the M2 subset is shown to ameliorate chronic inflammation of the kidneys (Y Wang et al., 2007). Depletion of infiltrating macrophages in a mouse model of IRI demonstrated impaired kidney recovery (Jang et al., 2008). Furthermore, tubular cell proliferation increased with macrophage administration during the repair phase of ischemic injury (Vinuesa et al., 2008). Regulatory T cells ($CD4^+$, $CD25^+$, $Foxp3^+$) are involved in immunosuppression, with the potential to mitigate immune responses. In the context of IRI, depletion of regulatory T cells using an anti-CD25 monoclonal antibody shows more severe renal damage. Adoptive transfer experiments of regulatory T cells into T-cell deficient mice showed significant protection in IRI, independent of other T-cell types (Kinsey et al., 2009). Over time as tubular integrity is restored, baseline levels of GFR are re-established.

1.2.3. Severity of AKI & Cell Death

The severity of AKI can be explained by the degree of cellular changes in renal tubular cells. Tubular cell injury can be viewed on a spectrum ranging from uninjured to sub-lethally injured and ultimately cell death (Venkatachalam et al., 1978). Under hypoxic conditions, the homeostasis of the renal microcirculation becomes disrupted (Aksu, Demirci, & Ince, 2010). Tubular cells experience varying levels of injury dependent on their location within the kidney network, metabolic ATP demand, ROS exposure and the quantity and duration of local perfusion (Lieberthal & Levine, 1996).

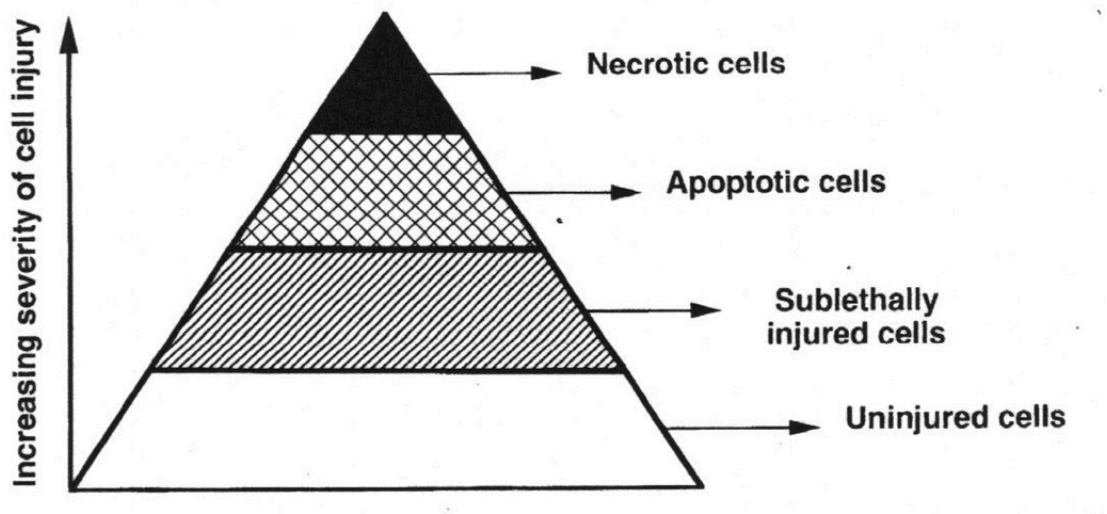

Figure 1.4 Tubular cell fate following renal injury. Renal tubular cells respond differently depending on the severity of the noxious stimulus. They can remain uninjured and viable or become sublethally injured with the potential to recover. Severe injury results in cell death via apoptosis, or even necrosis with extreme injury. (Modified from Lieberthal & Levine, 1996)

Following IRI, some tubular cells remain sub-lethally injured with the ability to repair and restore kidney function. The proportion of sub-lethally damaged cells depends on the duration and severity of ischemic insult (Lieberthal & Levine, 1996). Gene expression analysis following renal injury demonstrated upregulation of genes (eg: heat shock protein 70) that were associated injury adaptation (Supavekin et al., 2003). Differentiated outer medullary epithelial cells co-expressed Ki67 and red fluorescent protein two days following IRI, which indicates that cells that survive injury can undergo proliferative expansion. Furthermore, 66.9% of injured tubular epithelial cells had incorporated BrdU, which was significantly greater than the 3.5% of uninjured kidney. The main tubular cell repair mechanism is likely conducted by surviving damaged tubular epithelial cells that regenerate (Humphreys et al., 2008). In the proximal tubular region, newly formed cells distribute on the basement membrane and have a different gene

expression profile compared to already differentiated proximal tubules (Nony & Schnellmann, 2003).

The mode of renal tubular cell death in AKI has been characterized in many forms: apoptosis, necrosis and recently ferroptosis (Martin-Sanchez et al., 2016; Padanilam, 2003). Different forms of cell death are distinguishable by distinct morphological changes and biochemical mechanisms. Apoptosis is an ATP dependent form of programmed cell death, recognized by morphological features including shrinkage of the nucleus and cytoplasm, DNA fragmentation, chromatin condensation and breakdown into smaller apoptotic bodies for phagocytosis (Lieberthal & Levine, 1996). Cells undergoing apoptosis retain membrane integrity, keeping cellular/lysosomal contents contained intracellularly and avoiding injury to surrounding renal parenchymal cells. Tubular cells undergoing apoptosis expose phosphatidyl serine residues on the external plasma membrane, which signals for phagocytosis by macrophages (Padanilam, 2003).

A more severe form of tubular cell death is necrosis and involves an uncontained pro-inflammatory response that damages surrounding areas. The plasma membrane blebs and sheds the cytoplasmic contents into the surrounding environment, exacerbating the inflammatory response (Padanilam, 2003). A murine study looking at apoptosis and necrosis in renal injury demonstrated that less exposure to ischemia (eg: 15 minutes) resulted in only apoptosis and no sign of necrosis. Furthermore, longer duration of ischemia was associated with increased quantity of apoptosis and the subsequent manifestation of necrosis (Schumer et al., 1992). One way to study how these cells are dying is to monitor the dependency of ATP, since the type of cell death is associated with the amount of utilizable ATP. Apoptosis requires ATP, so it likely

occurs in the earlier phases of AKI prior to energy depletion. ATP also prevents onset of necrosis, but over time with depletion, necrosis becomes the dominant form. When ATP levels fall below 15% relative to baseline, necrosis is the predominant form of cell death. Alternatively, when ATP levels are reduced by 25–50% in proximal tubular region, apoptosis remains the principle mechanism (Lieberthal, Menza, & Levine, 1998; Linkermann et al., 2014).

Ferroptosis is an iron-dependent form of regulated cell death that is associated with lipid peroxidation of renal tubular cells (Martin-Sanchez et al., 2016). Iron is an essential element required for physiological functions in the body, including hematopoiesis, oxygen transportation, cellular respiration, cell cycle progression and DNA synthesis (Cammack, Wrigglesworth, & Baum, 1990). Total body iron content (3-4g) is distributed in red blood cells (RBC)/hemoglobin, myoglobin and macrophages, stored in ferritin and hemosiderins, bound to transferrins or remain free floating (~2mg) in circulation with excess stored in the liver (Kohgo, Ikuta, Ohtake, Torimoto, & Kato, 2008; J. Wang & Pantopoulos, 2011). Iron can be lost to the external environment through sloughing of skin, mucosal surfaces and the gastrointestinal tract (J. Wang & Pantopoulos, 2011). Dietary iron absorption in the duodenum is tightly regulated, as humans do not have a mechanism to actively excrete excess iron. Iron toxicity can be caused by excess catalytic non-transferrin bound iron that is available to generate ROS varieties. In severe cases of iron overload such as hemochromatosis, excess iron deposition, mainly the liver, heart, brain and endocrine system can be detrimental and contribute to organ damage (Kohgo, Ikuta, Ohtake, Torimoto, & Kato, 2008).

All patients undergoing cardiac surgery experience catalytic iron release. There are many potential sources of catalytic iron that contribute to the formation of ROS throughout cardiac surgery. An inevitable source of catalytic iron is extracorporeal circulation itself, with shear forces that physically weaken RBC structural integrity and elevate intravascular hemoglobin and catalytic iron (Leaf et al., 2015; Haase, Bellomo, & Haase-Fielitz, 2010; Davis, Kausz, Zager, Kharasch, & Cochran, 1999). Interestingly IRI alone, independent of CPB, increased hepatosplenic iron export through increased ferroportin expression in mice that underwent bilateral renal pedicle clamp ischemia for 26 minutes compared to sham mice (Scindia et al., 2015). Additionally, patients may undergo intraoperative blood transfusions that may contain senescent red blood cells with storage lesions, which can contribute to elevated iron levels upon lysis (Kim-Shapiro, Lee, & Gladwin, 2011). Notably, older red blood cells (stored for more than 14 days) were associated with a reduction in survival 6 months following cardiac surgery as compared to newer red blood cells (stored for 14 days or less) (Koch et al., 2008). Finally, rhabdomyolysis, the dissolution of skeletal muscle and content leakage into circulation, elevates myoglobin and catalytic iron and has been associated with AKI (Petejova & Martinek, 2014).

Catalytic iron remains unbound and is readily available for cellular uptake to participate in the Fenton reaction to generate highly reactive hydroxyl radicals that are capable of oxidative damage and destruction of cellular function and composition (Windsant et al., 2010; Petejova & Martinek, 2014; Leaf et al., 2015). Reactive oxygen species generated via the Fenton reaction are associated with lipid peroxidation and AKI in murine models (Linkermann et al., 2014; Martin-Sanchez et al., 2016). Ferroptosis was recently shown to be the primary form of cell death in a folic acid-induced AKI model (Martin-Sanchez et al., 2016). This model demonstrated that

inhibition of ferroptosis protected renal function, histological injury and cell death, whereas inhibition of apoptosis and necroptosis were ineffective (Martin-Sanchez et al., 2016). Indeed, inhibition of ferroptosis has added protective effects beyond necroptosis and necrosis inhibition alone in a murine model of severe renal IRI. All three forms of cell death have been implicated in AKI and ischemic tubular injury. However these should be viewed together as a spectrum of injury and not as separate entities, as the final outcome remains renal tubular injury and AKI (Lieberthal, Koh, & Levine, 1998).

1.3. Chapter 3: CSA-AKI Biomarker Research

A biomarker is a biological marker that can be measured and quantified to evaluate normal biological processes or pathophysiological processes (Strimbu & Tavel, 2010). In order for biomarkers to be clinically beneficial, they need to be non-invasive (eg: isolated from urine or blood), predictive of a specific medical condition with the ability to differentiate from similar disorders, significantly different between diseased and control groups within a therapeutic window of reversibility, efficiently measured using clinical assay platforms, able to monitor response to therapy, and both sensitive and specific (Devarajan, 2011; Nguyen & Devarajan, 2008). Additional knowledge of the source of a biomarker that localizes damage to a particular region of the kidney versus overall kidney deterioration would be ideal (Robinson, Pool, & Giffin, 2008). An improved understanding of the pathophysiological processes of certain regions can help determine targeted approaches to prevent or improve AKI outcomes.

Currently, there are no successful therapies shown to ameliorate AKI, which is largely attributed to late diagnosis. Serum creatinine levels increase to signify clinical AKI a few days following ischemic insult, after functional damage has already taken place (Chertow et al., 2005). Hence, efforts have been put into characterizing novel protein biomarkers that detect the early onset of AKI within a potential window of reversibility to facilitate therapeutic intervention prior to loss of kidney function (Ho, Dart, & Rigatto, 2014). Ideally, an optimal biomarker would be able to identify injury while it occurs or shortly afterwards (Ho, Tangri, et al., 2015). A CSA-AKI cohort is an excellent model to analyze potential biomarkers that may change prior to, during or after IRI, where the timing of IRI injury is clear. On average, 31% of patients undergoing cardiac surgery are diagnosed with AKI, which guarantees a study population that will consist of AKI

patients and non-AKI patients (Chertow et al., 1998; Gailiunas et al., 1980; R.M. et al., 1976). CSA-AKI biomarkers can be studied throughout the preoperative, intraoperative and postoperative periods.

1.3.1. Preoperative AKI Prediction Tools

Tools that identify early renal injury may be used to facilitate early therapeutic intervention for AKI; and indeed, could be used as an enrichment strategy for clinical trials evaluating novel AKI therapeutics (Roy-Chaudhury, 2017). Enrichment strategies decrease heterogeneity of a population by decreasing inter-patient and intra-patient variability while increasing the absolute effect size between patients with and without AKI (Freidlin & Korn, 2013). Preoperative clinical risk prediction models exist to predict likelihood of renal replacement therapy (RRT) or AKI (Kristovic et al., 2015). A number of models utilizing major preoperative risk factors were developed to predict RRT following cardiac surgery (Kristovic et al., 2015) A well-validated model is the Cleveland Clinic Score (Thakar score) (Thakar, Arrigain, Worley, Yared, & Paganini, 2005), which is also utilized to predict milder AKI phenotypes following cardiac surgery. The model generates a risk score using patient information such as gender, diabetes requiring insulin, preoperative creatinine and cardiac related data such as type of surgery and ejection fraction (Englberger et al., 2010; Wong, St Onge, Korkola, & Prasad, 2015).

Pre-cardiac surgery biomarkers that predict postoperative AKI may help enhance risk stratification, however, they have only shown moderate model discrimination at best. Preoperative biomarker serum brain natriuretic peptide demonstrated weak discrimination to predict AKI with AUC 0.60 in adults (Patel et al., 2012) and AUC 0.55 in children with mild

AKI versus AUC 0.57 in severe AKI cases (C.P. et al., 2014). Preoperative endogenous serum ouabain (EO) demonstrated AUC 0.75 for severe AKI in combined independent and validation cohorts (Bignami et al., 2013). Furthermore, Simonini et al confirmed that high preoperative serum EO values (EO >210 pmol/L) were associated with increased prevalence of AKI compared to normal levels (EO <133 pmol/L) (Simonini et al., 2014). Although EO demonstrated a reasonable discrimination for AKI, similar findings were seen in critically ill patients with longer ICU duration (Bignami et al., 2013). Furthermore, EO is released during stressful (eg: physical exercise) and pathological conditions (Bauer et al., 2005; Fuerstenwerth H., 2014), suggesting that EO is likely not specific to AKI.

1.3.2. Intraoperative Biomarkers

AKI onset following cardiac surgery remains incompletely characterized but is thought to originate in the intraoperative period from a combination of local inflammatory processes (Bonventre & Yang, 2011), oxidative stress (Bonventre & Yang, 2011; Zakkar, Guida, Suleiman, & Angelini, 2015), ischemic insult (Bonventre & Yang, 2011; Rosner, 2005) and inadequate iron regulation (Linkermann et al., 2014). Murine models demonstrate that AKI can be abrogated if treatment is initiated prior to extensive tubular injury (McIlroy, Farkas, Matto, & Lee, 2015; Mishra et al., 2004), which is believed to occur during the intraoperative period (Bonventre & Yang, 2011). The intraoperative period is when patients experience initial ischemia, followed by hypoxia and subsequent vascular and tubular injury. The ability to detect AKI intraoperatively or within a few hours following cardiac surgery gives the opportunity to evaluate novel AKI interventions. However, research on non-invasive AKI biomarkers of the intraoperative period is

limited. Evaluated intraoperative AKI biomarkers in adults following cardiac surgery include: urine neutrophil gelatinase-associated lipocalin (NGAL), urine N-acetyl-β-D-glucosaminidase (NAG), urine kidney injury molecule-1 (KIM-1), cystatin C and plasma (free) hemoglobin (Ho, Tangri, et al., 2015). However, these biomarkers only demonstrate modest discrimination for post-operative AKI (AUC<0.75) (Ho, Tangri, et al., 2015).

1.3.3. Postoperative Biomarkers

To date, the majority of cardiac surgery-associated AKI biomarker research has been focused on the postoperative period. However, it is difficult to draw conclusions from these studies, due to inconsistent model discrimination of the same biomarker across centers, variable sample collection time (ranging 0-24 hours post-operation) and heterogeneous AKI definitions (Ho, Tangri, et al., 2015). More importantly, this time point exceeds the potential window of reversibility, thus therapeutic intervention utilizing these biomarkers may be too late to change any adverse renal outcome. Moderate model discrimination is seen in most adult CSA-AKI studies with high variability across different centers. Urinary NGAL is the most comprehensively studied biomarker and has the greatest potential to detect AKI with a model discrimination as high as 0.91 (0.83-0.96) (Ho, Tangri, et al., 2015; Munir, et al., 2013).

1.3.4. Iron Regulation Biomarkers

In 1988, Walker et al released a seminal study that demonstrated the importance of catalytic iron in a gentamicin induced-AKI rat model. Treatment with iron chelator deferoxamine or a hydroxyl radical scavenger in these mice resulted in significantly improved renal histology compared to controls (Walker & Shah, 1988). Around the same time, another study demonstrated

that rats pre-treated with deferoxamine prior to reperfusion injury did not develop AKI (Paller, 1988). Deferoxamine is used in clinical practice to treat patients with iron overload disorders such as hemochromatosis (Anthon et al., 1995). Catalytic iron participates in redox reactions that generate highly reactive hydroxyl radicals that destroy tubular lipid bilayers and influence local inflammation by vasoconstriction (D. Basile et al., 2012; Leaf et al., 2015). Intraoperative catalytic iron measured at the end of CPB has also been found to be a univariate predictor of AKI in patients that required RRT or died post-surgery, $p=0.03$ (Leaf et al., 2015). Recently, a newly characterized form of cell death, ferroptosis has been strongly implicated in AKI following IRI (Xie et al., 2016). Taken together, iron regulation and pathways may play a critical role in AKI pathophysiology (Leaf et al., 2015).

Biomarkers involved in iron chelation or sequestration may be promising in the early detection of AKI. The most promising AKI biomarker to date remains NGAL, which is involved in iron regulation via binding of iron siderophores that are high-affinity iron chelators. Urine NGAL increases in patients that develop AKI following cardiac surgery (Bennett et al., 2008), suggesting iron-dependent pathways are important in renoprotection by preventing tubular injury (Bolignano et al., 2008; Mishra et al., 2005). Expanding on biomarker literature associated with the iron regulation mechanisms may help to identify earlier tubular injury in patients that develop AKI following cardiac surgery, particularly throughout the intraoperative period. Iron pathophysiology likely plays an important role in the intraoperative period, while patients are on CPB, which is known to elevate circulating iron levels, thus this remains an ideal time point to analyze other potential iron regulation proteins. Our biomarkers of interest are urine hepcidin-25, serum ferritin, serum transferrin saturation and urine lactotransferrin.

1.3.4.1. Hepcidin-25

Hepcidin-25 is an antimicrobial peptide that is abundant in cysteine residues that form intramolecular bonds generating a β-sheet configuration. It tightly regulates circulating iron concentrations by degrading and internalizing cellular iron transporter ferroportin displayed on hepatocytes, macrophages and enterocytes (Ganz & Nemeth, 2012; T. Ganz, 2007). Hepcidin is produced by the liver as an 84-amino acid pre-propeptide zymogen that is cleaved post-translation into the bioactive 25-amino acid form. Cleavage into a bioactive 25-amino acid form with a specific N-terminus is required for ferroportin interaction and intracellular iron sequestration (T. Ganz, 2007; Nemeth et al., 2006). Hepcidin-20 and -22 isoforms have been detected in human serum and urine, but only hepcidin-25 has been associated with ferroportin degradation (Addo et al., 2016; Maisetta et al., 2010; Park, Valore, Waring, & Ganz, 2001). The majority of hepcidin is bound to α_2-macroglobulin in the circulation, upwards of 90% (Huang et al., 2013). Hepcidin is freely filtered through the glomerulus and 97% is reabsorbed in the proximal region by megalin-dependent endocytosis (van Swelm et al., 2016; Peters et al., 2013).

The source of hepcidin-25 during IRI still remains unclear, however studies suggest local production in the kidneys. The liver is the main source of hepcidin, however, human and rat models show production in the cortical thick ascending limb and connecting tubules (Kulaksiz et al., 2005). During IRI, the distal tubules produced urine hepcidin-25 in a murine model (van Swelm et al., 2016). Human cardiac surgery models of AKI demonstrate that urine hepcidin-25 is significantly increased in non-AKI patients following cardiac surgery, while plasma hepcidin-25 levels remain the same (Prowle et al., 2012; Haase-Fielitz et al., 2011) Plasma hepcidin-25 increases in both AKI and non-AKI patients relative to baseline, however it remains the same at

6 and 24 hours from the start of CPB in both groups (Haase et al., 2010). Lower levels of urine hepcidin-25 are associated with the development of AKI in cardiac surgery cohorts (J Ho et al., 2011; Ho et al., 2009).

