INVESTIGATION OF URINARY NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN CONCENTRATION FOR THE DIAGNOSIS OF ISCHAEMIA – REPERFUSION INDUCED ACUTE KIDNEY INJURY IN DOGS

Jennifer Davis BVMS MANZCVS (anaesthesia and critical care)

This thesis is presented for the degree of Research Masters (with Training) of Murdoch University 2015
I declare that this thesis is my own account of my research and contains work which has not previously been submitted for a degree at any tertiary education institution.

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Jennifer Davis
ABSTRACT

Acute kidney injury (AKI) is a devastating potential consequence of renal ischaemia and reperfusion (I-R) subsequent to severe intra-operative hypotension and fluid resuscitation. Acute tubular epithelial damage is a common early histological abnormality in this syndrome. The high mortality rate associated with AKI in dogs is attributed in part to the limitations of current diagnostic techniques that can only detect AKI in the late stages when damage is irreversible. Early detection of renal tubular injury could improve outcome and might be possible by measuring urinary neutrophil gelatinase-associated lipocalin concentration (uNGAL) in at-risk dogs.

The objectives of this study were to establish a clinically relevant canine model of renal I-R injury, and use this model to determine changes in uNGAL within three hours of initiation of injury.

A pilot study was performed to establish the severity and duration of hypotension caused by haemorrhage, and duration of reperfusion, that produced histological evidence of acute tubular damage. Urine samples obtained during this pilot were used to determine optimal sample dilutions for use with a commercial dog NGAL immunoassay. Investigation into potential interference of synthetic colloid fluid solutions with the immunoassay was also performed. This experimental model was then used to determine changes in uNGAL in seven anaesthetised greyhound dogs. Urinary NGAL concentrations were measured before (T0) and immediately following (T1) haemorrhage, and hourly following fluid resuscitation (T2 - T4). After T4, dogs were euthanised, and renal tissue collected for histopathology. Statistical analysis for the main study was performed
using repeated measures one-way ANOVA and data presented are mean (95% confidence interval).

The pilot study showed that maintenance of mean arterial pressure (MAP) below 40 mmHg for one hour (ischaemic phase); followed by fluid resuscitation to maintain MAP > 60 mmHg for three hours (reperfusion phase) was required to ensure tubular damage was produced. Optimal dilution of urine samples for accurate NGAL measurement using the immunoassay was 1 in 1000. A 4% gelatin-based colloid solution did not interfere with the immunoassay. In the main study, histopathology confirmed renal tubular epithelial damage in all dogs. Urine NGAL increased from a mean of 12.1 (confidence interval 0 – 30.6) ng mL\(^{-1}\) at T0 to 122.0 (64.1 – 180.0) ng mL\(^{-1}\) by T3. Compared to T0, uNGAL was significantly higher at T3 (p = 0.016). Fold change in uNGAL at T3 was 24.2 (7.3 – 41.0).

Pressure-guided acute haemorrhage followed by colloid resuscitation produced a clinically relevant model of I-R AKI in dogs. Despite wide individual variation in baseline uNGAL, increases in uNGAL were observed in all dogs suggesting this biomarker has potential for detecting early tubular injury caused by renal I-R in this species.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>$C_{a}O_2$</td>
<td>Arterial oxygen content</td>
</tr>
<tr>
<td>$C_{cv}O_2$</td>
<td>Central venous oxygen content</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variance</td>
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<td>DO$_2$I</td>
<td>Oxygen delivery index</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Fig.</td>
<td>Figure</td>
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<tr>
<td>GEL</td>
<td>Succinylated bovine gelatin solution</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>I-R</td>
<td>Ischaemia – reperfusion</td>
</tr>
<tr>
<td>IRIS</td>
<td>International Renal Interest Society</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KIM-1</td>
<td>Kidney Injury Molecule 1</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil Gelatinase-associated Lipocalin</td>
</tr>
<tr>
<td>OER</td>
<td>Oxygen extraction ratio</td>
</tr>
<tr>
<td>$P_{a}O_2$</td>
<td>Partial pressure of arterial oxygen</td>
</tr>
<tr>
<td>$P_{cv}O_2$</td>
<td>Partial pressure of central venous oxygen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Qt</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>sCr</td>
<td>Serum creatinine concentration</td>
</tr>
<tr>
<td>sNGAL</td>
<td>Serum NGAL concentration</td>
</tr>
<tr>
<td>uNGAL</td>
<td>Urine NGAL concentration</td>
</tr>
<tr>
<td>UOP</td>
<td>Urine output</td>
</tr>
<tr>
<td>UNCR</td>
<td>Urine NGAL: urine creatinine ratio</td>
</tr>
<tr>
<td>UTI</td>
<td>Urinary tract infection</td>
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<tr>
<td>VO₂I</td>
<td>Oxygen consumption index</td>
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CHAPTER 1 - LITERATURE REVIEW