1.3.4.2. Ferritin

Ferritin is an iron binding protein found in both intracellular and extracellular compartments. Intracellular ferritin is a powerful iron binding protein that sequesters up to 4500 atoms until required for metabolic processes (Orino et al., 2001). Furthermore, ferritin is composed of H and L subunits that play a role in converting the oxidative state of reactive ferrous to ferric iron (Fe^{2+} to Fe^{3+}), via ferroxidase activity (Wang & Pantopoulos, 2011). Majority of ferritin is located intracellularly in the liver, but can be found in almost every cell in the body (Knovich, Storey, Coffman, Torti, & Torti, 2009). Serum ferritin is clinically measured to assess iron storage capacity, deficiency or overload (Wish, 2006). Commonly measured alongside serum iron and total iron-binding capacity (TIBC) to determine transferrin saturation, these indices give information on the state of systemic iron regulation.

Serum ferritin has not been well studied in AKI following cardiac surgery. One study demonstrated similar preoperative serum ferritin levels in both AKI and non-AKI groups in a cardiac surgery cohort (Davis et al., 1999). Additionally, Tuttle et al, showed similar serum ferritin results in both AKI and non-AKI groups (Tuttle et al., 2003). However, during cardiac surgery, serum ferritin levels were significantly elevated by POD 1 and at POD 3 in AKI patients that required RRT/died (Leaf et al., 2015).

1.3.4.3. Transferrin Saturation (TSAT)

Transferrin saturation (%) is the total amount of iron that is bound to proteins in blood and is represented by a ratio of serum iron to total iron-binding capacity. Transferrin is the main transporting protein that is produced by the liver to bind iron in circulation to prevent free iron-mediated toxicity. Thus, under physiological conditions, transferrin binds serum iron and results in 30% total TSAT (Tsagalis, 2011). Each molecule of transferrin can carry 2 molecules of iron bound in the cleft of N-terminal and C-terminal subdomains (Adams et al., 2007). It is clinically measured to assess patient iron status (deficiency or excess) and the balance between reticuloendothelial macrophage iron release and bone marrow uptake for erythropoiesis (Wish, 2006).

Iron pathophysiology has been strongly implicated in AKI, however transferrin saturation itself has seldom been studied in this context. One study showed that transferrin saturation was significantly increased during and following cardiac surgery in all patients relative to their baseline values. Transferrin saturation was not a univariate predictor of RRT and death following CPB (Leaf et al., 2015). However, catalytic iron levels in serum significantly increased at the end of CPB in AKI patients that required RRT or died post-surgery (Leaf et al., 2015). Speculating, iron-mediated free radical toxicity may potentially be avoided as elevated iron is bound by transferrin (Tsagalis, 2011).

1.3.4.4. Lactotransferrin

Lactotransferrin is an antimicrobial protein that belongs to the transferrin family and binds intravascular ferric iron (Farnaud & Evans, 2003), potentially playing a role in renoprotection

during IRI. The structural composition consists of two homologous lobes, that each binds a single ion of iron. Apo-lactotransferrin, the unsaturated form is active and readily binds iron, which results in a conformational state change from open to closed to eventually form holo-lactotransferrin, the saturated form (Massucci et al., 2004). Pancreatic acinar cells and neutrophils naturally produce lactotransferrin, however it is predominantly found in human breast milk and mucosal secretions with only trace amounts in serum (Sánchez, Calvo, & Brock, 1992).

Lactotransferrin has been studied in a potassium dichromate (PDC) induced-AKI rat model. Here, the pre-treatment of lactotransferrin in rats prior to AKI induction protected the kidneys (Hegazy, Salama, Mansour, & Hassan, 2016). However to date, no human IRI AKI studies have investigated lactotransferrin quantity or enzymatic activity. Mario Navarrete et al analyzed the temporal patterns of serine hydrolases in AKI and non-AKI patients in our cardiac surgery cohort (*Clinical Proteomics*, under review). In-gel activity-based protein profiling was performed on a nested case-control cohort that consisted of 8 AKI and 8 non-AKI patients to profile enzyme activity. Serine hydrolase activity was found in both groups at baseline, start of CPB, 1 hour CPB, arrival to ICU, POD 1 and POD 3-5. A consistent banding pattern was observed in non-AKI patients throughout IRI. However, AKI patients demonstrated more variability and intensity across the time points with unique bands appearing at 1 hour CPB and arrival to ICU. Thus affinity purification of active serine hydrolases with the Fp-TAMRA and phosphorofluoridate biotin probes (anti-FP-TAMRA antibody and streptavidin beads, respectively) was performed on pooled AKI samples at these 2 time points. Thirty-one active urine serine hydrolases were identified among other lipases, esterases, thioesterases, amidases, peptidases and proteases

involved in blood clothing, digestion, nervous system signaling and inflammation (Bachovchin & Cravatt, 2012). Lactotransferrin, an important iron binding protein, was selected for further validation of specific activity.

1.3.4.4.1. Activity-based protein profiling (ABPP)

A technique called activity-based protein profiling (ABPP) will be used to measure potential enzymatic activity of lactotransferrin. The use of ABPP to analyze proteins at the activity level along with quantitative changes is important to provide insight into renal pathophysiology. ABPP is a proteomic technique that can be used to characterize enzyme function. Lactotransferrin belongs to a large enzyme family of serine hydrolases, with serine residues in the active site that are composed of a catalytic dyad or triad that form a charge relay system (Wiedl et al., 2011). A site-directed probe covalently binds the catalytic site of active enzymes in their native biological state (Cravatt, Wright, & Kozarich, 2008). Probes have been designed for many enzyme classes including serine hydrolases, cysteine proteases, kinases and oxidoreductases among many others (Barglow & Cravatt, 2007).

Potential protein biomarkers of AKI have been quantified in murine and human models, but the enzymatic activity has seldom been studied. Proteins may exhibit compositional changes or pre-existing proteins may become activated in the intraoperative period as IRI is initiated. Navarrete et al previously identified 62 serine hydrolases using compositional analyses in normal urine and only 13 proteins were in the active state (Navarrete et al., 2013). This suggests that enzymatic activity may be independent of quantitative protein changes throughout IRI and may highlight another subset of potential biomarkers (*Clinical Proteomics*, under review).

2. RATIONALE, HYPOTHESIS & OBJECTIVES

2.1. Rationale

Acute kidney injury secondary to ischemia reperfusion injury is a major cause of clinical morbidity and mortality. Clinically, AKI is detected following an increase in serum creatinine, which reflects a loss of kidney function. In AKI following cardiac surgery, the potential therapeutic window of reversibility is thought to be during cardiopulmonary bypass when the kidneys experience IRI. To date, no effective therapies have been proven to ameliorate AKI. Thus, significant efforts have been made over the last decade to characterize novel protein biomarkers that are non-invasive and capable of early AKI detection within the therapeutic window of reversibility. Biomarkers that better detect AKI are lacking in terms of adequate discrimination and subsequent independent validation.

Iron regulation is an important modifier of renal ischemia reperfusion injury (IRI), but the role of iron sequestration throughout cardiac surgery remains unclear. The overarching goal of this Masters project is to characterize iron regulatory pathways in an adult cardiac surgery cohort in the context of clinical AKI predictors and in relation to clinical prediction alone. Additionally, we will expand on current literature of iron regulation during CPB, focusing on urine hepcidin-25, serum ferritin, serum transferrin saturation and urine lactotransferrin as potential non-invasive biomarkers for the early detection of AKI. Analyses will be conducted on a prospective observational cohort of adult cardiac surgery patients using serial urine and serum collected at: baseline, start of CPB, 1 hour CPB, arrival to intensive care unit (ICU), postoperative day (POD) 1 and 3-5; and AKI defined using the 2012 KDIGO criteria.

2.2. Hypothesis and Objectives

2.2.1. Part 1

The first goal of this project is to validate the diagnostic performance of urine hepcidin-25 in an independent cohort for early AKI prediction. Urine hepcidin-25 was previously characterized in a cardiac surgery cohort and was inversely associated with the development of AKI (J Ho et al., 2011; Ho et al., 2009). This study demonstrated that urine hepcidin-25 was elevated at POD 1 following CPB surgery and was inversely correlated with AKI. In our independent cardiac surgery cohort (n=306), we predicted the same kinetic changes of hepcidin-25 in both AKI and non-AKI groups across the six time points with a peak at POD 1. We hypothesized that hepcidin-25 would be an independent predictor of AKI at POD 1 in this independent cardiac surgery cohort.

2.2.2. Part 2

The second goal of this project is to characterize the diagnostic performance of iron-binding proteins in an adult cardiac surgery cohort (n=301) and determine if it provides additional discrimination compared to clinical prediction alone. The results from the first part of this project show the importance of iron regulation during CPB, thus we sought to characterize early markers of AKI at start of CPB and 1 hour CPB, which may facilitate AKI detection earlier than hepcidin-25. We wanted to better understand the pathophysiology of IRI in relation to iron pathways, particularly throughout the intraoperative period, a crucial time frame with therapeutic potential. The intraoperative iron-binding biomarkers of interest include serum ferritin and transferrin saturation. We hypothesized that serum ferritin and transferrin saturation will be

independent predictors of AKI during the intraoperative period in an independent cardiac surgery cohort.

2.2.3. Part 3

The third goal of this project is to analyze the enzymatic activity of lactotransferrin, another protein involved in iron regulation. Lactotransferrin was selected as a candidate from activity-based protein profiling (ABPP) research on AKI and non-AKI patients, completed by my colleague, Mario Navarrete (*Clinical Proteomics*, under review). Navarrete et al isolated activity labeled serine hydrolases, including lactotransferrin in AKI urine samples at time points 1 hour CPB and arrival to the ICU (*Clinical Proteomics*, under review). From this data, we hypothesized that lactotransferrin would be more active in AKI patients relative to non-AKI counterparts during the intraoperative and early postoperative periods.

3. MATERIALS AND METHODS

3.1. Adult Cardiac Surgery Model

The study protocol was approved by the University of Manitoba institutional review board, REB: HS15221 (H2012:097). All enrolled patients (n=380) provided written informed consent. Adult elective coronary artery bypass graft (CABG) and/or valve replacement/repair operations at St. Boniface General Hospital from June 2012- October 2014 were considered for inclusion. Patient demographics and comorbidities were abstracted from patient charts and the Manitoba Cardiac Surgery online database. Intraoperative and postoperative data collection included duration of the operation, pump and cross clamp time, urine output, blood pressure, inotrope support, blood products and potential nephrotoxic exposures (aminoglycoside, non-steroidal anti-inflammatories, angiotensin converting enzyme inhibitors, angiotensin receptor blockers and contrast).

3.1.1. Prospective Observational Cohort

Three hundred and eighty adult cardiac surgery patients were enrolled in the prospective observational cohort. The first set of exclusions included: twenty-one patients for off-pump surgeries (n=3); pre-existing dialysis dependence (n=3), deceased prior to surgery (n=1); and cancelled/delayed surgeries (n=14). Upon finalizing patient sample collection, additional patients (n=53 or n=58) were excluded for missed urine or serum bio-banking. Therefore, the final prospective observational cohort consisted of n=306 **[Figure 3.1]** for hepcidin-25 experiments and n=301**[Figure 3.2]** for other iron-binding proteins, serum ferritin and transferrin saturation experiments**.**

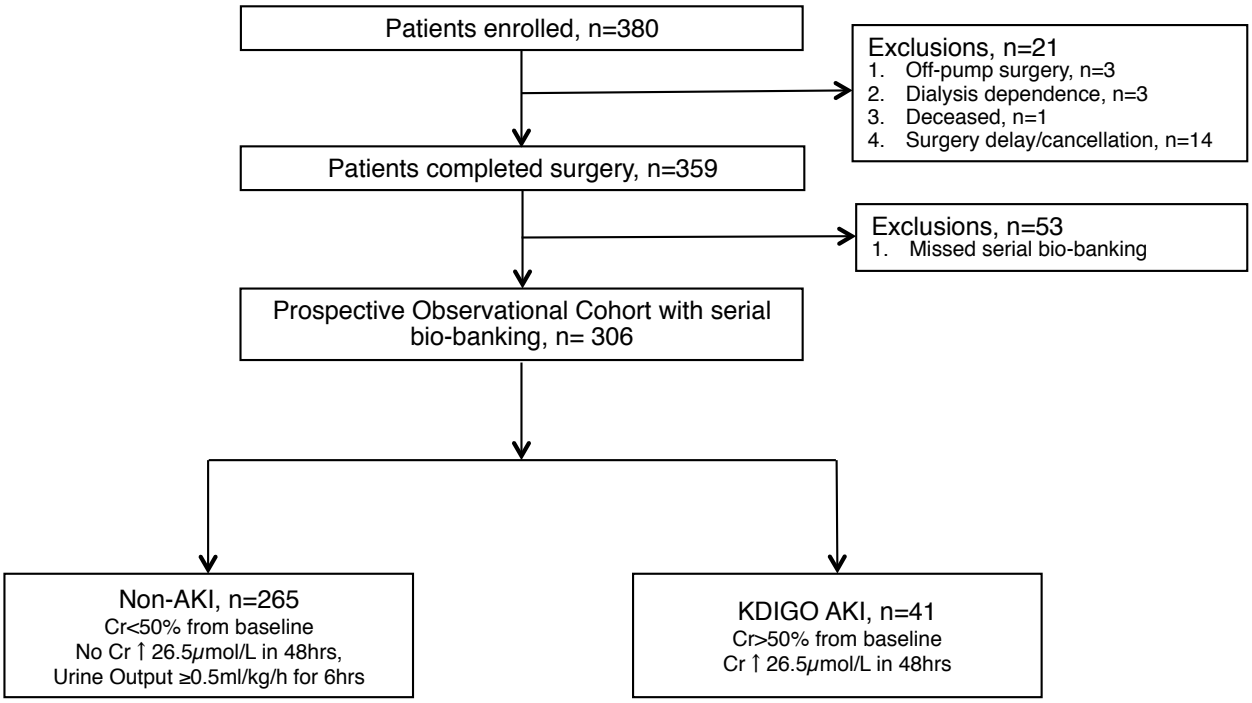

Figure 3.1 Prospective observational cohort of adult cardiac surgery patients analyzed for urine hepcidin-25, n=306. Three hundred eighty patients were initially enrolled in study, followed by first set of exclusions, n=21, for off-pump surgery, dialysis dependence, deceased and surgery delay or cancellation. Fifty-three more patients were excluded for missed sample serial bio-banking. KDIGO AKI criteria was used to determine patients that developed AKI following cardiac surgery. KDIGO, kidney disease improving global outcomes; AKI, acute kidney injury; Cr, creatinine.

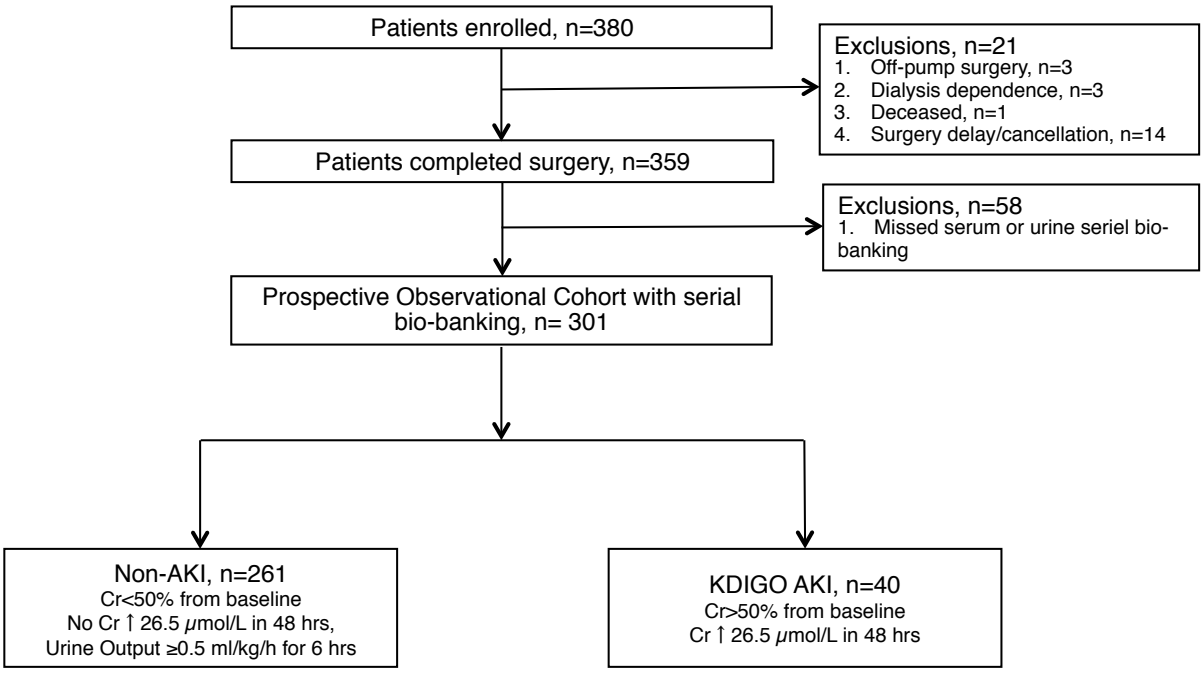

Figure 3.2 Prospective observational cohort of adult cardiac surgery patients analyzed for intraoperative serum iron handling indices, n=301. Three hundred eighty patients were initially enrolled in study, followed by first set of exclusions, n=21, for off-pump surgery, dialysis dependence, deceased and surgery delay or cancellation. Fifty-eight more patients were excluded for missed sample serial bio-banking. KDIGO AKI criteria was used to determine patients that developed AKI following cardiac surgery. KDIGO, kidney disease improving global outcomes; AKI, acute kidney injury; Cr, creatinine.

3.1.2. Nested Case-Control Cohort

A nested case-control cohort (n=42) was selected from the prospective observational cohort (n=306) to confirm the kinetics of urine hepcidin-25 elevation throughout IRI. Patients with chronic kidney disease with eGFR <60 ml/min 1.73m^2 and emergent surgeries were excluded. The nested case-control had 21 AKI patients who met the 2012 KDIGO serum creatinine criteria, and these were randomly matched (1:1) with 21 non-AKI patients with a serum creatinine rise <10% from baseline [**Figure 3.3**].

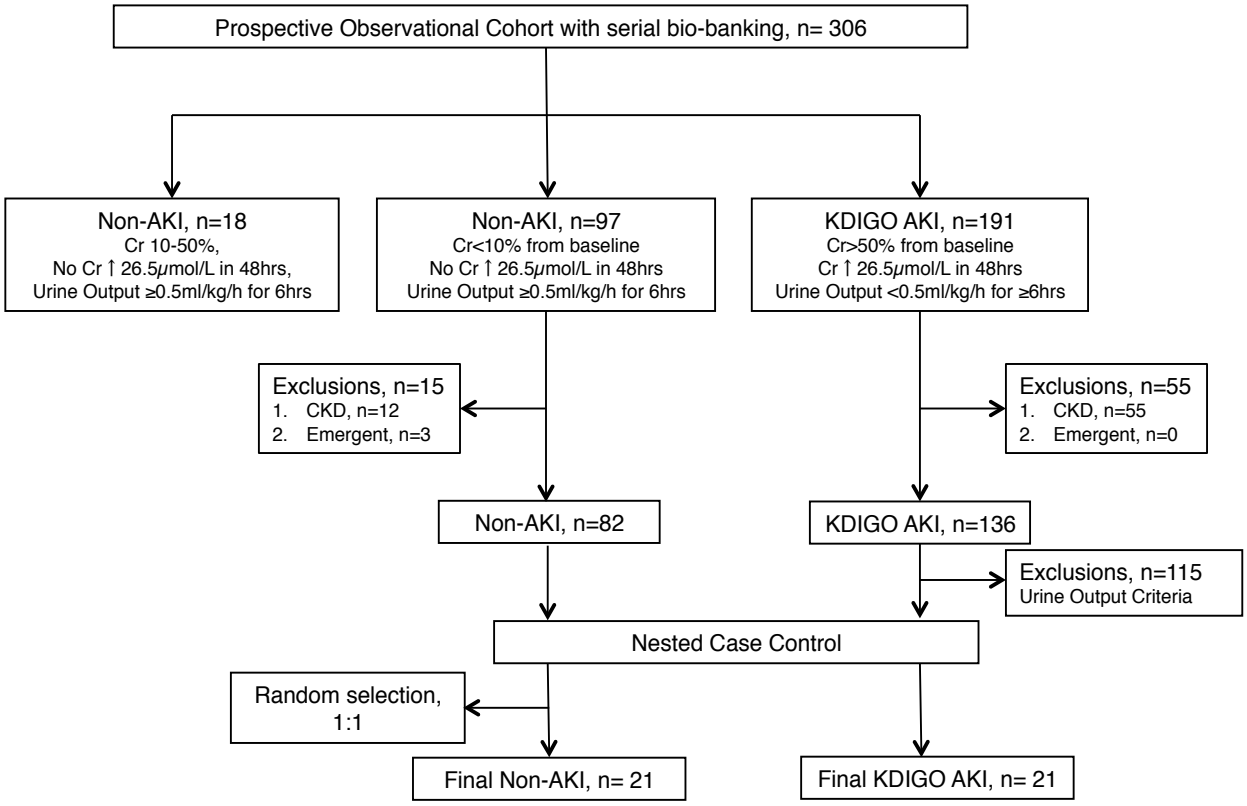

Figure 3.3 Derivation of adult cardiac surgery nested case-control for urine hepcidin-25 kinetics, n=42. KDIGO criteria was applied to the prospective observational cohort, n=306 to determine which patients developed AKI. The first set of exclusions, n=70, included all patients with CKD and all emergent cases. The urine output criteria was excluded to determine final AKI cohort, n=21, followed by 1:1 random selection of remaining non-AKI patients. KDIGO, kidney disease improving global outcomes; AKI, acute kidney injury; Cr, creatinine; CKD, chronic kidney disease.