INTRODUCTION

Acute kidney injury (AKI) is a syndrome that carries a high mortality rate in dogs, but unfortunately is difficult to diagnose sufficiently early to allow effective treatment. This review will describe the aetiology of AKI, the implications of the syndrome in dogs and current diagnostic techniques. The potential value of new diagnostic tests will also be explored.

ACUTE KIDNEY INJURY IN DOGS

Acute kidney injury is defined as an abrupt decline in renal function (Mugford et al. 2013). Previous terminology used to describe this process included “acute renal failure” and “acute tubular necrosis”; however these terms did not reflect that loss of kidney function is a multifactorial and dynamic process (Moore 2010; Ross 2011; Thoen & Kerl 2011). The kidney damage associated with the syndrome known as AKI is progressive and may, or may not, lead to renal failure (Thoen & Kerl 2011).

AETIOLOGY

There are many potential causes of AKI in dogs including ischaemia-reperfusion (I-R), nephrotoxicity, inflammation, infection, prolonged urinary tract obstruction, and hypercalcaemia (Ross 2011; Mugford et al. 2013). Ischaemia-reperfusion events are thought to be the most common cause of AKI in dogs (Vaden et al. 1997). Causes of ischaemia include reduced renal perfusion (associated with shock, non-steroidal anti-inflammatory drugs, anaesthesia, severe trauma or hypothermia), severe hypoxia, systemic inflammatory response syndrome (associated with sepsis, multiple organ dysfunction or acute pancreatitis) and vascular abnormalities.
(including renal vessel thrombosis, disseminated intravascular coagulopathy or vasculitis) (Vaden et al. 1997; Mugford et al. 2013). Given the aetiology, it is evident that patients undergoing general anaesthesia or hospitalisation in an intensive/critical care setting have an increased risk of developing AKI.

**IMPLICATIONS**

The reported percentage of dogs diagnosed with AKI that do not survive ranges from 56 – 65% according to several retrospective studies (Behrend et al. 1996; Vaden et al. 1997; Lee et al. 2011; Hsu et al. 2014). By the time it is diagnosed, AKI is associated with a long period of morbidity and therefore a long hospital stay. The costs of prolonged hospitalisation and advanced therapeutic options, such as peritoneal dialysis or haemodialysis, are large and unfortunately often lead to owners opting for euthanasia. The proportion of dogs diagnosed with AKI that were euthanised was 34% in one study and 38% in another (Behrend et al. 1996; Vaden et al. 1997). In dogs that survive, the development of chronic kidney disease (CKD) following AKI is common, with a frequency of 50% in one study (Vaden et al. 1997).

**FREQUENCY OF ISCHAEMIA-REPERFUSION INDUCED ACUTE KIDNEY INJURY**

The importance of an I-R event as a cause of AKI, with or without anaesthesia, has been identified in several studies. A previous (within two weeks) episode of general anaesthesia was identified as the cause in four of 29 dogs diagnosed with “hospital-acquired acute renal failure” in a single hospital over a nine year period (Behrend et al. 1996). However, the occurrence of AKI following anaesthesia in that hospital may actually have been much higher than that reported; as the authors chose only to diagnose AKI if renal azotaemia was present, possibly resulting in
many cases being missed that would fit the current definition of AKI (Behrend et al. 1996; Sharfuddin & Molitoris 2011).

In a separate study, a preceding ischaemic event was identified in more than 50% of dogs that developed acute renal failure (Vaden et al. 1997). In that study, acute renal failure was diagnosed by clinical signs, azotaemia, renomegaly (as detected by abdominal palpation, radiography or ultrasonography) and absence of data consistent with chronic renal failure.

Although not designed to investigate the frequency of canine AKI, a more recent veterinary study found that of 12 (30.7%) of 39 healthy dogs undergoing general anaesthesia to facilitate a variety of surgeries over a two year period were diagnosed with AKI on the basis of a post-operative increase in serum creatinine concentration (sCr) (Lee et al. 2012). Interestingly, dogs diagnosed with AKI had a significantly longer duration of general anaesthesia compared to those that did not develop AKI.

The above studies emphasise the importance of I-R as a cause of AKI in dogs. In order to develop methods of diagnosis and appropriate treatment, it is important to understand how I-R leads to renal damage. The following section outlines the pathophysiology of I-R induced renal injury.

**Pathophysiology of Ischaemia-Reperfusion Induced Acute Kidney Injury**

Although often simply referred to as “ischaemic AKI”; it is important to recognise that both ischaemia and subsequent reperfusion play a part in the development of AKI. During ischaemia there is a critical reduction in delivery of oxygen and other nutrients to kidney cells. Subsequent adenosine triphosphate depletion leads to metabolic and structural damage to renal tubular cells, resulting in disrupted kidney
function (Bonventre & Yang 2011; Ross 2011; Sharfuddin & Molitoris 2011). Reperfusion of the kidney during fluid resuscitation can augment the injury, mainly via production of reactive oxygen species (Collard & Gelman 2001). The main pathologic and functional abnormalities associated with I-R AKI are reduction in glomerular filtration rate (GFR), tubular obstruction, back leakage of filtrate across damaged tubular cells and haemodynamic alterations (vasoconstriction and renal medullary hypoxia) (Cowgill & Francey 2005). To understand the pathogenesis of these abnormalities we must consider the metabolic and structural changes that occur on a cellular level, as well as the haemodynamic changes and inflammation that occur.

The renal tubules, along with renal capillaries, comprise the working tissue mass of the kidney (Eaton & Pooler 2009). The tubules are responsible for reabsorption and secretion of water, solutes and toxins (Eaton & Pooler 2009). The tubules are classified into the proximal tubule which drains the glomerulus and runs from the cortex of the kidney into the outer medulla, Henle’s loop which consists of a descending thin limb, ascending thin limb and ascending thick limb and traverses into the inner medulla before travelling back to the cortex, the distal convoluted tubule, and finally the connecting tubule which leads to the collecting duct system of the nephron (Fig. 1.1) (Eaton & Pooler 2009).
Tubules consist of a single layer of epithelial cells resting on a basement membrane. The characteristics of epithelial cells vary depending upon location within the tubule and associated function (Cowgill & Francey 2005). The renal epithelium is highly specialised in order for the kidney to perform many energy dependent active functions. These functions include reabsorption of substances from the glomerular filtrate back into the blood, and secretion of substances from the blood to the tubular lumen via transcellular or paracellular transport (Cowgill & Francey 2005). For example, the epithelium of the proximal tubule contains a brush border that provides an increased surface area for improved absorption and intracellular transport of substances such as water, sodium and glucose (Clarkson & Fletcher 2011). This high metabolic workload means that the tubular
epithelium, particularly in the proximal tubules which have a high energy requirement and rely on oxidative catabolism, is particularly susceptible to hypoxic damage (Heyman et al. 2012). Oxidative catabolism is an oxygen dependent process by which energy substrates such as glucose are broken down to produce energy which is stored by cells in the form of adenosine triphosphate (ATP).

During ischaemia, ATP is depleted leading to increased free intracellular calcium concentrations that activate proteases and phospholipases leading to cell cytoskeleton damage and cell necrosis (Devarajan 2006; Ross 2011; Sharfuddin & Molitoris 2011). Adenosine triphosphate is also metabolised further into adenine nucleotides and hypoxanthine. Reactive oxygen species including hydrogen peroxide and superoxide are then generated during the conversion of hypoxanthine into xanthine during reperfusion (Devarajan 2006). Ischaemia also induces nitric oxide generation (Devarajan 2006). These reactive oxygen species may cause further cell damage via oxidation, peroxidation of lipids, DNA damage and apoptosis (Devarajan 2006). These reactive oxygen species also contribute to the development of a pro-inflammatory state and may contribute to ongoing vasoconstriction (Cowgill & Francey 2005; Sharfuddin & Molitoris 2011).