3.2. Urine Bio-banked Samples

Urine samples were obtained at six time points during and following cardiac surgery for each patient. The preoperative baseline sample was collected in the operating room following anesthesia, intraoperative samples were collected at start of CPB and 1 hour CPB, postoperative samples were collected upon arrival to ICU, POD 1 and POD 3,4 or 5 [**Figure 3.4**]. Urine (20 mL to 50mL) was collected directly from the Foley catheter placed alongside patient table following anesthetic induction until ICU discharge; ward patients provided a midstream catch. All samples were immediately placed on ice or stored in the refrigerator at 4°C. Samples were processed on same day of collection using centrifugation at 870 *g* for 6 minutes to separate out cellular components. Urine supernatant was collected in freezer-safe low retention cyrovials and stored at -80°C. Prior to all experiments, urine samples were aliquoted into small volumes (200µL- 1mL) to minimize freeze thaw cycles.

3.3. Serum Bio-banked Samples

Serum samples were obtained concurrent with urine samples at baseline (preoperative), start of CPB & 1 hour CPB (intraoperative), on arrival to ICU, POD 1 & POD 3-5 (postoperative) [**Figure 3.4**]. The anesthetist collected the baseline blood sample (10mL) sample following anesthetic induction and the perfusionist collected subsequent intraoperative samples. Upon arrival to the intensive care unit, ward nurses collected patient blood during regular morning routine. All samples were placed on ice or stored in refrigerator at 4°C and centrifuged at 1300*g* for 10 minutes. Centrifugation of blood separated out clotting factors from serum (supernatant). Serum supernatant was collected in freezer-safe low retention cyrovials and stored at -80°C.

Prior to all experiments, serum samples were aliquoted into small volumes (250µL- 500µL) to minimize freeze thaw cycles.

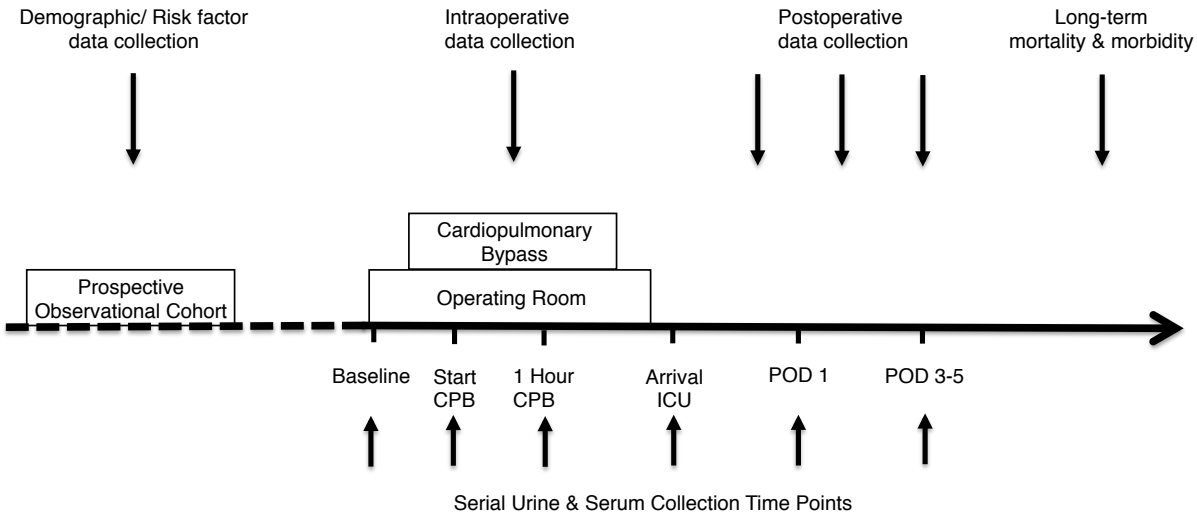

Figure 3.4 Patient population study design. To evaluate cardiac surgery-associated acute kidney injury, serial urine and serum samples were collected before, during and after ischemia reperfusion injury at: baseline (preoperative); start of cardiopulmonary bypass (CPB) & 1 hour CPB (intraoperative), arrival to the intensive care unit (ICU) & postoperative days (POD) 1 and POD 3-5 (postoperative).

3.4. Primary Outcome Variable

AKI was defined using the 2012 KDIGO criteria: serum creatinine (Cr) rise >50% from baseline and/or ≥26.5µmol/L increase within 48 hours (Kellum et al., 2012). Baseline serum creatinine was established using an average of two preoperative serum creatinine values gathered from patient charts. The KDIGO urine output criterion was not applied to determine AKI for a few reasons. Limited studies exist to validate the AKI urine output criterion and these data suggest that the urine output criteria require further refinement (Md Ralib, Pickering, Shaw, & Endre, 2013). Urine output can also be affected by other confounding renal conditions such as pre-renal

azotemia (Md Ralib et al., 2013). Accepting these inherent limitations, we chose to use the more broadly accepted serum creatinine definition of AKI from the KDIGO 2012 guidelines.

3.5. Primary Exposure Variables

3.5.1. Urine Hepcidin-25

Urine hepcidin-25 concentration was quantified using a commercially available competitive Enzyme-Linked Immunosorbent Assay (ELISA) kit (Penlab S-1337), according to manufacturer's instruction on a plate reader (Biotek Synergy 4 microplate reader, Gen 5 software, Fisher Scientific) using 650nm (blue) & 450nm (yellow) wavelengths. All plates were stored in 4°C refrigerator until required. The assay detection ranged from a sensitivity limit of 20pg/ml to upper limit of 25ng/ml.

All samples were run in duplicate on a clear 96-well plate. On day 1, the plate was set out at room temperature prior to experimentation. Polyclonal rabbit immunoglobulin anti-serum was rehydrated with EIA buffer and 25µl was added per well to plate. Plate was sealed and incubated for 1 hour at room temperature. To make the standard curve, human hepcidin-25 standard stock (1µg) was diluted with 1ml of sample diluent (0.085% BSA in 1X PBS and 0.05% Tween 20, pH 7.4) and briefly vortexed to ensure a homogenous mixture. Stock was serially diluted in tubes starting with 1/40, followed by ½ to generate standard curve dynamic range. Following 1 hour incubation, 50µl volumes of standard curve tubes were added onto the plate in duplicate. Then, 50µl patient urine samples were added onto the plate in duplicate. All urine samples were diluted 1/100 in sample diluent. Blank wells were coated with 50µl sample diluent. Plate was sealed and incubated at room temperature for 2 hours. Following incubation, biotinylated tracer (hepcidin-

25 competitor) was rehydrated with 5ml EIA buffer and 25µl was applied to each well. The biotinylated tracer is bound to streptavidin-conjugated horseradish peroxidase (HRP). The plate was sealed with plastic cover and incubated at 4°C overnight in an airtight moisture chamber.

On day 2, the plate was set out to reach room temperature then washed 5 times with EIA buffer on a plate washer machine (BioTek ELx405). Provided streptavidin-horseradish-peroxidase (HRP) stock was diluted 1/200 in EIA buffer. Following washing, the plate was manually blot dried on paper towels. Immediately, 100µl streptavidin-HRP was applied to all wells. Plate was incubated for 1 hour at room temperature. Again, plate was washed 5 times and blot dried using same method as above. Immediately, 100µl tetramethylbenzidine (TMB) substrate was added into all wells and progress of blue color formation was monitored and measured at 650nm every 30 minutes. Plate was incubated at room temperature for 120-180 minutes and covered with tinfoil. Once wells were noticeably blue, 100µl hydrochloric acid (HCl) stop solution was added, resulting in a color change to yellow. Plate was immediately read at 450nm. The duplicate intra-assay coefficient of variation (CV) was 3.2%. An internal control was included on each ELISA plate using normal urine spiked with hepcidin-25 (20ng/ml) in order to assess the plate-to-plate variation. The inter-assay CV of two different batches of plates from separate lots was observed to be 0.46% and 23.7%, respectively.

All urine samples were aliquoted into 500µl vials for creatinine measurements in the clinical laboratory using the Cobas C702 Analyzer (Roche Diagnostics). Urine sample was diluted 1 in 2.5 with saline, then placed into the Cobas C702 Analyzer for 10 minutes. The enzymatic colorimetric method works by converting creatinine to creatine using creatininase, followed by

addition of creatinase to produce sarcosine and urea. Superoxide dismutase converts sarcosine into glycine, formaldehye and hydrogen peroxide. Peroxidase further converts hydrogen peroxide to quinone imine chromogen that is measured at wavelength 546nm and is directly proportional to the creatinine concentration in the sample. Hepcidin-25 data are presented as corrected and uncorrected for dilutional factors using urine creatinine.

3.5.2. Cleveland Clinic Score (Thakar Score)

The Cleveland Clinic Score, commonly referred to as the Thakar score is a clinical risk prediction score for postoperative AKI (Thakar et al., 2005). It was originally developed to predict renal replacement therapy following cardiac surgery, but has since been validated to predict CSA-AKI (Wong et al., 2015; Englberger et al., 2010). Risk factors for scoring include: gender, congestive heart failure, left ventricular ejection fraction, use of an intra-aortic balloon pump, chronic obstructive pulmonary disease, previous cardiac intervention, emergent surgery status, type of surgery, diabetes and creatinine (Thakar et al., 2005). The Thakar score was calculated for each individual based on available pertinent variables in our study population.

3.5.3. Serum Ferritin and Transferrin Saturation (TSAT)

All serum samples were aliquoted into 500µl volumes for ferritin measurements in the clinical laboratory using Cobas e601/e602 Analyzer (Roche Diagnostics). A 10µL aliquot of serum sample was combined with a biotinylated monoclonal ferritin-specific antibody to isolate ferritin. A second monoclonal ferritin specific antibody labeled with ruthenium complex is added to form a sandwich complex. Streptavidin-coated microparticles are added to bind the ferritin sandwich complex. The reaction mixture was aspirated into the measuring cell where the microparticles

were magnetically captured onto electrode surface. All unbound substances were washed out. Voltage was added to the electrode to induce chemiluminescent emission and readouts on the photomultiplier.

Transferrin saturation is a ratio of iron to total iron-binding capacity in the sample. Iron was measured using the Cobas C702 Analyzer (Roche Diagnostics). The serum sample was combined with citric acid to allow for transferrin-Fe complex to dissociated into apotransferrin and Fe^{3+}. Ascorbate was added to reduce Fe^{3+} to Fe^{2+}. Fe^{2+} was combined with FerroZine reagent to form a colored complex that was measured at 570nm, which represents the iron concentration in the sample. TIBC was measured using the Ortho Vitros 4600 (Ortho-Clinical Diagnostics Inc). The serum sample was combined with $FeCl_3$ and iron-binding dye (chromazurol B), followed by acidic buffer (pH 4.5) to release iron from transferrin. A neutral buffer (pH 7.0) was added to the previous solution to increase the affinity of transferrin for iron from the dye-iron complex. The decrease in absorbance measured at 660nm of the colored dye-iron complex was directly proportional to the total iron-binding capacity of the serum sample. Transferrin saturation percentage (%) was calculated using serum iron and TIBC.

3.5.4. Lactotransferrin

3.5.4.1. Substrate Selection

The physiological endogenous substrate of human lactotransferrin is unknown. However, lactotransferrin is known to cleave *H. influenza* IgA1 protease at arginine rich regions (RRSRRSVR) and *H. influenza* Hap protein at arginine rich regions (VRSRRAAR) (Hendrixson et al., 2003). A synthetic fluorogenic substrate was selected based on literature that studied the

enzymatic activity of lactotransferrin. Bovine lactotransferrin hydrolyzed synthetic substrate Z-Phe-Arg 7-amido-4-methylcoumarin (Z-phe-arg-AMC) in a study testing the proteolytic activity of lactotransferrin among a variety of synthetic substrates (Massucci et al., 2004). According to the Uniprot Database, bovine and human lactotransferrin share the same active site, therefore substrate Z-phe-arg-AMC was selected for enzyme assays. Commercial substrate Z-phe-arg-AMC (Product Number A9521, Sigma-Aldrich) was purchased and stored in -20°C upon arrival. It is important to note that this substrate is not specific to lactotransferrin and other enzymes in the human body may potentially cross-react, including but not limited to: serglycin bound protease (Melo et al., 2011), cathepsin B, L (Barrett & Kirschke, 1981) and S (Sage et al., 2013), and kallikrein (Morita et al., 1977) **[Table 3.1]**.

Target Enzyme	Uniprot	Substrate Candidate	Cross-reactivity*
Lactotransferrin	PO2788	Z-Phe-Arg-AMC (7-Amino-4-methylcoumarin)	Serglycin Bound Protease
			Cathepsin B and L
			Cathepsin L
			Cathepsin S
			Kallikrein
*Other enzymes that can cleave substrate			

Table 3.1 Human lactotransferrin commercial substrate (Z-phe-arg-AMC) and potential cross-reactivity. This substrate is known to be cleaved by other enzymes which are found in the human body, including serglycin bound protease, cathepsin B, L and S and kallikrein.

3.5.4.2. Enzyme Selection

Highest catalytic activity of lactotransferrin is believed to occur in its 'apo'-form, with low iron saturation, and low catalytic activity is seen with more saturated forms (Massucci et al., 2004). Based on this phenomenon, affinity purified commercial human colostrum lactotransferrin with iron traces ≤400 mg/kg (Product Number 61326, Sigma-Aldrich) was purchased and stored in

4°C upon arrival. Lactotransferrin was reconstituted with water (1mg/1ml) and aliquoted into tubes to limit freeze thaw cycles and stored in -80°C until required.

3.5.4.2.1. Activity-Based Protein Profiling on SDS-PAGE

Activity-based protein profiling was performed on lactotransferrin to determine binding to the serine hydrolase activity probe using a two-day protocol. On day one, fluorophosphonate-tetramethyl-6-carboxyrhodamine (Fp–TAMRA) probe (2μM) (Product number 88318, Thermo Scientific, 3747 Meridian Rd., Rockford, IL 61101 USA), Tris buffer (50 μM) (pH 9), and lactotransferrin (0.2μg/μl) were combined. Additional ddH_20 was added to make up the difference up to 30μL in Eppendorf tubes. The negative control consisted of the same components excluding Fp-TAMRA probe. Tubes were incubated at 37°C for 90 minutes, followed by addition of 12 μL Laemmli loading buffer and 7.5 μL dithiothreitol (DTT) per tube. Tubes were boiled for 5 minutes on solid glass beads in water then set in -20°C freezer overnight.

On day two, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) running buffer was made using 475ml ddH_20 and 25ml 3-(N-morpholino) propanesulfonic acid (MOPS). SDS-PAGE apparatus was set up with Bolt 4-12% Bis-Tris plus SDS-PAGE gel (Product number NW04120BOX, Life technologies, CA 92008 USA) placed between black clamps. The reference ladder was loaded into the well followed by samples and controls and run at 120V for 80 minutes. The gel was removed from the apparatus and washed in ddH_20 water for 30 minutes on a shaking plate (with water change every 10 minutes), then scanned on gel reader (Alpha Innotech, Fluorchem Q®, USA). The same gel was further stained with Sypro ruby stain overnight. Sypro ruby stain was washed in ddH_20 water for 30 minutes on a shaking plate (with

water change every 10 minutes). The same gel was stained again with Coomassie blue stain overnight. Coomassie blue stain was washed in ddH_20 water for 30 minutes on a shaking plate (with water change every 10 minutes). The gel was scanned on gel reader to confirm equal protein load following each staining.

3.5.4.2.2. Lactotransferrin Enzyme Activity Assay

Commercial lactotransferrin and Z-phe-arg-AMC substrate were brought to room temperature prior to experimentation. To develop a positive control for lactotransferrin activity, 0.2M phosphate buffer (Molecular Cloning Handbook Vol. 3 Sambrook & Russell, pH=7.4) was put into a 96-well plate with black walls and a clear bottom. Human lactotransferrin was added to the wells at varying concentrations as suggested by Massucci et al (eg: 0.1μM). The difference in volume was filled with ddH_20 up to 200μl-250μl per well. The plate was sealed with a plastic cover and incubated in a 37°C dry chamber for 10 minutes. During this incubation, the substrate was reconstituted in dimethyl sulfoxide (DMSO) and added into wells using a multichannel pipette at varying concentrations following plate removal from chamber as suggested by Massucci et al (eg: 1-100μM. Km=50μM). Plate was immediately taken to plate reader and read at varying excitation and emission wavelengths (ex. 348nm-380nm and em. 440nm-460nm) in 37°C plate reader (Biotek Synergy 4 microplate reader, Gen 5 software, Fisher Scientific) for 30 minutes, with readings taken at 2 minute intervals. Blanks with identical contents of each sample excluding lactotransferrin were made for each condition to account for auto-hydrolysis of the substrate alone. Another secondary condition with 5mM EDTA and 1mM DTT was performed in attempt to improve the substrate and enzyme interaction by creating a reducing environment to

decrease the affinity of lactotransferrin for iron by changing the oxidative state of $Fe^{3+}$to Fe^{2+}. Reducing conditions also prevent oxidation of serine residues in the active site of lactotransferrin.

3.5.4.2.3. Lactotransferrin Enzyme Assay

Iron separation by dialysis and iron chelation using deferoxamine was performed to remove iron from commercial lactotransferrin in an attempt to get measurable lactotransferrin activity. **[Figure 3.5]** The first method was dialysis using an 8KDa dialysis bag. Lactotransferrin was placed into a dialysis bag and ends were closed with clamps. Dialysis bag was placed into a beaker filled with pyrophosphate buffer (pH 4) on low speed rotation overnight in the 4° cold room. In the following morning, the dialysis bag was transferred into phosphate-buffered saline (PBS) buffer (pH 7.2) and placed on low speed rotation in the 4° cold room. PBS buffer was changed at 4 hours following initial transfer and left on low speed rotation overnight in the 4° cold room. The second method was iron chelation using deferoxamine. The suggested ratio of iron to deferoxamine is 1:1 (Cooper et al., 1996), and one lactotransferrin molecule can carry 2 molecules of iron. Thus, 1:2 and 1:4 iron to deferoxamine ratio was used. Deferoxamine treatment was applied to one group of samples overnight and for 10 minutes prior to experimentation in a separate group of samples. Finally, these approaches were combined using dialyzed lactotransferrin and deferoxamine treatment overnight. ABPP using SDS-PAGE was performed to evaluate lactotransferrin activity, using same procedure as above. The exact concentration of lactotransferrin was unknown due to product loss from membrane absorption or adhesion during dialysis.

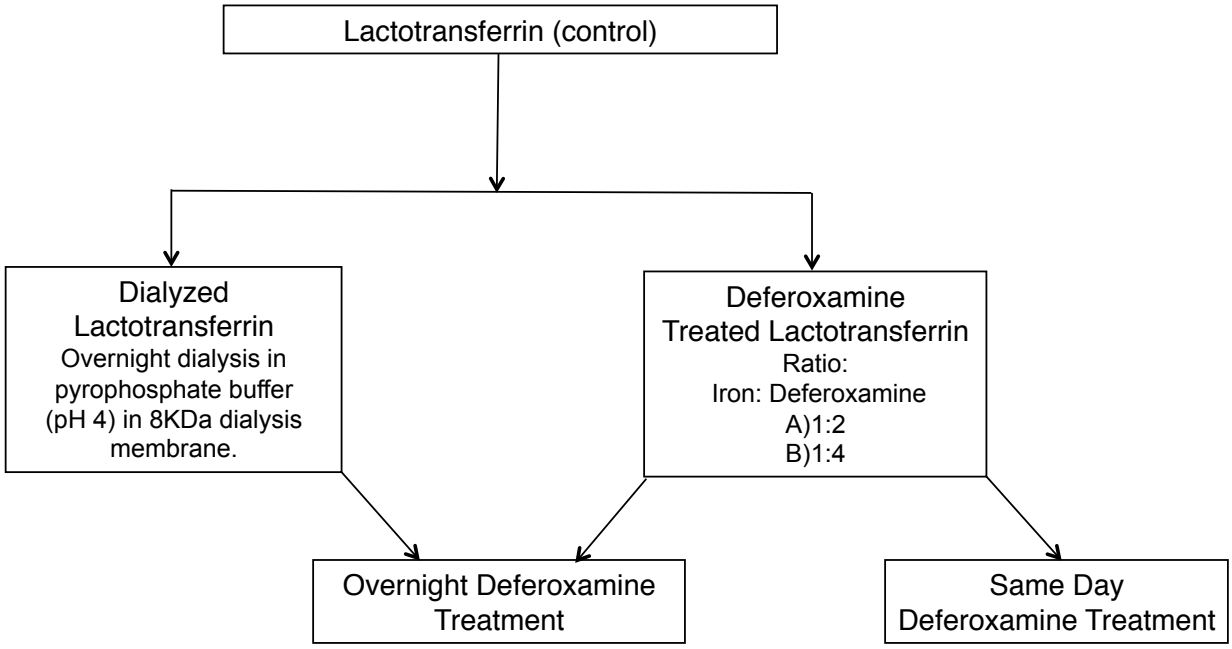

Figure 3.5 Iron separation from lactotransferrin using dialysis and deferoxamine treatment to activate lactotransferrin. Commercial lactotransferrin underwent dialysis using 8KDa dialysis membrane in pyrophosphate buffer, pH 4 overnight, followed by return to pH using PBS buffer, pH 7.2. Deferoxamine treatment was performed on lactotransferrin to chelate potential iron using iron:deferoxamine ratio 1:2 and 1:4. Overnight deferoxamine treatment was also applied on one group of dialyzed lactotransferrin samples.