Ischaemia also leads to alterations in the proteins of the basolateral membrane of renal tubular cells, such as Na, K - ATPase and integrins (Cowgill & Francey 2005; Devarajan 2006; Bonventre & Yang 2011). Loss of Na, K - ATPase results in impaired sodium reabsorption by the proximal tubule and altered cell polarity (Devarajan 2006). An increased fraction of sodium reaching the macula densa activates tubuloglomerular feedback resulting in afferent arteriole constriction and reduced GFR (Ross 2011). β-integrins play an important role in tubule epithelial cell integrity with their relocation from the basolateral to the apical membrane during AKI leading to detachment of tubular cells from the basement membrane (Devarajan 2006). This results in denuded areas of tubule that are unable to
perform their metabolic functions, and also tubular obstruction by the detached cells.

Disruption and redistribution of actin and actin depolymerising factor subsequent to cellular ATP depletion further contributes to tubular epithelial injury (Devarajan 2006). Combined with the effects of altered membrane proteins, these changes cause cellular swelling, loss of microvilli, vesicle formation, necrosis and apoptosis that can eventually lead to cast formation and obstruction of renal tubules (Cowgill & Francey 2005; Devarajan 2006; Sharfuddin & Molitoris 2011). Depletion of ATP also causes disruption of cell-to-cell interactions via loss of tight and adherent junctions resulting in back leakage of tubular filtrate, flattened epithelial cells and further cell detachment from the basement membrane (Ross 2011; Sharfuddin & Molitoris 2011).

**HAEMODYNAMIC ALTERATIONS**

During normal physiological conditions, the renal cortex receives the majority of renal blood flow, while the medulla receives only approximately 10% (Munshi et al. 2011). Ischaemia causes a regional rather than uniform pattern of reduced perfusion, with a disproportionate reduction of flow to the outer medulla further reducing flow to that area (Bonventre & Yang 2011; Munshi et al. 2011). Under normal physiological conditions, autoregulation allows the kidney to maintain constant blood flow (and therefore constant GFR) despite changes in perfusion pressure in the range of 80 – 180 mmHg (Eaton & Pooler 2009; Munshi et al. 2011). However, following ischaemic injury, increased renal vascular resistance due to an intense and persistent vasoconstriction causes further reduction in renal blood flow (Devarajan 2006; Sharfuddin & Molitoris 2011). Vasoconstriction is exacerbated by endothelial cell injury that results in increased tissue levels of local vasoconstrictive mediators and increased sympathetic nervous system activity (Bonventre & Yang 2011). Vasoconstrictive mediators include endothelin-1,
angiotensin II, thromboxane A2, prostaglandin H2, leukotrienes and adenosine (Cowgill & Francey 2005; Devarajan 2006; Bonventre & Yang 2011). At the same time there is a reduced response to, and decreased abundance of, mediators of vasodilation including acetylcholine, bradykinin and nitric oxide (Cowgill & Francey 2005; Bonventre & Yang 2011). Endothelial cell injury also exacerbates the inflammatory response initiated during AKI, and increases microvasculature permeability (Bonventre & Yang 2011; Munshi et al. 2011; Sharfuddin & Molitoris 2011).

INFLAMMATORY RESPONSE

Inflammation and leukocyte activation are recognised as major mediators of ongoing cellular damage and hypoxia subsequent to ischaemic renal injury (Devarajan 2006; Sharfuddin & Molitoris 2011). Injured tubular epithelial cells release pro-inflammatory mediators (cytokines) that exacerbate the inflammatory cascades initiated by damaged vascular endothelium (Devarajan 2006). These cytokines include TNF-α, interleukin (IL)-6, IL-1β, IL-8, C-C motif chemokines and TGF β (Bonventre & Yang 2011; Sharfuddin & Molitoris 2011). Injured tubule cells also affect T lymphocyte activity via expression of Toll-like receptors, complement and complement receptors and co-stimulatory molecules (Bonventre & Yang 2011). Inflammatory cells including neutrophils and macrophages accumulate in the kidney following ischaemia; these cells produce cytokines and reactive oxygen species thus exacerbating tubule cell damage (Devarajan 2006; Bonventre & Yang 2011).