3.6. Statistical Analysis

Data was analyzed using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). Descriptive statistics are expressed as a median [interquartile range] or number [percentage]. Non-parametric Mann-Whitney U Test was used to compare continuous variables. Chi-squared or Fisher's exact test was used to compare categorical variables. Multiple imputation was used on missing hepcidin-25 covariate data. The p-value was calculated for each characteristic comparison between AKI/non-AKI and study population/excluded patients to demonstrate the probability of observed findings given that the null hypothesis is true (eg: the sample observations are purely attributable to chance). The alternative hypothesis states that there is a difference between compared groups for a specific characteristic. An alpha of 0.05 was used as the significance cutoff with a value less than (or equal to) 0.05 showing rejection of the null hypothesis. However, it is important to evaluate all results not just for their statistical significance, but to determine if they are also clinically significant. Not all statistically significant results reflect true biological significance, especially within larger samples sizes. Claims of significance are inflated when a single test is performed which inevitably causes selection bias and over-interpretation of the p-value (Altman & Krzywinski, 2017). To critically control the number of false positives, gene discovery studies use a genome-wide p-value threshold of $<5\times10^{-8}$, (Panagiotou et al., 2012).

Univariate logistic regression-derived odds ratios [95% confidence interval] were calculated in the prospective observational cohort to estimate the association between the outcome of postoperative AKI and all exposure variables, including urine hepcidin-25, urine hepcidin-25:Cr, ferritin, transferrin saturation, TIBC and iron, individually. In the nested case-controlled analysis, differences in urine hepcidin-25 concentrations between AKI and non-AKI groups and across all

time points were determined by linear mixed modeling. A hepcidin-25 internal control was incorporated into linear mixed modeling to control for plate-to-plate variation.

A multivariate logistic regression model was developed for postoperative AKI using the stepwise selection method, with consideration of all patient characteristics. Finally, three or four exposure variables were modeled into multivariate analysis in relation to the outcome of AKI prediction. The primary exposure variable was urine hepcidin-25 concentration at POD 1. Given the high inter-assay CV, a hepcidin-25 internal control was included as a continuous variable on the final multivariate analysis to control for plate-to-plate variation. Additional multivariate logistic regression models were produced using clinical prediction with intraoperative iron handling biomarkers, serum ferritin and transferrin saturation. Their ability to predict postoperative AKI were assessed and compared to a base clinical prediction model alone (Thakar Score). Enriched model 1 included the Thakar Score, intraoperative serum ferritin and transferrin saturation, while enriched model 2 incorporated these variables with urine hepcidin-25 at POD 1.

The combined area under the receiver operating characteristic curve (AUC) was determined in the final multivariate models. The AUC is generated using the true positive rate (sensitivity) in conjunction with the false positive rate (1-specificity) to demonstrate the diagnostic ability of AKI using variables. The ideal AUC value depicts the highest sensitivity and highest specificity with a maximum value of 1.0, however this value is rarely achieved and either sensitivity, specificity, or both is sacrificed. An AUC value of 0.5 represents no discriminatory power to determine the outcome (Fan, Upadhye, & Worster, 2006). An important advantage of using this statistical analysis is the ability to analyze a small diseased group in comparison to those without,

independent of disease prevalence. Additionally, the sensitivity can be analyzed at any point along the curve. The AUC may give meaningful information for biomarker performance, however any confounding variables must be determined and integrated into the data interpretation, as the AUC analysis does not take this into consideration. Specificity is most accurate with a diverse spectrum of disease with varying levels of severity in the diseased group (Hajian-Tilaki, 2013).

The model goodness-of-fit and complexity was evaluated using the Hosmer-Lemeshow test and Akaike information criterion (AIC). AIC was generated to determine the balance between goodness-of-fit and model complexity and allow for model quality comparison. Each enriched model was compared to the base model to examine improvement in discrimination (ΔAUC, integrated discrimination improvement (IDI)) and reclassification (net reclassification index (NRI)). The IDI and NRI reclassify AKI and non-AKI patients with the addition of new variables. Spearman's rank correlation coefficients (ρ) were calculated between each of the predictor variables. Multi-collinearity was assessed using a multiple linear regression model. Two variables were considered highly correlated if $| \rho | > 0.7$ and collinear if the variance inflation factor was > 10.

Finally, a supplemental analysis of hepcidin-25 was performed excluding patients that developed AKI on or before POD 1 (n=6) for sensitivity purposes. Youdens's J index (range 0-1), which represents the maximum potential effectiveness of a biomarker (Ruopp, Perkins, Whitcomb, & Schisterman, 2008), was determined using maximum sensitivity and specificity values to determine strength of the multivariate models.

4. RESULTS

4.1. Chapter 1. Urine Hepcidin-25

4.1.1. Prospective Observational Cohort Study Population

The prospective observational cohort characteristics were compared between AKI and non-AKI patients [**Table 4.1**]. Characteristics that differed between AKI and non-AKI groups included: age, p=0.03; eGFR, p<0.0001; baseline creatinine, p<0.0001; Thakar score, p<0.0001; diabetes, p<0.0001; and a previous history of hospitalized congestive heart failure, p=0.001; EuroSCORE II, p=0.0001. Urine hepcidin-25 and hepcidin-25:Cr at POD 1 were significantly higher in patients that did not develop AKI [urine hepcidin-25, p=0.0003 and hepcidin-25:Cr p=0.004].

Table 4.1 Study population characteristics of prospective observational cohort, n=306. Observational comparisons were made between AKI and non-AKI groups on preoperative, intraoperative and postoperative characteristics.

Characteristic	Non-AKI (n=265)	AKI (n=41)	p-value
Pre-operative			
Age (years)	66 (59 – 71)	70 (64 – 75)	0.03
Male	204 (77%)	27 (66%)	0.14
Baseline eGFR (mL/min/1.73 m^2)	82 (66 – 97)	61 (43 – 76)	<0.0001
Baseline creatinine (mg/dL)	84 (72 – 100)	102 (85 – 141)	<0.0001
Thakar Score	2 (1 – 2)	3 (2 – 5)	<0.0001
Diabetes mellitus	77 (29%)	25 (61%)	<0.0001
Chronic obstructive pulmonary disease	22 (8%)	2 (5%)	0.75
Hospitalized congestive heart failure	20 (8%)	11 (27%)	0.001
Previous myocardial infarction	99 (37%)	12 (29%)	0.32
Previous CABG	8 (3%)	2 (5%)	0.34
Peripheral arterial disease	18 (7%)	6 (15%)	0.12
Amputation or peripheral arterial disease bypass	2 (1%)	0 (0%)	1.00
Previous cerebrovascular accident	12 (5%)	4 (10%)	0.26
Previous transient ischemic attack	10 (4%)	1 (2%)	1.00
European system for cardiac operation risk (%)	1.4% (0.8-2.5%)	3.0% (1.5 - 5.6%)	0.0001
Type of surgery (Isolated CABG)	169 (64%)	23 (56%)	0.34
Intra-operative			
Pump time (minutes)	95 (72 – 131)	97 (82 – 144)	0.14
Cross-clamp time (minutes)	64 (45 – 92)	61 (45 – 95)	0.57
Operating room duration (minutes)	262 (220 – 339)	261 (203 – 352)	0.74
Intraoperative urine output (mL)	630 (455 – 970)	628 (197 – 1749)	0.25
Post-operative			
Urine hepcidin-25 (ng/mL)	1599 (680– 2753)	628 (197 – 1749)	0.0003
Urine hepcidin-25:Cr (µg/mmoL)	121 (65 – 208)	67 (29 – 134)	0.004

Values expressed as median (Interquartile Range) or N (percent). Continuous variables compared using Mann-Whitney Test, categorical variables compared using Chi-Square or Fisher's Exact Test. Missing covariate values were estimated via multiple imputation. eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft; Cr, creatinine.

4.1.2. Sensitivity Analysis for Study Population Versus Excluded Patients

The baseline characteristics of patients who were excluded for missing urine samples (n=53) were compared to the final study population (n=306), and no differences were found between these groups [**Table 4.2**]. However, the incidence of AKI in the two groups differed, 13.4% in the study population and 26.4% in the excluded patient population, p=0.015. This sensitivity analysis was performed to ensure that the patient characteristics for the missed urine bio-banking group were similar to the analyzed population.

Table 4.2 Study population, n=306, and excluded patients, n=53, characteristics. Preoperative and intraoperative characteristics were compared between the study population and excluded patients.

Characteristic	Study Population (n=306)	Excluded Patients (n=53)	p-value
Pre-operative			
Age (years)	67 (60 – 72)	69 (63 – 74)	0.13
Male	230 (75%)	39 (74%)	0.81
Baseline eGFR (mL/min/1.73 m^2)	79 (63 – 94)	80.0 (60 – 95)	0.49
Baseline creatinine (mg/dL)	85 (72 – 102)	80 (65 – 108)	0.38
Thakar Score	2 (1 – 3)	2 (1 – 3)	0.26
Diabetes mellitus	102 (33%)	17 (32%)	0.86
Chronic obstructive pulmonary disease	24 (8%)	5 (9%)	0.78
Hospitalized congestive heart failure	32 (11%)	9 (17%)	0.17
Previous myocardial infarction	111 (36%)	17 (32%)	0.56
Previous CABG	9 (3%)	1 (2%)	1.00
Peripheral arterial disease	25 (8%)	5 (9%)	0.79
Amputation or peripheral arterial disease bypass	2 (1%)	0 (0%)	1.00
Previous cerebrovascular accident	17 (6%)	2 (4%)	1.00
Previous transient ischemic attack	11 (4%)	1 (2%)	1.00
European system for cardiac operation risk (%)	1.5% (0.9% - 3.0%)	1.7% (1.2% - 3.0%)	0.10
Type of surgery (Isolated CABG)	192 (63%)	31 (59%)	0.56
Intra-operative			
Pump time (minutes)	95 (74 – 132)	90 (73 – 138)	0.54
Cross-clamp time (minutes)	64 (45 – 92)	62 (40 – 85)	0.53
Operating room duration (minutes)	262 (216 – 339)	253 (193 – 312)	0.11
Intraoperative urine output (mL)	618 (450 – 960)	550 (420 – 900)	0.58

Values expressed as median (Interquartile Range) or N (percent). Continuous variables compared using Mann-Whitney Test, categorical variables compared using Chi-Square or Fisher's Exact Test. Missing covariate values were estimated via multiple imputation. eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft.

4.1.3. Nested Case-Control Comparison

A nested case-control cohort was used to determine the kinetic change of hepcidin-25 across time points before, during and after IRI. The nested case-control patient characteristics were largely balanced between AKI and non-AKI groups [**Table 4.3**]. However, patients who developed postoperative AKI had a significantly lower baseline eGFR [76.4 (68.9-79.2) mL/min/1.73m^2 versus 86.4 (71.3-103.8) mL/min/1.73m^2, p=0.03] and a higher incidence of diabetes relative to non-AKI counterparts [10 (47.6%) versus 3 (14.3%), p=0.02].

Table 4.3 Patient characteristics for nested case-control population, n=42. Preoperative and intraoperative characteristics were compared between AKI and non-AKI groups.

Characteristic	Non-AKI (N=21)	AKI (N=21)	p-value
Pre-operative			
Age (years)	66 (59 - 70)	70 (62 - 75)	0.27
Male	19 (90.5%)	15 (71.4%)	0.24
Baseline eGFR (mL/min/1.72 m^2)	86.4 (71.3 - 103.8)	76.4 (68.9 - 79.2)	0.03
Baseline creatinine (mg/dL)	84 (68 - 97)	85 (74 - 100)	0.31
Thakar Score	1 (0 - 2)	2 (1 - 3)	0.12
Diabetes mellitus	3 (14.3%)	10 (47.6%)	0.02
Chronic obstructive pulmonary disease	2 (9.5%)	2 (9.5%)	1.00
Hospitalized congestive heart failure	3 (14.3%)	2 (9.5%)	1.00
Previous myocardial infarction	9 (42.9%)	5 (23.8%)	0.19
Previous CABG	0 (0.0%)	0 (0.0%)	1.00
Peripheral arterial disease	3 (14.3%)	2 (9.5%)	1.00
Amputation or peripheral arterial disease bypass	0 (0.0%)	0 (0.0%)	1.00
Previous cerebrovascular accident	0 (0.0%)	1 (4.8%)	1.00
Previous transient ischemic attack	1 (4.8%)	1 (4.8%)	1.00
European system for cardiac operation risk evaluation	1.2% (0.8% - 2.0%)	1.1% (1.0% - 2.1%)	0.57
Type of surgery (Isolated CABG)	12 (57.1%)	11 (52.4%)	0.76
Intra-operative			
Pump time (min)	93 (74 - 116)	108 (82 - 144)	0.16
Cross-clamp time (min)	55 (44 - 89)	78 (50 - 99)	0.12
Operating room duration (min)	253 (222 - 282)	271 (230 - 315)	0.28
Intraoperative urine output (mL)	615 (400 - 1005)	570 (470 - 925)	0.94

Values expressed as median (Interquartile Range) or percent. Continuous variables compared using Mann-Whitney Test, categorical variables compared using Chi-Square or Fisher's Exact Test. eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft.

4.1.4. Kinetics of Urine Hepcidin-25 Elevation throughout Cardiac Surgery

Urine hepcidin-25 was analyzed before, during and after IRI in the nested case-control cohort to validate kinetic change across time points. There were no differences in urine hepcidin-25 at baseline between patients that subsequently developed AKI versus those that did not. Urine hepcidin-25 increased following cardiac surgery and peaked at POD 1 in all patients compared to baseline ($p<0.0001$), which is consistent with previous studies (J Ho et al., 2011; Prowle et al., 2012). Urine hepcidin-25 remained significantly elevated in all patients at POD 3-5 ($p<0.0001$). Surprisingly, urine hepcidin-25 did not differ between AKI and non-AKI patients in the nested case-controlled cohort [**Figure 4.1**]. Finally, due to the observed ELISA plate-to-plate variability, the internal control was incorporated into the linear mixed model analysis ($p=0.06$). After accounting for this variability, there was only a trend for higher urine hepcidin-25 in non-AKI versus AKI patients.

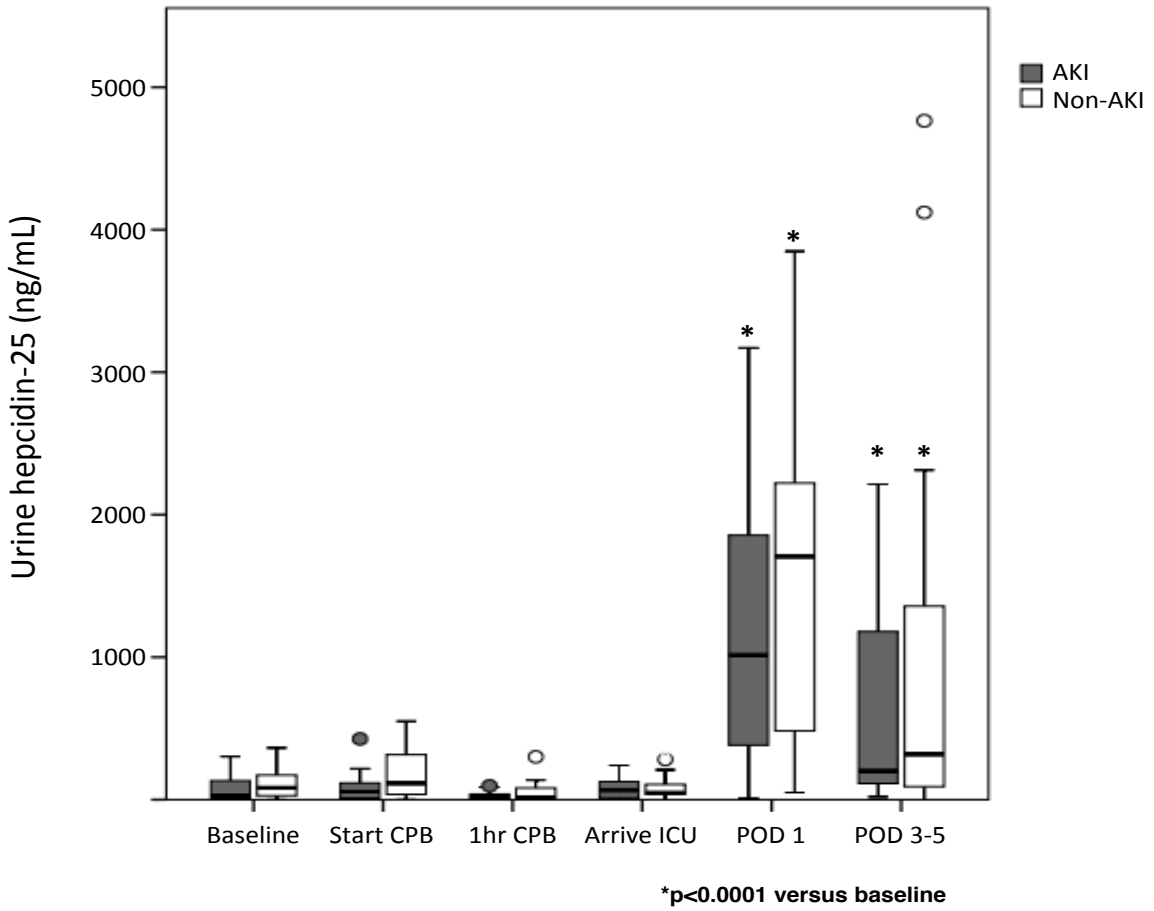

Figure 4.1 Kinetics of urine hepcidin-25 throughout cardiac surgery. A nested case controlled analysis, n=42, confirms elevation of urine hepcidin-25 at POD 1 compared to baseline ($p<0.0001$) in all patients. CPB, cardiopulmonary bypass; ICU, intensive care unit; POD, postoperative day.

4.1.5. Univariate Predictors of AKI

The patient characteristics of the prospective observational cohort (n=306) were evaluated by univariate logistic regression in AKI versus non-AKI patients [**Table 4.4**]. Univariate predictors for AKI development were baseline renal function as determined by eGFR [OR 0.95, 95%CI 0.94-0.97, p<0.0001] and baseline creatinine [OR 1.02, 95%CI 1.01-1.03, p<0.0001]; Thakar score [OR 1.63, 95% CI 1.35-1.97, p<0.0001]; diabetes [OR 3.82, 95%CI 1.93-7.54, p<0.0001] and a previous history of hospitalized congestive heart failure [OR 3.82. 95%CI 1.72-8.47, p=0.0005]. Urine hepcidin-25 and hepcidin-25:Cr at POD 1 were both univariate predictors for the subsequent development of AKI [urine hepcidin-25: OR 0.61, 95%CI 0.45-0.83, p=0.002 and hepcidin-25:Cr OR 0.65, 95%CI 0.46-0.94, p=0.02], with increased urine hepcidin-25 associated with a lower likelihood of developing AKI.

Table 4.4 Univariate predictors of AKI, n=306. Univariate comparisons were made between AKI and non-AKI groups on preoperative, intraoperative and postoperative characteristics.