ALTERED GENE EXPRESSION

Transcriptomic studies have displayed altered expression of a large number of genes during renal I-R; including neutrophil gelatinase-associated lipocalin (NGAL) that is highly up regulated in the distal tubule and kidney injury molecule-
1 (KIM-1) that is up regulated in the proximal tubule (Ichimura et al. 1998; Mishra et al. 2003; Supavekin et al. 2003). The precise roles of NGAL and KIM-1 are unclear; however they appear to be involved in the recovery process of AKI by reducing cell damage and inflammation (Bonventre & Yang 2011). Other protective proteins up regulated in the proximal tubule during ischaemic AKI include heme oxygenase and heat shock proteins (Bonventre & Yang 2011).

TEMPORAL PHASES OF ISCHAEMIA-REPERFUSION ACUTE KIDNEY INJURY

AKI can be described as occurring in four different stages: initiation, extension, maintenance and recovery (Cowgill & Francey 2005; Devarajan 2006; Ross 2011; Mugford et al. 2013). The initiation phase occurs during and immediately after an ischaemic insult to the kidney when parenchymal damage is initiated and tubule cells suffer sub lethal damage (Cowgill & Francey 2005; Ross 2011). Intervention at this stage can potentially halt further progression of injury and reverse damage that has already occurred (Cowgill & Francey 2005). During the extension phase there is a continuation of ischaemia, hypoxia and inflammation; cell damage continues and functional change such as alterations to GFR and tubule reabsorption occur (Cowgill & Francey 2005; Ross 2011). Clinical signs and laboratory changes associated with AKI may still not be evident during the extension phase; however, by this stage renal damage may not be reversible (Cowgill & Francey 2005). The maintenance phase is entered when a critical degree of epithelial damage has occurred; during this stage, AKI is detectable using clinical signs and traditional laboratory tests. Removal of the inciting cause and instigation of treatment at this point will not alter existing damage or increase speed of recovery (Cowgill & Francey 2005; Mugford et al. 2013). The final stage is the recovery phase where renal tissue is repaired and normal function restored over a period of weeks to
months. In many cases, complete recovery does not occur and scar formation and long-term renal dysfunction persists (Cowgill & Francey 2005).

As renal injury is only reversible during the early stages of AKI, there is a need for better methods to diagnose AKI earlier so inciting causes can be removed and appropriate treatment instigated earlier. The next section outlines the tests that are available and their inherent limitations.

**CURRENT METHODS FOR DIAGNOSIS OF CANINE ACUTE KIDNEY INJURY**

Conventional tests for diagnosis of acute renal failure can be divided into those that measure GFR (sCr, creatinine clearance, blood urea nitrogen (BUN) concentration), those that assess renal tubular function (urine output (UOP), urinalysis, urine microscopy and fractional excretion of electrolytes) and those indicating renal tubular injury (enzyme excretion, histopathology). These tests are used alongside assessment of clinical signs such as sudden onset lethargy, depression, dehydration, anorexia, vomiting and weakness (note that these signs may be difficult to detect in animals already hospitalised for other critical illnesses) (Cowgill & Francey 2005).

**MEASURES OF GLOMERULAR FILTRATION RATE**

The gold standard method to measure GFR involves calculation of the rate of clearance of a substance from the plasma which is eliminated from the body only by glomerular filtration without any tubular reabsorption or secretion (e.g. inulin, iohexol and radiolabelled molecules). Unfortunately these methods can be complicated and expensive precluding their use in clinical veterinary practice. Instead, exogenous substances that are similarly freely filtered by the glomerulus with minimal tubular reabsorption or secretion are used as surrogate markers of GFR.
The combination of an increase in BUN concentration and sCr is referred to as azotaemia, and reflects a reduction in the rate of filtration of these substances by the glomerulus. Blood urea nitrogen is a by-product of protein metabolism. Unfortunately BUN concentration is a poor surrogate for GFR as although it is largely filtered by the glomerulus, there is also variable tubular reabsorption of BUN. It is also a non-specific marker of GFR as serum levels depend on several extra-renal factors including nutrition and hydration status, gastrointestinal bleeding, liver function and steroid administration (Cowgill & Francey 2005; Venkataraman & Kellum 2007; Moore 2010; Cowgill & Langston 2011).

Serum creatinine results from the breakdown of creatine and creatine phosphate by skeletal muscle and the liver (Braun et al. 2003). It is a more reliable surrogate for GFR because it is freely filtered by the glomerulus and not reabsorbed. However, a small amount does undergo tubular secretion which may account for 10-40% of creatinine clearance during reductions in GFR (Moore 2010). Unfortunately sCr can also be influenced by extra-renal factors such as muscle mass, hydration status, nutrition status, exercise, liver function and drugs (Moore 2010; Moore et al. 2012). The administration of large volumes of intravenous fluids, as occurs in the anaesthesia and intensive care setting, may artificially lower sCr (Moore et al. 2012).

There are major limitations associated with the use of sCr for the diagnosis of AKI. It can take up to 24 hours for a detectable increase in sCr to occur following renal injury (Moore et al. 2012). This means that 65-75% of renal functional mass may have been lost before sCr increases above the reference interval (Delanghe & Speeckaert 2011). As a result, AKI may not be detected until extensive injury has occurred, and at a time that is too late to allow useful therapeutic intervention. A further limitation relates to analytical error associated with the measurement of creatinine concentrations. Most laboratories utilise Jaffé’s method to quantify sCr,
and the accuracy of this method can be reduced by the presence of several substances within the sample, including cephalosporin or bilirubin. As a result this method may overestimate sCr by up to 45% in healthy dogs (Braun et al. 2003; Delanghe & Speeckaert 2011).

MEASURES OF RENAL TUBULAR FUNCTION

Urine output monitoring
A reduction in UOP, defined as either oliguria (< 1 mL kg\(^{-1}\) hour\(^{-1}\)) or anuria (no urine production), has good positive predictive value for diagnosing AKI (Cowgill & Francey 2005; Venkataraman & Kellum 2007). However, due to the propensity for therapeutic interventions (fluid therapy, diuretics, dopamine) to artificially elevate UOP, it has poor negative predictive value and sensitivity (Venkataraman & Kellum 2007). Normal UOP was found in six of 29 dogs diagnosed with AKI in one study, and in 11 of 99 dogs diagnosed with acute renal failure in another (Behrend et al. 1996; Vaden et al. 1997). Another limitation of UOP monitoring is the requirement for indwelling urinary catheter placement. These are not routinely placed in veterinary patients and carry a risk of complications such as infection (Thoen & Kerl 2011).

Urine specific gravity
The measurement of urine specific gravity has conventionally been thought to be an important diagnostic test for AKI in small animal patients. The production of isosthenuric urine (specific gravity of 1.008-1.018) is thought to reflect reduced concentrating ability of the renal tubules (Cowgill & Francey 2005). Interestingly, the use of urine specific gravity to diagnose AKI is not emphasised in human medicine, likely because it is heavily affected by oral fluid intake and treatments such as intravenous fluid therapy and diuretics (Thoen & Kerl 2011). In addition,
numerous endogenous and exogenous substances including artificial colloids, glucose or protein may affect refractometric measurements of specific gravity.

**Urinalysis and microscopy**

Further analysis of urine for abnormalities such as glycosuria and proteinuria may be performed; however, these are nonspecific findings in AKI because they can also result from extra-renal disease. Urine microscopy can demonstrate the presence of granular and hyaline casts formed during tubule cell damage. Despite good positive predictive value the presence of casts has poor sensitivity as demonstrated in a retrospective study where only 28 of 99 dogs with acute renal failure had microscopic evidence of urine casts (Vaden *et al.* 1997). A further limitation of urine microscopy is that casts are readily affected by urine storage – both within the body (i.e. urinary bladder) and between collection and analysis (Cowgill & Francey 2005).

**Fractional Excretion of Electrolytes**

Tests to calculate the fractional excretion of electrolytes are used with the premise that alterations to renal tubular function will result in abnormal handling of certain electrolytes (e.g. sodium). Unfortunately the clinical utility of these tests in veterinary patients is limited due to considerable intra- and inter- individual variation, lack of agreed reference intervals and dependency of results on dietary electrolyte intake (Heiene & Lefebvre 2007).