Characteristic	Odds Ratio	95%CI	OR p-value
Pre-operative			
Age (years)	1.03[a]	0.99 – 1.07	0.09
Male	0.58	0.28 – 1.17	0.14
Baseline eGFR (mL/min/1.73 m^2)	0.95	0.94 – 0.97	<0.0001
Baseline creatinine (mg/dL)	1.02	1.01 – 1.03	<0.0001
Thakar Score	1.63	1.35 – 1.97	<0.0001
Diabetes mellitus	3.82	1.93 – 7.54	<0.0001
Chronic obstructive pulmonary disease	0.57	0.13 – 2.51	0.45
Hospitalized congestive heart failure	3.82	1.72 – 8.47	0.0005
Previous myocardial infarction	0.69	0.34 – 1.42	0.32
Previous CABG	1.65	0.34 – 8.04	0.44
Peripheral arterial disease	2.22	0.83 – 5.94	0.11
Amputation or peripheral arterial disease bypass	-	-	-
Previous cerebrovascular accident	2.28	0.70 – 7.44	0.22
Previous transient ischemic attack	0.64	0.08 – 5.12	0.67
European system for cardiac operation risk (%)	1.06	0.99 – 1.13	0.07
Type of surgery (Isolated CABG)	0.73	0.37 – 1.41	0.35
Intra-operative			
Pump time (minutes)	1.34[b]	0.93 – 1.92	0.12
Cross-clamp time (minutes)	1.10[b]	0.77 – 1.58	0.86
Operating room duration (minutes)	1.03[b]	0.85 – 1.24	0.74
Intraoperative urine output (mL)	0.57[c]	0.25 – 1.29	0.19
Post-operative			
Urine hepcidin-25 (ng/mL)	0.61[d]	0.45 – 0.83	0.002
Urine hepcidin-25:Cr (µg/mmoL)	0.65[e]	0.46 – 0.94	0.02

Odds ratio: [a] Age (per year); [b] Time (per hour); [c] Intraoperative urine output (per L); [d] Urine hepcidin-25 (per 1000 ng/ml); [e] Urine hepcidin-25:Ucr (per 100 µg/mmol)

4.1.6. Multivariate Predictors of AKI – Urine hepcidin-25

A combined multivariate model was developed using patient characteristics: baseline eGFR, diabetes mellitus and urine hepcidin-25. This model demonstrated AKI prediction with an AUC 0.82 [95%CI 0.75-0.88] and reasonable goodness-of-fit (Hosmer-Lemeshow p=0.90) [**Table 4.5**]. Here, urine hepcidin-25 was inversely associated with AKI [OR 0.67, 95%CI 0.50-0.95, p=0.02] on multivariate analysis, with higher concentrations associated with a lower likelihood of developing AKI. It is important to note that the Thakar score did not remain in the model at the end of stepwise logistic regression.

Table 4.5 Urine hepcidin-25 is inversely and independently associated with AKI on multivariate analysis. Higher urine hepcidin-25 concentrations at POD 1 are associated with a lower likelihood of developing AKI. The model, including eGFR, diabetes mellitus and urine hepcidin-25 showed discrimination AUC 0.82.

Characteristic	Odds Ratio	95% CI	p-value
Baseline eGFR (per mL/min/1.72 m^2)	0.96	0.95 – 0.98	<0.01
Diabetes mellitus	3.08	1.47 – 6.45	<0.01
Urine hepcidin-25 (per 1000 ng/mL)	0.67	0.50 – 0.95	0.02

Final multivariable logistic regression model developed using stepwise selection method. Missing covariate values estimated via multiple imputation. Outcome: AKI Defined as Cr>50% from baseline or a ≥26.5 µmol/L within 48 hours.
Area Under the ROC Curve = 0.82 (0.75 – 0.88). Hosmer-Lemeshow p-value = 0.90. eGFR, estimated glomerular filtration rate

4.1.7. Sensitivity Analysis of Hepcidin-25 Inter-assay Variability

In order to control for the observed plate-to-plate variability, the multivariate analysis was repeated including the internal quality control as a variable in a sensitivity analysis. Urine hepcidin-25 remained a predictor of AKI [OR 0.70, 95%CI 0.51-0.96, p=0.03]. The combined AKI prediction model using baseline eGFR, diabetes mellitus and urine hepcidin-25 still demonstrated an AUC 0.83 [95%CI 0.76-0.89) and reasonable goodness-of-fit (Hosmer-Lemeshow, p=0.72), however slightly lower [**Table 4.6**].

Table 4.6 Urine hepcidin-25 is inversely and independently associated with AKI, after controlling for plate-to-plate variability. Again, higher urine hepcidin-25 concentrations at POD 1 are associated with a lower likelihood of developing AKI. The model, including eGFR, diabetes mellitus, internal control and urine hepcidin-25 showed discrimination AUC 0.83.

Characteristic	Odds Ratio	95% CI	p-value
Baseline eGFR (per mL/min/1.73 m^2)	0.96	0.94 – 0.98	<0.0001
Diabetes mellitus	3.61	1.68 – 7.74	0.001
Internal Control (per 100 Units)	0.41	0.17 – 0.95	0.04
Urine hepcidin-25 (per 1000 ng/mL)	0.70	0.51 – 0.96	0.03

Final multivariable logistic regression model developed using stepwise selection method. Missing covariate values estimated via multiple imputation.
Outcome: AKI Defined as Cr>50% from baseline or a ≥26.5 µmol/L within 48 hours.
Area Under the ROC Curve = 0.83 (0.76 – 0.89) Hosmer-Lemeshow p-value = 0.72. eGFR, estimated glomerular filtration rate.

4.1.8. Sensitivity Analysis Excluding Patients with AKI before POD 1

The goal was to explore if urine hepcidin-25 was an early predictor of AKI that precedes clinical detection. Thus, we excluded patients who developed AKI before POD 1 (n=6), and repeated the multivariate analysis with adjusted prospective observational cohort (n=300). Urine hepcidin-25 remained inversely associated with AKI [OR 0.64, 95%CI 0.44-0.95, p=0.03] in the revised model. Furthermore, the combined clinical predictor and biomarker model with baseline eGFR, diabetes mellitus, urine hepcidin-25 and the internal control still showed good AKI prediction with an AUC 0.84 (0.77– 0.91). Finally, the internal control for plate-to-plate variability was not statistically significant in this model (p=0.11) [**Table 4.7**].

Table 4.7 Urine hepcidin-25 is inversely and independently associated with AKI, after excluding patients that developed AKI on or before postoperative day 1. Again, higher urine hepcidin-25 concentrations at POD 1 are associated with a lower likelihood of developing AKI. The model, including eGFR, diabetes mellitus and urine hepcidin-25 showed discrimination AUC 0.84.

Characteristic	Odds Ratio	95% CI	p-value
Baseline eGFR (per mL/min/1.73 m^2)	0.96	0.94 – 0.98	<0.01
Diabetes mellitus	3.17	1.39 – 7.19	<0.01
Internal Control (per 100 Units)	0.49	0.20 – 1.19	0.11
Urine hepcidin-25 (per 1000 ng/mL)	0.64	0.44 – 0.95	0.03

Final multivariable logistic regression model developed using stepwise selection method. Missing covariate values estimated via multiple imputation.
Outcome: AKI Defined as Cr>50% from baseline or 26.5 µmol/L in 48 hours.
Area Under the ROC Curve = 0.84 (0.77 – 0.91) Hosmer-Lemeshow p-value = 0.46. eGFR, estimated glomerular filtration rate.

4.1.9. Sensitivity & Specificity Analysis of AKI Prediction Model

The maximum cutoff was 16% with sensitivity 0.66 and specificity 0.78 for multivariate model including baseline eGFR, diabetes and urine hepcidin-25 **[Table 4.8]**. Sensitivity and specificity values were maximized according to the Youden's J statistic. The Youden's J index was reasonable with 0.44.

Table 4.8 Table Enriched model sensitivity and specificity maximum cutoffs determined by maximum Youden's J index. Multivariate model including including baseline eGFR, diabetes and urine hepcidin-25 demonstrated a maximum cutoff of 16% with sensitivity 0.66 and specificity 0.78.

Probability Cutoff	Sensitivity	Specificity	Youden's J Statistic
Multivariate Model #1			
0%	1.00	0.00	0.00
10%	0.76	0.63	0.39
16% (Maximum Cutoff)	**0.66**	**0.78**	**0.44**
20%	0.56	0.84	0.40
30%	0.37	0.93	0.30
40%	0.27	0.96	0.22
50%	0.20	0.98	0.17
60%	0.15	0.99	0.14
70%	0.05	1.00	0.04
80%	0.00	1.00	0.00
90%	0.00	1.00	0.00
100%	0.00	1.00	0.00

4.2. Chapter 2. Iron Handling Biomarkers – Serum Ferritin and Transferrin Saturation

4.2.1. Prospective Observational Cohort Study Population

The prospective observational cohort characteristics were compared between AKI and non-AKI patients [**Table 4.9**]. Characteristics that differed between AKI and non-AKI groups included: age, $p=0.044$; eGFR, $p<0.0001$; baseline creatinine, $p<0.0001$; Thakar score, $p<0.0001$; diabetes, $p<0.0001$; and hospitalized congestive heart failure, $p=0.006$. Serum ferritin at start of CPB and 1 hour CPB were significantly higher in patients that did not develop AKI [start CPB, $p=0.005$; 1 hour CPB, $p=0.001$]. Urine hepcidin-25 and hepcidin-25:Cr at POD 1 were also significantly higher in patients that did not develop AKI [urine hepcidin-25, $p<0.001$ and hepcidin-25:Cr $p=0.002$].

Table 4.9 Study population characteristics of prospective observational cohort, n=301. Observational comparisons were made between AKI and non-AKI groups on preoperative, intraoperative and postoperative characteristics.

Characteristic	Non-AKI (n = 261)	AKI (n = 40)	p-value
Pre-operative			
Age (years)	66 (60 – 71)	70 (63 – 75)	0.044
Male	198 (76%)	27 (68%)	0.26
Baseline eGFR (mL/min/1.73 m^2)	82 (66 – 96)	62 (42 – 77)	<0.001
Baseline creatinine (mg/dL)	84 (72 – 100)	102 (85 – 143)	<0.001
Thakar Score	2 (1 – 3)	3 (2 – 5)	<0.001
European system for cardiac operation risk (%)	1.4 (0.9 – 2.5)	3.0 (1.4 – 6.0)	<0.001
Diabetes mellitus	76 (29%)	25 (63%)	<0.001
Chronic obstructive pulmonary disease	22 (8%)	2 (5%)	0.75
Hospitalized congestive heart failure	21 (8%)	10 (25%)	0.006
Previous myocardial infarction	97 (37%)	12 (30%)	0.38
Previous CABG	7 (3%)	2 (5%)	0.34
Peripheral arterial disease	19 (7%)	6 (15%)	0.099
Amputation or peripheral arterial disease bypass	2 (1%)	0 (0%)	–
Previous cerebrovascular accident	12 (5%)	4 (10%)	0.24
Previous transient ischemic attack	9 (3%)	1 (3%)	0.76
Type of surgery (Isolated CABG)	165 (63%)	23 (58%)	0.49
Intra-operative			
Pump time (minutes)	94 (73 – 130)	98 (82 – 147)	0.110
Cross-clamp time (minutes)	64 (45 – 90)	62 (45 – 96)	0.57
Operating room duration (minutes)	258 (218 – 336)	266 (207 – 355)	0.98
Intraoperative urine output (mL)	640 (445 – 970)	525 (453 – 853)	0.24
Serum ferritin Start CPB (μg/L)	142 (87 – 264)	78 (46 – 176)	0.005
Serum ferritin 1 Hour CPB (μg/L)	180 (110 – 315)	100 (60 – 200)	0.001
Serum iron (μmol/L)	11 (9 – 14)	10 (8 – 13)	0.38
TIBC (μmol/L)	35 (32 – 39)	34 (31 – 38)	0.74
Transferrin saturation Start CPB (%)	28 (21 – 37)	24 (20 – 41)	0.19
Transferrin saturation 1 Hour CPB (%)	31 (24 – 40)	28 (23 – 41)	0.59
Post-operaive			
Urine hepcidin-25 (μg/L)	1568 (686 – 2733)	623 (152 – 1803)	<0.001
Urine hepcidin-25:Cr (μg/mmoL)	120 (64 –208)	63 (23 – 130)	0.002

Values expressed as median (Interquartile Range) or frequency (percent). eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft; Cr, creatinine; TIBC, total iron-binding capacity.

4.2.2. Sensitivity Analysis for Study Population Versus Excluded Patients

In order to determine the generalizability of findings, we compared the characteristics of patients that were excluded for missed urine and serum bio-banking (n=58) with the final study population (n=301). The baseline characteristics of patients who were excluded for missing urine samples were compared to the final study population and no differences were observed **[Table 4.10].** However, the incidence of AKI in the two groups differed, 13.3% in the study population and 25.6% in the excluded patient population, p=0.015.

Table 4.10 Study population, n=301, and excluded patients, n=58, characteristics. Preoperative and intraoperative characteristics were compared between AKI and non-AKI groups.

Characteristic	Study Population (n = 301)	Excluded Patients (n = 58)	p-value
Pre-operative			
Age (years)	67 (60 – 72)	68 (61 – 74)	0.183
Male	225 (75%)	44 (76%)	0.86
Baseline eGFR (mL/min/1.73 m^2)	78 (63 – 93)	80 (63 – 96)	0.34
Baseline creatinine (mg/dL)	85 (72 – 102)	80 (65 – 109)	0.32
Thakar Score	2 (1 – 3)	2 (1 – 3)	0.46
European system for cardiac operation risk(%)	1.5 (0.9 – 3.0)	1.5 (1.1 – 3.0)	0.21
Diabetes mellitus	101 (34%)	18 (31%)	0.71
Chronic obstructive pulmonary disease	24 (8%)	5 (9%)	0.80
Hospitalized congestive heart failure	31 (10%)	9 (16%)	0.25
Previous myocardial infarction	109 (36%)	19 (33%)	0.62
Previous CABG	9 (3%)	1 (2%)	1.00
Peripheral arterial disease	25 (8%)	5 (9%)	1.00
Amputation or peripheral arterial disease bypass	2 (1%)	0 (0%)	–
Previous cerebrovascular accident	16 (5%)	3 (5%)	1.00
Previous transient ischemic attack	10 (3%)	1 (3%)	1.00
Type of surgery (Isolated CABG)	188 (62%)	35 (60%)	0.76
Intra-operative			
Pump time (minutes)	95 (74 – 132)	92 (71 – 138)	0.64
Cross-clamp time (minutes)	64 (45 – 92)	63 (40 – 93)	0.64
Operating room duration (minutes)	260 (216 – 338)	262 (195 – 326)	0.27
Intraoperative urine output (mL)	620 (450 – 955)	573 (420 – 965)	0.76

Values expressed as median (Interquartile Range) or frequency (percent). Continuous variables compared using Mann-Whitney U Test, categorical variables compared using Chi-Squared Test. eGFR, estimated glomerular filtration rate; CABG, coronary artery bypass graft.

4.2.3. Iron Handling Indices at Start of CPB and 1 Hour CPB

Serum ferritin, transferrin saturation, iron and total iron-binding capacity (TIBC) were compared in AKI versus non-AKI patients **[Figure 4.2].** Intraoperative serum ferritin at the start of CPB and 1 hour CPB was significantly lower in AKI than non-AKI counterparts, p=0.005 and p=0.001 respectively. Intraoperative transferrin saturation at the start of CPB and 1 hour CPB was not significantly different in AKI compared to non-AKI counterparts, p=0.19 and p=0.59, respectively. Additionally, iron and TIBC showed no significant difference between AKI and non-AKI at both start of CPB and 1 hour CPB.

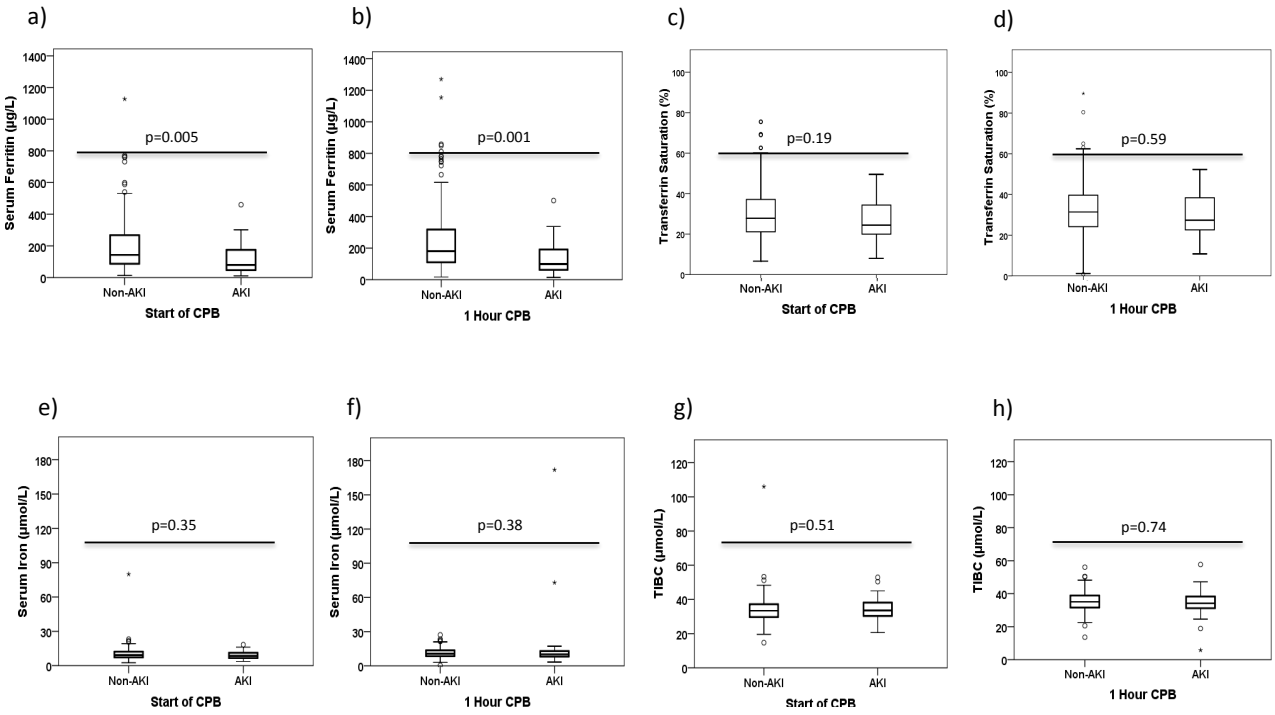

Figure 4.2 Intraoperative start of CPB and 1 Hour CPB serum ferritin, transferrin saturation, iron and TIBC. Lower serum ferritin levels are associated with AKI patients a) start of CPB (p=0.005) and b) 1 hour CPB (p=0.001). No difference in transferrin saturation, iron and TIBC between AKI and non-AKI patients at both start of CPB and 1 hour CPB; c) p=0.19, d) p=0.59, e) p=0.35, f) p=0.38, g) p=0.51 h) p=0.74. CPB, cardiopulmonary bypass; TIBC, total iron-binding capacity.

4.2.4. Univariate Predictors of AKI

Patient characteristics were evaluated by univariate logistic regression in AKI versus non-AKI patients [**Table 4.11**]. Univariate predictors for AKI included preoperative eGFR [OR 1.05, 95%CI 1.03-1.07, p<0.001] and baseline creatinine [OR 1.02, 95%CI 1.01-1.03, p<0.001] and EuroSCORE II [OR 1.06, 95%CI 0.99-1.03, p<0.001]; diabetes mellitus [OR 4.06, 95%CI 2.03-8.12, p<0.001]; and hospitalized congestive heart failure [OR 3.76, 95%CI 1.63-8.82, p=0.006]. The Thakar score was a univariate predictor of AKI [OR 1.62, 95%CI 1.34-1.96, p<0.001]. Total intraoperative packed red blood cell transfusion was a significant predictor of AKI with more units associated with greater likelihood of developing AKI [OR 1.27, 95% CI 1.07-1.50, p=0.007]. Intraoperative serum ferritin was a predictor of AKI, with higher levels associated with a lower likelihood of developing AKI [start CPB: OR 0.62, 95%CI 0.44-0.86, p=0.005; 1 hour CPB: OR 0.62, 95%CI 0.45-0.84, p=0.002]. As expected, postoperative day 1 urine hepcidin-25 and hepcidin-25:Cr were predictors of AKI [urine hepcidin-25: OR 0.62, 95%CI 0.45-0.85, p=0.002] and hepcidin-25:Cr: OR 0.63, 95%CI 0.43-0.92, p=0.015] with higher levels associated with a lower likelihood of developing AKI. Serum transferrin saturation, iron and TIBC were not significant univariate predictors at either start CPB or 1 hour CPB.

Table 4.11 Univariate predictors of postoperative AKI, n=301. Univariate comparisons were made between AKI and non-AKI groups on preoperative, intraoperative and postoperative characteristics.