**MEASURES OF RENAL TUBULAR INJURY**

**Urinary enzyme excretion**

Renal tubular cell injury results in leakage and increased urinary excretion of enzymes (Goldstein 2011). Increased serum activity of these enzymes has little impact on urine activity because they are too large to be filtered by the glomerulus (Clemo 1998). Enzymes are present in the highest concentrations in the most
metabolically active part of the renal tubule (i.e. the proximal convoluted tubule), and as mentioned earlier it is this portion that is most susceptible to ischaemic damage (Brunker et al. 2009). Two of the most commonly studied enzymes are Y-glutamyl transpeptidase, found in the brush border of the proximal tubule, and N-acetyl-β-D-glucosaminidase (NAG), found in the lysosomes of the proximal tubule (Ward 1975; Brunker et al. 2009; Goldstein 2011). Several studies have found increased urinary activities of these enzymes to be more sensitive markers of acute tubular injury than GFR, azotaemia or clinical signs (Goldstein 2011). However baseline values for the urine activities of these enzymes varies greatly making it difficult to determine a canine reference interval, and studies have shown marked overlap in urine Y-glutamyl transpeptidase activities between dogs with and without AKI (Heiene et al. 1991; Goldstein 2011). This limits the diagnostic utility of these tests to dogs in which baseline values have already been determined. There is also a wide circadian variation for urine activities of some of these enzymes; limiting the value of testing a single spot urine sample (Heiene et al. 1991). Increased urinary activity of enzymes may only be seen for a limited period following renal tubular damage as excretion varies with type and severity of renal damage (Clemo 1998). In summary, measurement of urinary enzyme activity at a single time point appears to be of limited diagnostic value for AKI.

**Histopathology**

Following percutaneous or laparoscopic biopsy techniques, histopathological examination of kidney tissue may be performed (Cowgill & Langston 2011). The goal of renal biopsy in a suspected case of AKI is to clarify the underlying aetiopathogenesis, as well as to assess severity and prognosis (Lees & Bahr 2011). Alterations to renal tubule histology that occur during I-R AKI have been well documented by various studies investigating renal I-R injury through experimental rodent models (Jablonski et al. 1983; Williams et al. 1997; Moosavi et al. 2009;
Tirapelli et al. 2009). The abnormalities appreciated during histological examination of tubules exposed to I-R reflect the pathophysiology of this type of injury as already discussed. The main tubular epithelial cell changes include: vacuolisation, pigmentation, pyknosis, necrosis, apoptosis and thinning or loss of the brush border (Williams et al. 1997; Devarajan 2006; Moosavi et al. 2009; Tirapelli et al. 2009). Damaged epithelial cells may detach from the basement membrane; this is visible as denuded areas of basement membrane and as exfoliated cells present within the tubular lumen (Tirapelli et al. 2009). Exfoliated cells may then combine with mucoproteins to become granular or cellular casts evident in the tubular lumen. Most of these changes can be discerned by examination of haematoxylin-eosin-stained sections of renal tissue using light microscopy. However different methods including special stains and electron microscopy may allow further characterisation of renal I-R injury. Many experimental studies formulate histopathology scoring systems to grade the severity of tubular damage based on some or all of the expected abnormalities (Jablonski et al. 1983; Moosavi et al. 2009; Tirapelli et al. 2009). In veterinary medicine, renal biopsy is rarely performed, likely due to financial constraints, unfamiliarity with techniques in obtaining and interpreting the biopsy, and concerns relating to the potential for exacerbation of renal injury during biopsy collection (e.g. anaesthesia-induced reduction in renal perfusion, or direct renal injury). Unfortunately renal histopathology following AKI in veterinary patients is more commonly performed in a post mortem setting.

**SCORING SYSTEMS**

Diagnosis of AKI in human patients is based on scoring systems such as RIFLE (Risk, Injury, Failure, Loss, End-stage) and AKIN (Acute Kidney Injury Network). These systems use changes in sCr and UOP to detect AKI and assign a severity grade (Mishra et al. 2003). While conventional diagnosis of AKI in veterinary
patients has long involved identification of azotaemia and a low urine specific
gravity, scoring systems similar to those used in people have recently been
investigated (Thoen & Kerl 2011; Lee et al. 2011). These single-centre veterinary
studies have generally detected an association between increasing severity score
and survival time; however these scores are designed to predict severity and
outcome rather than to detect AKI as early as possible. The tests used in the
scoring systems (sCr +/- UOP) have inherent limitations, as already discussed.
These studies do help to illustrate that trends of values such as sCr are of more use
that absolute values. The Veterinary Acute Kidney Injury staging system (Thoen &
Kerl 2011) classifies dogs as having stage 1 AKI if sCr is increased by >150%, or
by at least 26.5 µmol L⁻¹, from baseline. Only 21% of dogs classified to stage 1 in
that study had a sCr above the laboratory reference interval; in other words an
increase from baseline rather than absolute value of sCr allowed for diagnosis of
AKI in these dogs. A problem with relying on trends in these parameters to
diagnose AKI in veterinary patients is that we very rarely have access to blood
samples collected prior to injury (Ross 2011).