Characteristic	Odds Ratio	95% CI	OR p-value
Pre-operative			
Age (years)	1.03	0.99 – 1.06	0.129
Male	0.66	0.32 – 1.36	0.26
Baseline eGFR (mL/min/1.73 m^2)	1.05	1.03 – 1.07	<0.001
Baseline creatinine (mg/dL)	1.02	1.01 – 1.03	<0.001
Thakar Score	1.62	1.34 – 1.96	<0.001
European system for cardiac operation risk (%)	1.06	0.99 – 1.03	<0.001
Diabetes mellitus	4.06	2.03 – 8.12	<0.001
Chronic obstructive pulmonary disease	0.57	0.13 – 2.53	0.75
Hospitalized congestive heart failure	3.76	1.63 – 8.82	0.006
Previous myocardial infarction	0.73	0.35 – 1.49	0.38
Previous CABG	1.91	0.38 – 9.54	0.34
Peripheral arterial disease	2.25	0.84 – 6.02	0.099
Amputation or peripheral arterial disease bypass	–	–	–
Previous cerebrovascular accident	2.31	0.71 – 7.54	0.24
Previous transient ischemic attack	0.72	0.09 – 5.82	0.76
Type of surgery (Isolated CABG)	0.79	0.40 – 1.55	0.49
Intra-operative			
Total PRBC transfusion (units)	1.27	1.07 – 1.50	0.007
Pump time (minutes)	1.36[a]	0.95 – 1.96	0.094
Cross-clamp time (minutes)	1.05[a]	0.71 – 1.54	0.82
Operating room duration (minutes)	1.06[a]	0.87 – 1.27	0.58
Intraoperative urine output (mL)	0.95[b]	0.87 – 1.03	0.192
Serum ferritin Start CPB (μg/L)	0.62[c]	0.44 – 0.86	0.005
Serum ferritin 1 Hour CPB (μg/L)	0.62[c]	0.45 – 0.84	0.002
Serum iron (μmol/L)	0.97	0.94 – 1.01	0.116
TIBC (μmol/L)	1.01	0.96 – 1.06	0.56
Transferrin saturation Start CPB (%)	1.23[d]	0.91 – 1.67	0.176
Transferrin saturation 1 Hour CPB (%)	1.14[d]	0.99 – 1.31	0.073
Post-operative			
Urine hepcidin-25 (μg/L)	0.62[e]	0.45 – 0.85	0.002
Urine hepcidin-25:Cr (μg/mmoL)	0.63[f]	0.43 – 0.92	0.015

Odds Ratio: [a] Time (per 60 minutes) [b] Intraoperative urine output (per 100mL) [c] Serum ferritin (per 100 μg/L) [d] Transferrin saturation (per 10%) [g] Urine hepcidin-25 (per 1000 μg/L) [h] Urine hepcidin-25:Cr (per 100 μg/mmoL).

4.2.5. Multivariate Predictors of AKI – Serum ferritin and transferrin saturation

A baseline clinical model using the Thakar score predicted the development of postoperative AKI [OR 1.62, 95%CI 1.34-1.96, p<0.001]. Enriched model 1 (adding serum ferritin and transferrin saturation) demonstrated that intraoperative serum ferritin and transferrin saturation at 1 hour CPB predicted postoperative AKI, after adjusting for Thakar score [serum ferritin OR 0.66, 95% CI 0.48-0.91, p=0.012 and transferrin saturation OR 1.26 95% CI 1.02-1.55, p=0.035] [**Table 4.12**]. As different iron sequestering proteins may be linked by the same causal pathways, we next sought to determine if they were independent predictors of AKI. Urine hepcidin-25 at POD 1 remained an independent predictor of AKI after adjusting for Thakar score, serum ferritin and transferrin saturation [OR 0.68, 95% CI 0.48-0.95, p=0.026] in enriched model 2 [**Table 4.12**].

Table 4.12 Multivariate analyses of iron handling measures of AKI prediction. Serum ferritin, transferrin saturation, urine hepcidin-25 and Thakar score were predictors of AKI on multivariate analysis.

Model Type	Odds Ratio	95% CI	p-value
Base Model (pre-operative clinical prediction)			
Thakar Score	1.62	1.34 - 1.96	<0.001
Enriched Model 1 (clinical prediction + Fe handling 1 hour CPB)			
Thakar Score	1.62	1.33 - 1.98	<0.001
Serum ferritin (per 100 μg/L)	0.66	0.48 - 0.91	0.012
Transferrin saturation (per 10%)	1.26	1.02 - 1.55	0.035
Enriched Model 2 (clinical prediction + Fe handling 1 hour CPB + urine hepcidin-25 POD 1)			
Thakar Score	1.51	1.23 - 1.85	<0.001
Serum ferritin (per 100 μg/L)	0.67	0.49 - 0.92	0.013
Transferrin saturation (per 10%)	1.34	1.06 - 1.69	0.014
Urine hepcidin-25 (per 1000 μg/L)	0.68	0.48 - 0.95	0.026

Logistic regression was performed using the Thakar AKI risk prediction score as a base clinical model, which demonstrated good discrimination [AUC 0.72; 95%CI 0.62-0.81] **[Table 4.13].** Enriched model 1 demonstrated better discrimination [AUC 0.76; 95% CI 0.67-0.85] compared to the Thakar score **[Table 4.13].** Further improvement in the model discrimination was seen with the addition of urine hepcidin-25 [AUC 0.80; 95% CI 0.72-0.87]. The model sensitivity and specificity were 0.78 and 0.63, respectively. Finally, the addition of iron handling biomarkers improved the overall quality of the model as demonstrated by the lower AIC for enriched models 1 and 2 compared to the base model (200.0, 195.1 vs. 213.5) **[Table 4.13].**

Table 4.13 Multivariate model performance. Base model, Thakar score alone demonstrated AUC 0.72. AKI discrimination improved in enriched model 1 to AUC 0.76 and further improvement was seen in enriched model 2 with AUC 0.80.

Diagnostic Test	Base Model (Thakar)	Enriched Model #1	Enriched Model #2
Global p-value	<0.001	<0.001	<0.001
Discrimination (AUC (95% CI]))	0.72 (0.62 - 0.81)	0.76 (0.67 - 0.85)	0.80 (0.72 - 0.87)
Akaike Information Criterion	213.5	200.0	195.1
Goodness of Fit (Hosmer-Lemeshow test statistic (p-value]))	2.76 (0.60)	11.41 (0.180)	15.09 (0.057)
Sensitivity	0.63	0.53	0.78
Specificity	0.75	0.92	0.63

Maximum cutoff used to generate sensitivity and specificity values. AUC, Area under the Receiver Operating Characteristic.

4.2.6. Model Performance: Net reclassification index (NRI) and Integrated discrimination improvement (IDI)

Intraoperative iron-binding proteins improved preoperative clinical prediction as measured by the absolute difference in AUC and IDI (enriched model 1: ΔAUC 0.05, 95%CI 0.01-0.09, p=0.011; IDI 0.08, 95%CI 0.02-0.15, p=0.011). Enriched model 1 was more likely to correctly reclassify subjects according to the observed outcomes (NRI= 0.20), however the difference was not statistically significant (p=0.25). Furthermore, addition of urine hepdicin-25 in enriched model 2 signaled superior model discrimination relative to clinical prediction alone, and by a higher margin than enriched model 1, measured by both absolute difference in AUC and by IDI (ΔAUC 0.08, 95%CI 0.03-0.13, p=0.002; IDI 0.10, 95%CI 0.03-0.17, p=0.003). This enriched model 2 was also more likely to correctly reclassify subjects (NRI 0.50, 95%CI 0.18-0.82, p=0.003) [**Table 4.14**].

Table 4.14 Multivariate model comparison of independent predictors of AKI using net reclassification index and integrated discrimination improvement.

Model Comparison (Enriched Model #1 vs. Base Model)	**Value**	**95% CI**	**p-value**
Δ AUC	0.05	0.01 - 0.09	0.011
Net Reclassification Index	0.20	-0.14 - 0.53	0.25
Integrated Discrimination Improvement	0.08	0.02 - 0.15	0.011
Model Comparison (Enriched Model #2 vs. Base Model)	**Value**	**95% CI**	**p-value**
Δ AUC	0.08	0.03 - 0.13	0.002
Net Reclassification Index	0.50	0.18 - 0.82	0.003
Integrated Discrimination Improvement	0.10	0.03 - 0.17	0.003

AUC, Area under the Receiver Operating Characteristic.

4.2.7. Correlation of Iron Binding Proteins

As serum ferritin, serum transferrin saturation and urine hepcidin-25 are all markers of iron binding or sequestration; we sought to determine if they were highly correlated in a sensitivity analysis. A correlation matrix was generated which demonstrated that these variables were not highly correlated and none were collinear. Therefore, no decision was taken to remove any variables from the models [**Table 4.15**].

Table 4.15 Correlation matrix of iron handling variables serum ferritin, urine hepcidin-25 and transferrin saturation (Spearman's Rank Correlation Coefficient**).**

Variable	Serum ferritin	Urine hepcidin-25	Transferrin saturation
Serum ferritin	-	ρ = 0.19	ρ = 0.27
Urine hepcidin-25	ρ = 0.19	-	ρ = 0.19
Transferrin saturation	ρ = 0.27	ρ = 0.19	-
Spearman's rank correlation coefficients (ρ) demonstrate relationship between variables. Multiple linear regression with AKI as a continuous variable was used to determine multi-collinearity. Variables were considered highly correlated if |ρ| > 0.7 and collinear if the variance inflation factor was > 10.			

4.2.8. Sensitivity Analysis Excluding Patients with AKI before POD 1

As the goal was to identify early predictors of AKI before clinical detection, we sought to exclude patients that developed AKI prior to the measurement of serum ferritin and transferrin saturation at 1 hour CPB and urine hepcidin-25 at POD 1. Six patients developed KDIGO AKI prior to POD 1, therefore enriched model 2 multivariate analysis was repeated on adjusted prospective observational cohort (n=295). **[Table 4.16]** Serum ferritin and urine hepcidin-25 remained independent predictors of postoperative AKI, however serum transferrin saturation did not. The overall model still showed good AKI prediction with AUC 0.81 (0.73 – 0.87).

Table 4.16 Multivariate analyses and model performance of iron handling measures for AKI prediction excluding patients that developed KDIGO AKI prior to POD 1, n=295. Serum ferritin, urine hepcidin-25 and Thakar score remained predictors of AKI on multivariate analysis and model discrimination remained reasonable with AUC 0.81.

Model Type	**Odds Ratio**	**95% CI**	**p-value**
Enriched model #2 (clinical prediction + Fe handling 1 hour CPB + urine hepcidin-25 POD 1)			
Thakar Score	1.52	1.22 - 1.89	<0.001
Serum ferritin (per 100 μg/L decrease)	1.63	1.09 - 2.42	0.017
Transferrin Saturation (per 10% increase)	1.22	0.86 - 1.71	0.27
Urine hepcidin-25 (per 1000 μg/L decrease)	1.52	1.02- 2.25	0.039
Diagnostic Test		**Enriched Model #2**	
Global p-value		<0.001	
Discrimination (AUROC (95% CI))		0.81 (0.73 – 0.87)	
Akaike Information Criterion		173.7	
Goodness of Fit (Hosmer-Lemeshow test statistic (p-value))		11.84 (0.158)	
Sensitivity		0.59	
Specificity		0.84	
Model Comparison (Enriched Model #2 vs. Base Model)	**Value**	**95% CI**	**p-value**
Δ AUROC	0.08	(0.02 – 0.13)	0.009
Net Reclassification Index	0.08	(0.02 – 0.14)	0.006
Integrated Discrimination Improvement	0.06	(0.01 – 0.11)	0.030

Maximum cutoff used to generate sensitivity and specificity. AUC, Area under the Receiver Operating Characteristic

4.2.9. Sensitivity & Specificity Analysis of AKI Prediction Models

The maximum cutoff was determined for enriched model 1 and enriched model 2 to determine sensitivity and specificity. **[Table 4.17]** Enriched model 1 had a maximum cutoff of 24%, with sensitivity 0.53 and specificity 0.92. Enriched model 2 had a maximum cutoff of 10%, with sensitivity 0.78 and specificity 0.63. Sensitivity and specificity values were maximized according to the Youden's J statistic. The Youden's J index was reasonable in enriched model 1 with 0.45 and in enriched model 2 with 0.41.

Table 4.17 Enriched model sensitivity and specificity maximum cutoffs determined by maximum Youden's J index. In enriched model 1, a maximum cutoff of 24% gave sensitivity 0.53 and specificity 0.92. In enriched model 2, a maximum cutoff of 10% gave sensitivity 0.78 and specificity 0.63.

Probability Cutoff	Sensitivity	Specificity	Youden's J Statistic
Enriched Model #1			
0%	1.00	0.00	0.00
10%	0.73	0.60	0.35
20%	0.55	0.86	0.41
24% (Maximum Cutoff)	**0.53**	**0.92**	**0.45**
30%	0.43	0.94	0.37
40%	0.25	0.98	0.23
50%	0.20	0.99	0.19
60%	0.15	0.99	0.14
70%	0.05	1.00	0.05
80%	0.03	1.00	0.03
90%	0.03	1.00	0.03
100%	0.00	1.00	0.00
Enriched Model #2			
0%	1.00	0.00	0.00
10% (Maximum Cutoff)	**0.78**	**0.63**	**0.41**
20%	0.55	0.84	0.39
30%	0.43	0.94	0.37
40%	0.33	0.97	0.30
50%	0.20	1.00	0.20
60%	0.15	1.00	0.15
70%	0.05	1.00	0.05
80%	0.05	1.00	0.05
90%	0.05	1.00	0.05
100%	0.00	1.00	0.00

4.3. Chapter 3. Positive Control Development for Lactotransferrin Assay

4.3.1. Activity-based Protein Profiling

An activity-based probe Fp-TAMRA was used in an SDS-PAGE gel to directly assess the functional state of the enzyme lactotransferrin (Product Number 61326, Sigma-Aldrich). The well with Fp-TAMRA probe showed a notable band around 80KDa, which indicates that lactotransferrin reacts with the Fp-TAMRA probe. As expected, the negative control without Fp-TAMRA probe showed no band at 80KDa. The protein load per well was consistent, demonstrated by Sypro ruby and Coomassie blue staining **[Figure 4.3].**

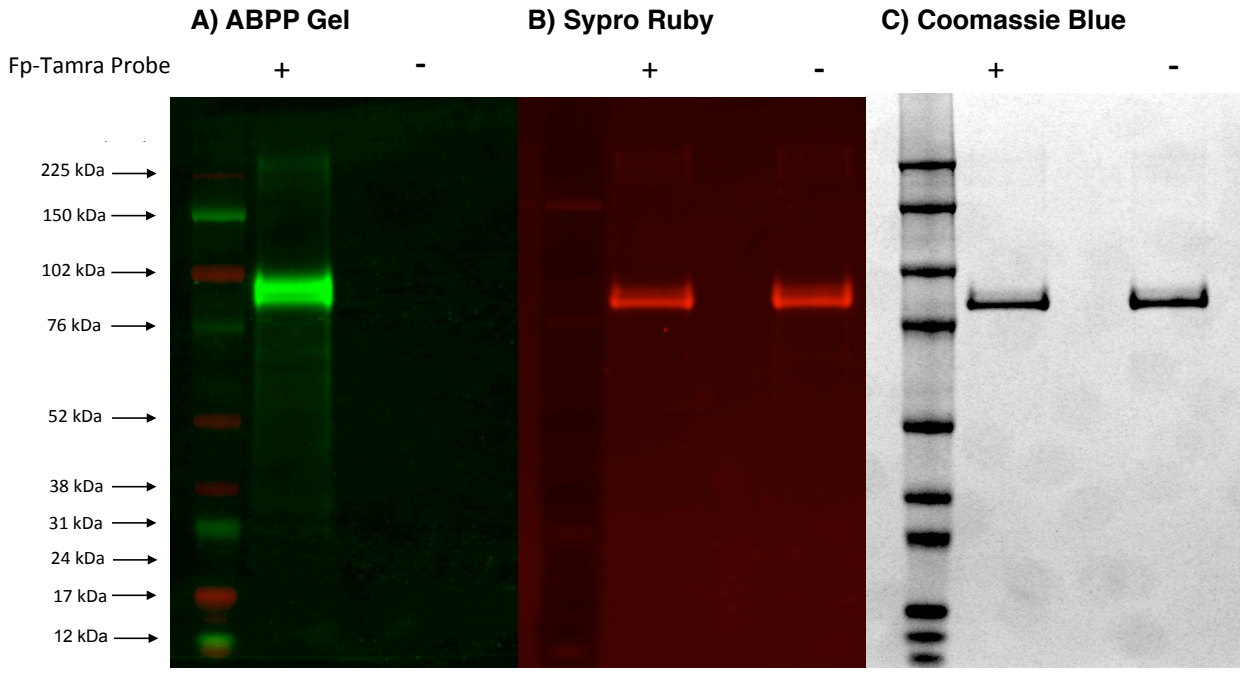

Figure 4.3 Demonstration of probe reactivity with commercial lactotransferrin, Product Number 61326, Sigma-Aldrich. A) ABPP using FP-TAMRA probe reacts with commercial lactotransferrin. B) Sypro Ruby Stain to confirm equal protein load per well. C) Coomassie Blue staining to confirm equal protein load per well. ABPP, activity-based protein profiling; FP-TAMRA, fluorophosphonate- tetramethyl-6-carboxyrhodamine; KDa, kilodalton.

4.3.2. Enzyme Assay Performance

Multiple enzyme assays were performed using lactotransferrin and substrate (Z-phe-Arg-AMC) at different concentrations. **[Table 4.18]** The concentration of the substrate ranged from 20µM to 100µM, based around optimal concentration of 50µM and the concentration of the enzyme was 0.1µM. Assays were run at optimal pH 7.4 and physiological temperature 37°C. Plates were run for 30 minutes or longer, with fluorescence measured at 2-minute intervals and all conditions showed no activity, demonstrated by a straight line (no slope) or downwards sloping line. Enzyme assay conditions were modified with EDTA and DTT, but no activity was observed. A graphical representation of a lactotransferrin enzyme assay performed in reducing conditions is displayed **[Figure 4.4].** The downward slope indicates photobleaching of the fluorophore AMC.

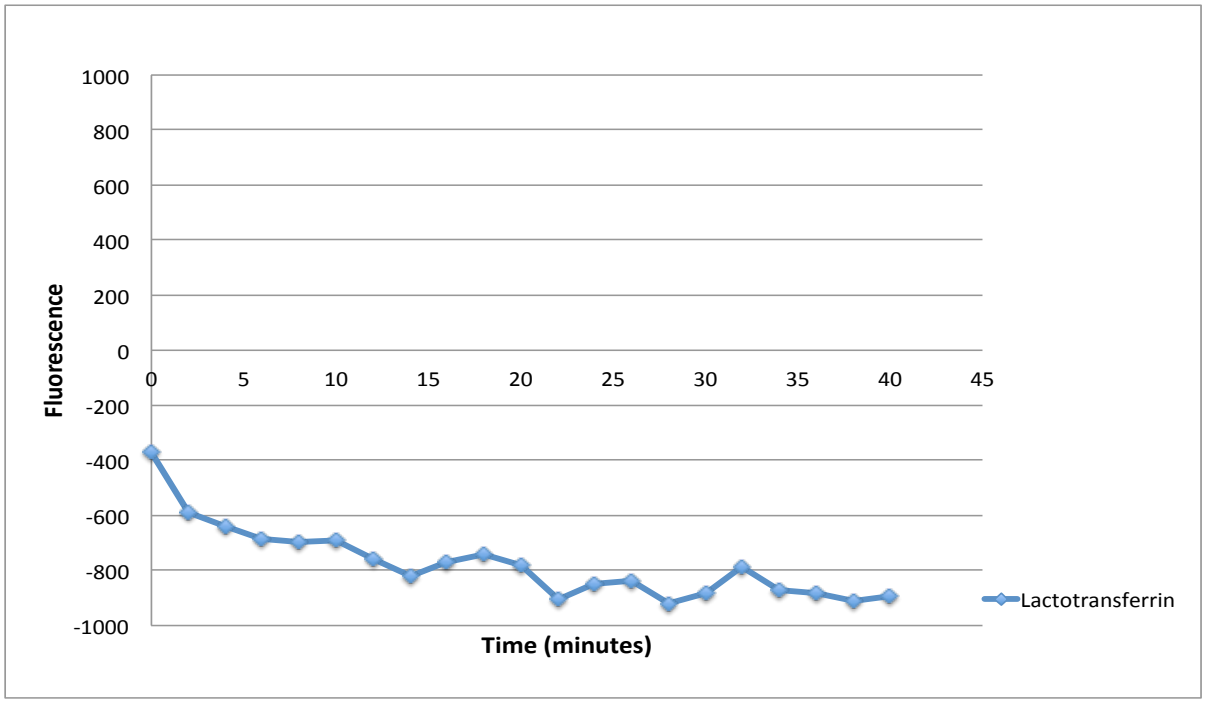

Figure 4.4 Enzyme assay performance of commercial lactotransferrin in reducing conditions using substrate Z-phe-arg-AMC. Substrate concentration, 50µM and enzyme concentration, 0.1µM at pH 7.4 and 37°C was performed in duplicate and averaged over 40 minutes. The slope displayed represents the result of: (lactotransferrin+ Z-phe-arg-AMC+ buffer) - (water + Z-phe-arg-AMC+ buffer). The downward slope indicates possible photobleaching of substrate fluorophore AMC.

Table 4.18 Enzyme assay conducted with varying substrate and lactotransferrin concentrations. No activity was seen in any condition. The addition of EDTA and DTT had no impact on activity.

Enzyme Assay Conditions	Activity
Lactotransferrin (0.1µM) Substrate (20-100µM) 200mM phosphate buffer (pH=7.4)	ND
Lactotransferrin (0.1µM) Substrate (50µM) 200mM phosphate buffer (pH=7.4) 5mM EDTA 1mM DTT	ND
EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; ND, not detected.	

4.3.3. Iron Separation to Obtain Detectable Lactotransferrin Activity

As no lactotransferrin activity was detected in the previous enzyme assays, we performed iron removal steps with the goal to determine if the presence of iron may be interfering with the assay. Two different methods were used to separate iron and lactotransferrin. The first was dialysis overnight using an 8KDa dialysis membrane and the second method was same-day and overnight iron chelation using deferoxamine. In the ABPP gel, all conditions with Fp-TAMRA application show a band around 80KDa, indicating a reaction between lactotransferrin and Fp-TAMRA probe. Conditions included dialyzed lactotransferrin, overnight and same-day deferoxamine treatment of lactotransferrin and the combination of overnight deferoxamine treatment on dialyzed lactotransferrin. The intensity of the activity band was similar in all conditions. Untreated lactotransferrin was used for the control, combined with and without Fp-TAMRA probe **[Figure 4.5].** Coomassie blue staining on the same gel show that protein load was consistent in all wells. Despite efforts to remove iron from lactotransferrin, no intensity change was observed between control and iron separation conditions on the ABPP gel.

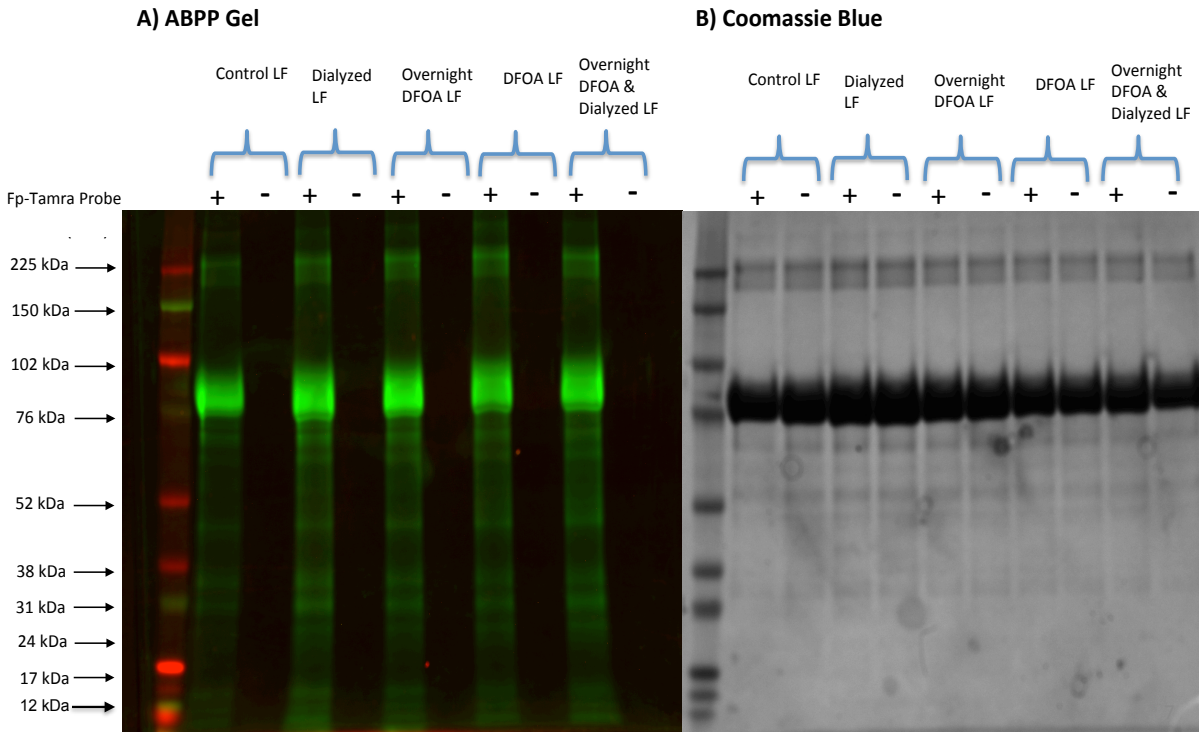

Figure 4.5 Dialysis and iron chelation using deferoxamine of lactotransferrin. a) ABPP gel shows band around 80KDa, indicating lactotransferrin and FP-TAMRA probe reaction in all conditions. b) Coomassie blue stain was used to show protein load consistency in all the wells. KDa, kilodalton; LF, lactotransferrin; DFOA, deferoxamine; FP, flourophosphonate.

4.3.4. Lactotransferrin Enzyme Assay Following Iron Separation Methods

Dialyzed lactotransferrin, overnight and same-day deferoxamine treated lactotransferrin and dialyzed lactotransferrin combined with overnight deferoxamine treatment were analyzed in an enzyme assay. **[Table 4.19]** The concentration of the substrate was 50μM, and the concentration of lactotransferrin control was 0.1μM. Assays were run at optimal pH 7.4 and physiological temperature 37°C. Plates were run for 30 minutes, with fluorescence measured at 2-minute intervals and all conditions showed no activity, demonstrated by a straight line (no slope) or downwards sloping line, which indicates photobleaching of the fluorophore AMC.

Table 4.19 Enzyme assay for lactotransferrin that underwent dialysis treatment and/or deferoxamine chelation to separate iron. No activity was observed in any condition.

Enzyme Assay Conditions	Activity
Lactotransferrin (0.1μM) Substrate (50μM) 200mM phosphate buffer (pH=7.4)	ND

5. DISCUSSION

5.1. CSA-AKI Model

Iron regulation and sequestration mechanisms throughout cardiac surgery remain unclear, however, iron regulatory pathways are known to be a significant factor in renal ischemia reperfusion injury. Our research focused on markers of iron regulation in a prospective observational cohort of adult cardiac surgery patients that developed acute kidney injury secondary to ischemia reperfusion injury following cardiopulmonary bypass.

A prospective observational study design is an unselected cohort that allows for unbiased data collection of a specific exposure within a chosen time frame (Song & Chung, 2010). Scientific 'evidence' has been classified into a widely accepted hierarchical system to interpret biomedical research findings to approach clinical decisions. The overall goal is to organize different study designs and quality of the literature into a hierarchy to establish recommendations for clinical practice (Petrisor & Bhandari, 2006). A large well-defined prospective observational cohort study or systematic review with meta-analysis ranks high whereas expert opinion ranks the lowest in evidence-based medicine, which includes diagnostic research. Randomized control trials are at the peak of the hierarchy of evidence-based medicine, however this is not always appropriate or possible to do (Centre for Evidence Based Medicine. [Accessed Oct.10, 2017]; Available at http://www.cebm.net).

A clinical cardiac surgery cohort is a well-defined model to identify diagnostic and therapeutic targets to ameliorate AKI because the IRI insult has predictable timing; is relatively standardized; and patient follow-up in-hospital for outcomes highly feasible. Baseline/control data is readily

available and captured to study the patient population and AKI outcomes. The timing of AKI onset in a cardiac surgery model is believed to be in the intraoperative period during IRI. Therefore, serial urine and serum samples were collected for each patient at: baseline, start of CPB, 1 hour CPB, arrival to ICU, POD 1 and POD 3-5. Multiple time points before, during and following cardiac surgery allow for a comprehensive evaluation of iron regulation kinetics.

The onset and progression of AKI can be studied from an iron regulation perspective to better understand the pathophysiology throughout IRI. The iron regulation markers of interest for this project included urine hepcidin-25, serum ferritin, serum transferrin saturation and urine lactotransferrin. The first part of this project independently validates hepcidin-25 in a prospective observational adult cardiac surgery cohort (Choi et al., *Can J Kid Health Dis*, accepted Aug 04, 2017). We previously characterized urine hepcidin-25 as an early marker for AKI at POD 1 and demonstrated that it is inversely associated with AKI (J Ho et al., 2011; Ho et al., 2009). The second part of this project focuses on iron binding proteins serum ferritin and transferrin saturation that change in an earlier time frame of cardiac surgery, the intraoperative period. The clinical risk prediction Thakar score was analyzed to determine improvement in overall AKI risk prediction using additional biomarkers. The third part of this project evaluates the enzymatic activity of commercial lactotransferrin with the goal to develop a positive control for a urine lactotransferrin activity assay. Lactotransferrin was identified as a promising candidate in AKI patients using activity-based protein profiling by colleague Mario Navarrete (*Clinical Proteomics*, under review).

5.2. Potential Biomarkers of AKI

The kidneys are a vital human organ required for blood filtration, blood pressure regulation, water balance, mineral absorption and disposal and hormone and urine production. A variety of acute and chronic kidney conditions can alter structure and function, resulting in temporary to potentially irreversible tubular damage. Biomarkers can be measured and monitored to evaluate changes in pathophysiological processes that lead to AKI (Strimbu & Tavel, 2010). An ideal biomarker should be non-invasive, highly sensitive and specific for AKI, and clinically measured and monitored with ease (Devarajan, 2011; Nguyen & Devarajan, 2008). Potential non-invasive proteins, urine hepcidin-25, serum ferritin, serum transferrin saturation and urine lactotransferrin that regulate iron sequestration may act as biomarkers for the early detection of AKI in a human model of IRI. The performance of non-invasive biomarkers to better detect AKI have been studied in both children and adults in a variety of clinical settings, including the intensive care unit, post coronary angiography and cardiac surgery (Coca, Yusuf, Shlipak, Garg, & Parikh, 2009; Kellum et al., 2012). However, biomarkers that can detect the early onset of AKI are yet to be translated into clinical practice.

Currently, clinical AKI is diagnosed using serum creatinine, however, detection is delayed following irreversible insult and injury (Kellum et al., 2012; Chertow et al., 2005). The ability to detect the early onset of AKI following cardiac surgery using biomarkers may improve patient morbidity and mortality. Extensive AKI biomarker research has been conducted over the past decade in the postoperative period. However, these studies have limitations including, inconsistent discrimination of a biomarker between different center studies and more importantly AKI detection outside the potential therapeutic window of reversibility (Ho, Tangri, et al., 2015).

In terms of AKI secondary to IRI following cardiac surgery, many biomarker candidates have been studied but the majority do not show significant changes until the postoperative period, which is believed to be outside the potential therapeutic window of reversibility (Ho et al., 2014). These biomarkers show moderate discrimination at best when measured 24 hours post-cardiac surgery (Ho, Tangri, et al., 2015). Intraoperative AKI biomarkers show weaker discrimination with the majority of research focused on NGAL, which shows inconsistencies between different study populations (Nguyen & Devarajan, 2008).

5.2.1. Urine Hepcidin-25

An independent validation of urine hepcidin-25 as a biomarker to better detect AKI was performed in 306 patients that underwent CPB surgery. The principle finding was that urine hepcidin-25 was inversely associated with AKI in an independent, prospective observational adult cardiac surgery cohort. The low risk nature of our cohort was one potential reason for why there was no statistical difference in urine hepcidin-25 between the AKI and non-AKI groups of the nested case control cohort. Urine hepcidin-25 levels peaked in both AKI and non-AKI patients at POD1 in the nested-case controlled cohort, therefore POD 1 was chosen for further univariate and multivariate model analyses using the full prospective observational cohort. Importantly, a combined multivariate model with clinical baseline eGFR, diabetes mellitus and urine hepcidin-25 demonstrated an AUC 0.82 for the subsequent development of AKI. These results were congruent with our previous urine hepcidin-25 study (J Ho et al., 2011) and other single center studies (Haase-Fielitz et al., 2011; Prowle et al., 2012), with the extension of these results by demonstrating that they outperformed clinical prediction alone (Thakar score). Indeed, urine hepcidin-25 was inversely associated with AKI at POD 1 in another cohort (n=301) that

was based on the same overall patient population. Urine hepcidin-25 at POD 1 combined with intraoperative ferritin and transferrin saturation were each independent predictors of AKI, with model performance AUC 0.80, which outperformed clinical prediction alone.

Hepcidin-25 is an endogenous, antimicrobial hepatic peptide that indirectly regulates circulating iron by keeping it sequestered intracellularly by degrading ferroportin, the only known iron exporter located predominantly on hepatocytes, macrophages and enterocytes, but also detected on the basolateral side of renal tubular cells (Ganz & Nemeth, 2012; Scindia et al., 2015). Ferroportin expression in the spleen and liver is increased during IRI, increasing circulating iron (Scindia et al., 2015). Hepcidin is freely filtered through the glomerulus and reabsorbed at the proximal tubule via megalin-dependent endocytosis (van Swelm et al., 2016; Peters et al., 2013); the fractional excretion is negligible with up to 97% reabsorbed in physiological conditions (Swinkels et al., 2008). Negligible amounts of hepcidin-25 were excreted in urine of all nested-case control patients prior to cardiac surgery with significant elevation at POD 1 following IRI. While the source of the observed urine hepcidin-25 in our model is unknown, the hepcidin gene, renal Hamp1 mRNA expression was increased after 4 hours in mice with hemoglobin induced AKI relative to controls. Furthermore, hepcidin was locally synthesized in the distal tubules in mice with AKI (van Swelm et al., 2016). Kidney immunostaining of patients with hypernephroma show local hepcidin production in the thick ascending limb, connecting tubule and distal regions (Kulaksiz et al., 2005). Interestingly, increased ferroportin expression and iron deposition was seen in the distal region suggesting hepcidin presence for renoprotection and not in the proximal region where hepcidin-25 is reabsorbed and thus absent (van Swelm et al., 2016). These findings are consistent with cardiac surgery models of AKI which demonstrated increased

urine hepcidin-25 and increased fractional excretion of hepcidin-25 in non-AKI patients, in the absence of substantive changes to plasma/serum hepcidin-25, even after excluding chronic kidney disease patients (Prowle et al., 2012; Haase-Fielitz et al., 2011).

As urine hepcidin-25 begins at similar levels pre-operation and is increased in AKI and non-AKI patients following IRI, we previously postulated that it is a renoprotective response which may be mediated via the intracellular sequestration of iron to limit oxidative stress, free radical damage and renal injury (Ho et al., 2009). Since then, Scindia et al have found hepcidin to be highly protective in a murine model of renal IRI. Notably, the administration of exogenous hepcidin resulted in a significant reduction in tubular injury, apoptosis, renal oxidative stress and neutrophil infiltration, while simultaneously improving renal function (Scindia et al., 2015). Furthermore, these findings are highly consistent with van Swelm et al. who demonstrated that the administration of hepcidin-25 decreased markers of kidney injury in a murine model of hemoglobin-mediated AKI (van Swelm et al., 2016). While hepcidin-25 is renoprotective in animal models, its underlying mechanisms in CSA-AKI remains unknown as there are conflicting observations regarding the role of apoptosis and renal ferroportin (Scindia et al., 2015; van Swelm et al., 2016). Additionally, it is tempting to speculate that the lack of consistently observed inhibition of apoptosis by hepcidin-25 administration (Scindia et al., 2015; van Swelm et al., 2016) may be due to activation of alternative cell death pathways, such as ferroptosis; but this cannot be clarified in our human model of IRI-AKI. Notably, these observations suggest that hepcidin-25 may act via alternative pathways to prevent renal tubular epithelial cell death (Scindia et al., 2015; van Swelm et al., 2016). These murine model findings coincide with our

iron handling results, suggesting that iron-dependent pathways are significant in renal injury, alongside other conventional forms of cell death.

Interestingly, we identified active urinary dipeptidyl peptidase IV in AKI patients with the activity-based protein profiling (*Clinical Proteomics*, under review), which may degrade hepcidin-22 to hepcidin-20 (Schranz et al., 2009). Taken together, we speculate that elevation of urine hepcidin-25 following cardiac surgery is the result of increased distal nephron production in response to IRI, but it remains to be determined if luminal enzyme activity plays an in-vivo regulatory role by inactivating hepcidin-25. The intersection of these findings with the observed renoprotective effect of hepcidin in murine models of renal IRI and hemoglobin-mediated AKI, all suggest that iron handling is a crucial and potentially modifiable factor of cardiac surgery associated-AKI.

5.2.2. Serum Ferritin and Transferrin Saturation

To further investigate iron regulation in AKI, we looked at intraoperative serum ferritin and transferrin saturation. The diagnostic performance of iron handling biomarkers serum ferritin and transferrin saturation was evaluated at an earlier time point throughout IRI compared to hepcidin-25. Serum ferritin and transferrin saturation were quantified in the intraoperative period and analyzed in conjunction with clinical prediction alone. The principal finding of this study is that intraoperative prediction of AKI following cardiac surgery is feasible with iron-binding proteins using readily available clinical assays. The Thakar score was evaluated in 301 patients that underwent CPB surgery showing reasonable AKI prognosis with AUC 0.72. Model discrimination was identical in a similar study investigating patients that underwent isolated

CABG surgery in northern New England (Brown et al., 2007) and comparable to a cardiac surgery cohort at the Cleveland Clinic Foundation with AUC 0.81 (Thakar et al., 2005). Upon multivariate analysis, clinical prediction using the Thakar score and intraoperative serum ferritin and transferrin saturation distinguishes AKI development with an AUC 0.76. This model discrimination improved with the addition of POD 1 urine hepcidin-25 to AUC 0.80 and preceded the clinical detection of AKI. Each of these markers is involved in iron-binding and regulation; however, it is notable that they are not highly correlated and are each independent predictors of AKI. Taken together, these data suggest that different iron regulatory pathways are activated in response to IRI to collectively mitigate the impact of catalytic iron release on CPB, which is a known inciting factor for AKI (Scindia et al., 2015; van Swelm et al., 2016).

Ferritin is an iron binding protein that sequesters up to 4500 atoms of iron intracellularly until required for metabolic processes (Orino et al., 2001). Ferritin is also found in circulation and reflects the size of body iron stores (Knovich, Storey, Coffman, Torti, & Torti, 2009) and is used to determine iron deficiency or overload (Wish, 2006). While it is possible that intraoperative ferritin reflects an acute phase response (Paparella, Yau, & Young, 2002), we speculate that higher ferritin levels are associated with increased iron-binding capacity to mitigate the effects of catalytic iron release (Leaf et al., 2015; Scindia et al., 2015). Indeed, increased intraoperative ferritin was observed in non-AKI patients whereas increased inflammation would have been anticipated in AKI patients. Our serum ferritin findings add to previous literature that looked at preoperative levels prior to induction of anesthesia and intraoperative levels at induction of anesthesia in adult cardiac surgery patients. Tuttle et al measured pre-anesthesia (preoperative) serum ferritin levels in AKI (158±119μg/L) and non-AKI (163±125μg/L) patients and found no

significant difference between the groups (Tuttle et al., 2003). Serum ferritin at the induction of anesthesia prior to CPB was measured by Davis et al, showing no significant difference in AKI (133±138μg/L) versus non-AKI (196±137μg/L) (Davis et al., 1999). Average intraoperative ferritin levels measured at 1 hour CPB in our cohort were significantly higher in non-AKI patients with 180 μg/L (110-315) compared to 100μg/L (60-200) in AKI patients. Preoperative serum ferritin is not associated with AKI development (Tuttle et al., 2003), whereas increased ferritin at the end of CPB and POD 1 and 3 is associated with RRT requirement or death (Leaf et al., 2015). Taken together, lower serum ferritin levels in the earlier intraoperative period may be associated with impaired renoprotection due to a lower capacity to bind labile iron**.**

Transferrins are important iron-binding intravascular transport proteins with 30% of the total iron binding sites occupied under physiological conditions. Transferrins have a protective scavenger role of sequestering labile iron as they have a high iron-binding turnover rate of 10 times per day (Cazzola et al., 1985; Elsayed, Sharif, & Stack, 2016). Transferrin saturation increases with elevated iron levels as additional binding sites become occupied. Leaf et al showed that transferrin saturation was not a univariate predictor of RRT and death following CPB, however a significant increase was found in both groups at the end of CPB and in the postoperative period relative to baseline. Transferrin saturation remained similar in AKI and non-AKI groups preoperatively, at the end of CPB and throughout the postoperative period (Leaf et al., 2015). We anticipated that transferrin saturation would increase in the intraoperative period between the start and 1 hour CPB as free iron is released during CPB (Haase et al., 2010; Leaf et al., 2015); however this was not the case. This one-hour window may have been insufficient to observe a significant increase in transferrin saturation, and indeed peak catalytic iron release has been

observed at the end of CPB (Leaf et al., 2015). Nevertheless, higher transferrin saturation was an independent predictor for the development of AKI for milder phenotypes upon multivariate analysis in this study and this may reflect an adaptive response to minimize iron-mediated renal tubular toxicity (Linkermann et al., 2014).

5.2.3. Lactotransferrin

As mentioned above, novel serine hydrolase candidates were identified using Fp-TAMRA and phosphorofluoridate biotin probe labeling, which included iron-binding proteins lactotransferrin, haptoglobin and transferrin. Lactotransferrin was selected for further validation using enzyme assays. The goal was to develop a urine lactotransferrin enzyme activity assay for evaluation of our AKI cohort. Commercial human lactotransferrin (Product Number 61326, Sigma-Aldrich) was purchased to be the positive control for enzyme assay development. Unfortunately, we were unable to develop a working enzyme activity assay using commercial lactotransferrin and synthetic substrate Z-phe-arg-AMC. The ABPP results indicated Fp-TAMRA probe interaction with untreated, dialyzed and deferoxamine treated lactotransferrin. However, no activity was measured in the activity assays. One possible factor for the lack of observed enzyme activity is incompatibility of the substrate with commercial human lactotransferrin. A better alternative to commercially purchased lactotransferrin may be lactotransferrin isolated from human colostrum using the Sharma et al protocol (Sharma, Paramasivam, Srinivasan, Yadav, & Singh, 1999). Lactotransferrin was extracted and purified from bovine colostrum using the Sharma et al protocol, and demonstrated weak activity when combined with substrate Z-phe-arg-AMC (Massucci et al., 2004). Ultimately, we were unable to develop a positive control and functional

enzyme assay for lactotransferrin. Therefore, further patient analyses could not be conducted and no conclusions about lactotransferrin activity could be drawn.

5.3. Iron Pathophysiology in IRI

All cardiac surgery patients experience an element of renal tubular injury related to IRI and hypoxia (Windsant et al., 2010), that contributes to hemoglobin and catalytic iron release (Leaf et al., 2015) and subsequent activation of different renoprotective mechanisms (Haase et al., 2010). The mechanical forces exerted during extracorporeal circulation weakens RBC structural integrity and induces hemolysis (Haase et al., 2010), with increasing CPB duration correlated with increased intravascular hemoglobin release (Mamikonian et al., 2014). Elevated free hemoglobin levels are associated with AKI following on-pump repair of aortic aneurysms (Windsant et al., 2010). Intraoperative blood transfusions consist of senescent red blood cells that can disintegrate and contribute to elevated iron levels (Kim-Shapiro et al., 2011). RBC's gradually degrade in storage over time and can result in weakened structural integrity and increased hemolysis following blood transfusion (Ho, Sibbald, & Chin-Yee, 2003; Kim-Shapiro et al., 2011) while myoglobin release may also contribute to the circulating labile iron pool (Benedetto et al., 2010).

Murine models link catalytic iron with nephrotoxicity in ischemia reperfusion (Baliga, Ueda, & Shah, 1993), rhabdomyolysis (Baliga, Zhang, Baliga, & Shah, 1996) and hemoglobinuria (Paller, 1988) AKI models. This phenomenon is associated with tubular lipid peroxidation, which is characteristic of an iron-dependent form of cell death known as ferroptosis (Martin-Sanchez et al., 2016). Ferroptosis has recently been demonstrated to play a key role in AKI-induced cell

death (Linkermann et al., 2014; Martin-Sanchez et al., 2016). Indeed, inhibition of ferroptosis has added protective effects beyond necroptosis and necrosis inhibition alone in a murine model of severe renal IRI (Linkermann et al., 2014). Furthermore, ferroptosis was the primary mediator of cell death and renal injury in folic acid-induced AKI with protected renal function, histological injury and cell death by inhibiting ferroptosis (Martin-Sanchez et al., 2016). These findings are intriguing in that they delineate an alternative iron-dependent pathway to renal injury that is shared between different types of AKI.

Ferroptotic cell death is the consequence of the dysfunction of intracellular glutathione dependent antioxidant mechanisms and the increase in intracellular iron (Bogdan, Miyazawa, Hashimoto, & Tsuji, 2016). Ischemia reperfusion injury is linked to glutathione depletion and subsequent glutathione peroxidase depletion, a key enzyme in lipid peroxidation regulation (Friedmann Angeli et al., 2014; Linkermann et al., 2014). Catalytic iron, particularly in the ferrous state, plays an important role in the development and severity of AKI (Leaf et al., 2015). Iron release is an inevitable consequence of cardiac surgery that requires the support of CPB. Patients that develop severe AKI following cardiac surgery have significantly elevated iron levels that peak at the end of CPB which remain elevated by POD 1 (Leaf et al., 2015). Renal IRI alone can induce increased serum iron levels, with associated kidney iron accumulation (Scindia et al., 2015). Urine catalytic iron levels were significantly higher 8 hours post-cardiac surgery with a peak at 24 hours in patients that developed AKI relative to their non-AKI counterparts (Akrawinthawong et al., 2013). Systemic catalytic iron increases significantly in all patients that undergo cardiac surgery as compared to homeostatic levels, which results in intracellular iron

accumulation and storage. Evidently, iron chelation and regulation is crucial during the intraoperative and postoperative times to mitigate lipid peroxidation and renal tubular damage.

Intracellularly, the Fenton redox reaction has the potential to generate highly reactive hydroxyl radicals that are capable of oxidative cellular damage and cell death (Leaf et al., 2015; Xie et al., 2016). Our findings on iron regulation biomarkers: urine hepcidin-25, serum ferritin and transferrin saturation can be summarized in relation to ferroptosis and iron related renal tubular damage, although a direct link cannot be experimentally studied in this prospective observational cohort. Intracellular iron remains in a non-toxic form when bound to intracellular ferritin in physiological conditions (Orino et al., 2001). We found that transferrin saturation levels were higher in our AKI patients by 1 hour CPB relative to the non-AKI patients, which suggests sequestration of circulating iron and tubular iron uptake via transferrin receptors to alleviate further oxidative stress (Zhang, Meyron-Holtz, & Rouault, 2007). High levels of intracellular iron contribute to ferroptotic cell death, however Scindia et al demonstrated that pre-administration of hepcidin prior to IRI increased renal H-ferritin, which is responsible for ferroxidase activity that converts iron to the ferric form and iron storage (Orino et al., 2001). This phenomenon is congruent with our urine hepcidin-25 findings showing higher levels in our non-AKI patients compared to AKI counterparts, suggesting a form of renoptrotection that is different from the primary function of ferroportin degradation. Lastly, serum ferritin levels were lower in patients that developed AKI in the earlier intraoperative period, which may be associated with a lower capacity to bind labile iron systemically.

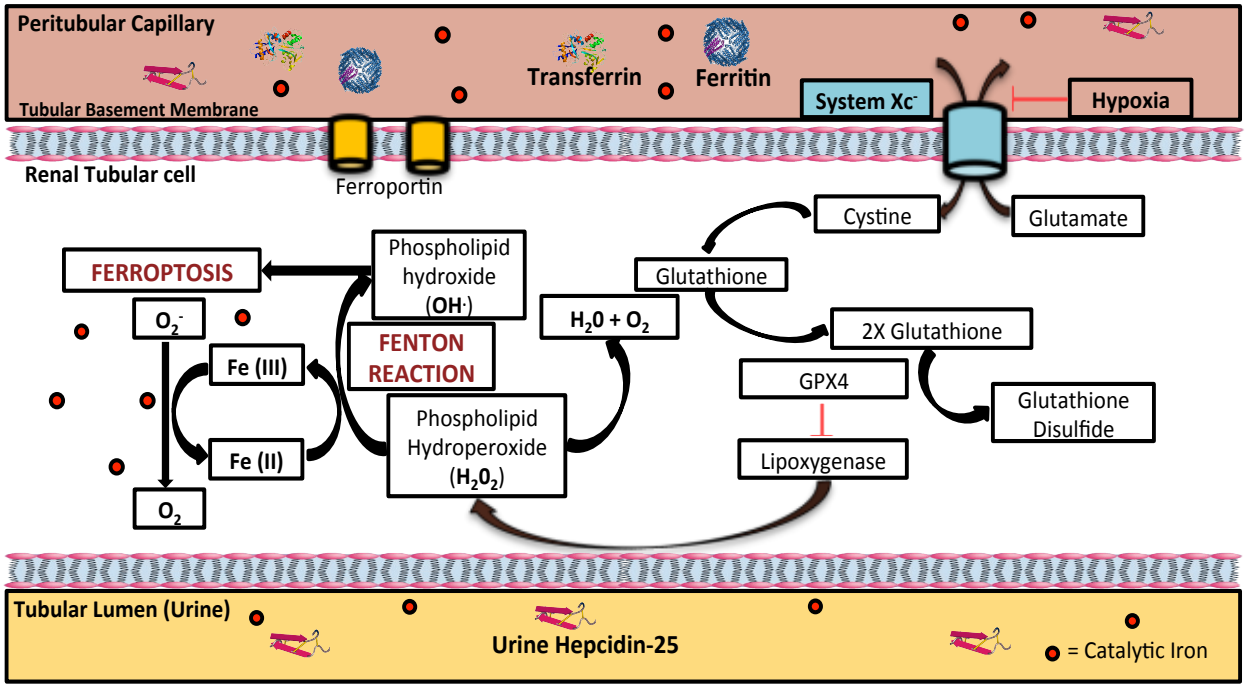

Figure 5.1 Renal cellular iron pathophysiology during AKI. Urine hepcidin-25 may play a renoprotective role from the luminal side and serum ferritin and transferrin saturation may play a renoprotective role from the basolateral side to mitigate the effects of increased systemic iron and iron related injury/damage during CPB surgery that may result in AKI. O_2, oxygen; Fe, iron; H_2O_2, hydrogen peroxide; GPX4, glutathione peroxidase; OH, hydroxyl; H_2O, water.

5.4. Study Limitations

Our study had several limitations that we acknowledge. First, this is strictly an observational study. While we speculate that serum ferritin, transferrin saturation and hepdicin-25 identify distinct iron homeostatic mechanisms that may be protective in IRI to minimize renal oxidative stress/tubular injury, we cannot prove causality. Second, this is a single center observational study. Further validation in other independent cohorts and multi-center settings is required. However, this was a large, unselected, consecutive adult cardiac surgery population with a broad inclusion criterion. Third, hepcidin-25 is limited as an early, non-invasive biomarker by its quantification using a polyclonal antibody-based ELISA assay. ELISA antiserum was composed of polyclonal rabbit IgG that was collected from the same master bleed, however the production

lots were different. This resulted in significant plate-to-plate variation between lots, which we identified with our internal controls. We accounted for this variability in the multivariate analysis and demonstrated that it did not substantially impact the overall findings. While a hepcidin-25 monoclonal antibody sandwich ELISA has been developed (Butterfield et al., 2010), these antibodies were not available at the time of our experiments. Clinically applicable biomarkers require a robust, reproducible assay that is readily translatable to clinical laboratories and conform to GLP guidelines (Hirt-Minkowski, De Serres, & Ho, 2015; Ho, Rush, & Nickerson, 2015). Additionally, hepcidin-25 peaks later at POD 1, similar to KIM-1, and therefore has more limited utility for early diagnosis compared to biomarkers that peak earlier. Nevertheless, these cohorts provided useful observations regarding the consistent response of urine hepcidin-25 as a marker of AKI and helps shed insight into the pathophysiology of human renal IRI. Fourth, we focused exclusively on iron handling biomarkers to better predict AKI. Iron regulation is likely part of a larger pathological picture of IRI, however, crucial to AKI onset and progression. Further analysis of other categories of biomarkers involved in AKI pathogenesis is required to fully understand mechanism and isolate potential targets for therapeutic intervention. Fifth, our cardiac surgery cohort consisted of lower risk elective patients that demonstrated lower to intermediate Thakar scores averaging around 3(2-5) for AKI patients and 2 (1-3) for non-AKI patients. Therefore, the rates of renal replacement therapy were very low. Our results should be validated in higher risk cohorts. Sixth, our study population and excluded patient population had different AKI incidence rates. These missed samples resulted in a lower risk cohort for analyses. Lastly, substrate Z-phe-arg-AMC was the best available synthetic option according to the literature (Massucci et al., 2004), however using this substrate and commercial lactotransferrin, we were unable to develop a functioning activity assay. The physiological substrate remains

unknown and Z-phe-arg-AMC shows weak activity when combined with bovine lactotransferrin (Massucci et al., 2004). Ultimately, the source and production of commercial lactotransferrin may not have been ideal for the purpose of an activity assay.

6. CONCLUSION

Our study demonstrated that serum ferritin, transferrin saturation and urine hepcidin-25 were each independent predictors of AKI development, suggesting that maintaining intraoperative iron homeostasis is a key and potentially modifiable factor in AKI. These findings are consistent with another well characterized iron binding protein, NGAL, which has been demonstrated to be renoprotective as part of an NGAL: iron: siderophore complex that up-regulates heme-oxygenase (Mori et al., 2005). Taken together, these findings suggest that hepcidin-25, serum ferritin and transferrin saturation may be part of a coordinate response involving different iron-binding proteins that act to mediate the response to human renal IRI. Monitoring iron handling biomarkers throughout and following IRI may enhance current clinical risk prediction for AKI following cardiac surgery, with the potential for modifiable therapeutic intervention.

We showed that urine hepcidin-25 is elevated following cardiac surgery and independently and inversely associated with AKI. These findings independently validate the existing literature in a prospective, observational cohort of adult cardiac surgery patients (Haase-Fielitz et al., 2011; J Ho et al., 2011; Ho et al., 2009; Prowle et al., 2012), and extend them to show that a combined clinical and biomarker model with baseline eGFR, diabetes and hepcidin-25 has an AUC 0.83 for the subsequent development of AKI. These findings are consistent with animal models that demonstrate hepcidin-25 is renoprotective in AKI. The addition of iron handling proteins serum ferritin and transferrin saturation outperformed and improved clinical prediction alone in patients that developed AKI following cardiac surgery. Additionally, our intraoperative and early postoperative iron binding proteins preceded current clinical detection of AKI using serum creatinine. Early intraoperative AKI prediction is feasible using FDA-validated quantitative

assays that are readily available in clinical laboratories. These data could inform targeted AKI prevention strategies, such as avoidance of nephrotoxic medications and strict fluid management protocols. However, in order to move this field forward, clinical trials are required to evaluate novel, specific AKI therapeutics. These data could be used as an enrichment strategy to recruit patients at high risk of developing AKI.

Overall, our data offer the intriguing possibility that extracorporeal removal of catalytic iron during CPB may protect against AKI. Indeed, FDA-approved cartridges with immobilized deferoxamine have been developed for use in series with dialysis to maximize the intra-dialytic removal of aluminum (Anthon et al., 1995) and are effective for clearing non-transferrin bound iron (C.M. et al., 1999); this suggests that such cartridges could be used in series with the CPB circuit. While AKI pathophysiology is multifactorial in nature, targeted and minimally invasive interventions such as this may mitigate the effects of IRI and improve outcomes. Trials of targeted interventions to remove labile iron, such use of immobilized deferoxamine cartridges may be useful. Notably, the results of a clinical trial evaluating the utility of systemic iron chelation in AKI with deferoxamine are pending (NCT00870883). Alternatively, more restrictive hemoglobin transfusion thresholds (<8.0 g/dL;<5.0mmol/L) could be considered (Carson et al., 2016). A clinical trial (NCT02042898) measuring renal function using a decreased restrictive hemoglobin threshold (from <8.0 g/dL;<5.0mmol/L to <7.5 g/dL;<4.7mmol/L) is ongoing, which may help mitigate vascular catalytic iron increase and subsequent AKI.

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8. APPENDIX

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: **Biomarkers for the early detection of cardiac surgery associated acute kidney injury (CSA-AKI)**

Investigators: Dr. R. Arora & Dr. C. Rigatto
St. Boniface Hospital
409 Tache Avenue
Winnipeg, Manitoba R2H 2A6

Sponsor: Sections of Cardiac Surgery and Nephrology, St. Boniface Hospital

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this study and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study
The purpose of the study is to assess how cardiac surgery affects the kidneys by testing blood and urine samples. The study will involve about 600 patients from St. Boniface Hospital**.**

Study procedures
It has already been determined that you will be having cardiac surgery. You will have consented to the surgical procedure using a separate process. The research in which you are being asked to participate will not impose any additional requirements on you other than as follows:

Main Study: If you agree to take part, we would take samples of your blood and urine to see if we can detect kidney injury. 10 mls (about 2 teaspoons) of blood will be collected each time, using the same methods of collecting routine blood samples before, during and after the surgery. A total of 60 mls (about 4 tablespoons) of blood would be collected for the study.

50 mls (about one fifth of a cup) of urine will be collected each time from a catheter which is inserted into the bladder prior to your cardiac surgery. This urinary catheter insertion is part of the usual preparation for your cardiac surgery.

Your study blood and urine would be collected at the following times:

- Upon your arrival to the Operating Room prior to surgery
- At the start of your surgery
- 1 hour after surgery begins
- Upon arrival to the post recovery ICU (approx. 6 hours after surgery)
- Morning of post-operative day 1
- Morning of post-operative day 3-5 (done once on only one of these days)

Thirty (30) days after surgery, the study staff will call you at home to see how you are feeling and ask about your general health status. No other tests or procedures would be required and your participation would end after the 30 day follow up call.

Additional Components:
Long term outcomes: As part of this research, we would like to know what happens to your health status several years after the surgery. We would like to link the study database to provincial databases containing information on hospitalizations and vital statistics. This will be done at 3 and 5 years after the surgery. To make this link possible, we need to record your Personal Health Identification Number (PHIN) as part of the study. The PHIN information will be kept for 5 years and then destroyed. If you do not want us to use your PHIN in this way, then you can opt out of this part of the study, in which case your PHIN would not be stored and would not be used for the database linkages described above. You can also opt out of this part at any time if you change your mind.

Long term storage of study samples and study data: We would like to store blood and urine samples and study data for up to 25 years. The samples and data will be identified only by a study number and no personal identifiers will be present to link those study results to you as an individual. These data can be used for future research into outcomes of cardiac surgery. The samples may be tested in future for additional biomarkers related to cardiac, kidney and related disease. If you do not wish your data and samples kept for up to 25 years, you can opt out of this part of the study. You can also opt out later if you change your mind.

<u>Risks and Discomforts</u>

The risks associated with your cardiac surgery are separate from this research study and will be explained in a separate consent form.

Blood may be drawn from your arm by venipuncture (needle stick) the standard method for taking blood from a vein in your arm by needle stick. If you have a tube in your arm for taking blood, then this may be used instead. Risks associated with drawing blood by venipuncture include pain, bruising or swelling at the site of the blood draw. Infection at the site of the blood draw is also possible, although very unlikely. We will attempt to take any blood samples at the same time samples are being taken as part of your routine care at St. Boniface Hospital, to avoid any additional needle-stick. It is possible that an additional needle-stick may be required.

There are no risks associated with providing a urine sample.

The risks associated with data collection are minimal. Your study related data will be entered into a password protected database and kept in a secure location at St. Boniface Hospital, accessible only by members of the research team. Personal information is needed for the operation of the database and in the case you choose to withdraw from participation.

You are not waiving any of your legal rights by signing this consent form or releasing the investigator from their legal and professional responsibilities.

Benefits

You may not benefit from participation in this research; however, the study should contribute to a better understanding of changes in kidney function during and after cardiac surgery.

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you.

Payment for participation

You will not be paid for your participation in this study.

Confidentiality

Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. St. Boniface Hospital staff involved with your care will review/copy medical information that may reveal your identity. The Health Research Ethics Board at the University of Manitoba and St. Boniface Hospital may also review your research-related records for quality assurance purposes. If the results of the study are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave St. Boniface Hospital. By signing the attached Informed Consent Form you consent to direct access to your medical records.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care at this site.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff:

Investigator:	Dr. Claudio Rigatto	Tel No.	204-237-2121
Investigator:	Dr. Rakesh Arora	Tel No.	204-258-1031
Research Coordinator:	Mr. Brent Gali	Tel No.	204-235-3521
Research Assistant:	Mr. Michael Pereira	Tel No.	204-235-3521

This study has been reviewed and approved by the University of Manitoba Bannatyne Campus Health Research Ethics Board. For any questions about your rights as a research participant, you may contact: Health Research Ethics Board, University of Manitoba at 204-789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Consent

1) I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in research study.

2) By checking the appropriate choice below, I am indicating my wish to participate in the following additional components of the study;

   a) I agree to the use of my PHIN for the assessment of long term outcomes

   ☐ YES ☐ NO

   b) I agree to the storage of my blood and urine samples and study data for up to 25 years. I understand that these samples and information will be stripped of any identifiers linking them to me as an individual.

   ☐ YES ☐ NO

3) I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the study, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

4) I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

 Initials of Subject: _____

5) I understand that authorized staff at St. Boniface Hospital may wish to review my medical records. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations. By signing this document, I give permission for such review and data collection, and grant direct access to my medical records.

6) By signing and dating this document, I am aware that none of my legal rights are being waived.

_____
Participant printed name

_____ _____ _____
Participant signature **Date** **Time**

_____ _____
Printed Name of Authorized Third Party (if applicable) **Relationship to Participant**

_____ _____ _____
Signature of Authorized Third Party (if applicable) **Date** **Time**

I confirm that I have explained the purpose, duration etc of this study, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

_____ _____
Printed Name of person obtaining consent **Role in Study**

_____ _____ _____
Signature of person obtaining consent **Date** **Time**

_____ _____
Printed Name of Witness or Translator (if applicable) **Role in obtaining consent**

_____ _____ _____
Signature of Witness or Translator (if applicable) **Date** **Time**

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

 Initials of Subject: _____