The International Renal Interest Society (IRIS) has recently proposed an AKI
staging system (Table 1.1). Patients are initially assigned to grade 1 - 6 depending
on sCr, and then further sub-graded according to UOP and the requirement for
renal replacement therapy. Grade 1 represents patients with sCr within the normal
canine reference interval, but evidence of AKI from history, clinical findings, other
laboratory findings or imaging. The aim of the IRIS system is to improve early
recognition, treatment stratification and prediction of outcome in veterinary
patients with AKI.
<table>
<thead>
<tr>
<th>AKI grade</th>
<th>Blood creatinine concentration</th>
<th>Clinical description</th>
<th>Subgrade</th>
</tr>
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| **Grade I** | < 140 μmol L\(^{-1}\) | Non Azotaemic AKI:  
  a. Documented AKI: (Historical, clinical, laboratory, or imaging evidence of acute kidney injury, clinical oliguria/anuria, volume responsiveness), and/or  
  b. Progressive non azotaemic increase in blood creatinine; ≥0.3 mg dL\(^{-1}\) (≥26.4 μmol L\(^{-1}\)) within 48 hours  
  c. Measured oliguria (<1 mL kg\(^{-1}\) hour\(^{-1}\)) or anuria over 6 hours | Each grade of AKI is further sub graded as:  
1. Non oliguric or oligoanuric  
2. Requiring renal replacement therapy |
| **Grade II** | 141 – 220 μmol L\(^{-1}\) | Mild AKI:  
  a. Documented AKI and static or progressive azotaemia  
  b. Progressive azotaemic increase in blood creatinine; ≥0.3 mg dL\(^{-1}\) (≥26.4 μmol L\(^{-1}\)) within 48 hours), or volume responsiveness‡  
  c. Measured oliguria (<1 mL kg\(^{-1}\) hour\(^{-1}\)) or anuria over 6 hours | |
| **Grade III** | 221 – 439 μmol L\(^{-1}\) | Moderate to Severe AKI:  
  a. Documented AKI and increasing severities of azotaemia and functional renal failure | |
| **Grade IV** | 440 – 880 μmol L\(^{-1}\) |  | |
| **Grade V** | > 880 μmol L\(^{-1}\) |  | |

**Table 1.1** Summary of the International Renal Interest Society’s 2013 “Grading of acute kidney injury” (Cowgill & Langston 2011).
THE FUTURE FOR ACUTE KIDNEY INJURY DIAGNOSIS

Given the pathophysiology of AKI, it is evident that the window of intervention to prevent ongoing damage following an ischaemic insult is short and occurs early in the syndrome. In order to detect patients in this early phase, a reliable diagnostic test is required. However, as discussed, it is clear that no such test exists.

It has been suggested that the ideal test would be non-invasive, rapid and easy to perform and robust, as well as sensitive and specific for the diagnosis of AKI (Devarajan 2010a). The search for the ideal marker has been a busy area of research in the human medical field for the last decade, with transcriptomic studies shifting interest towards novel biomarkers of renal structural damage, such as urine and serum concentrations of NGAL and KIM-1 (Moore et al. 2012). Veterinary interest has been slow to develop with only a handful of published studies investigating the use of these biomarkers in small animal species. The summary of a recent study into a staging system for canine AKI highlighted the importance of veterinary investigation into biomarkers such as NGAL concentration (Thoen & Kerl 2011). The following section will summarise current knowledge regarding this biomarker.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN

STRUCTURE, ORIGIN AND FUNCTION

Neutrophil gelatinase-associated lipocalin is a 45-kDa member of a family of small, secreted glycoproteins known as lipocalins, first identified in human granulocytes during a search for a marker of neutrophils (Flower 1996; Xu & Venge 2000). Lipocalins are a diverse family of proteins with a wide range of structures and functions, and common to all lipocalins are molecular recognition abilities such as binding to hydrophobic ligands, binding to cell-surface receptors
and formation of macromolecular complexes (Flower 1996). Lipocalins share a common ‘folding pattern’ consisting of a cup-shaped pouch inside a barrel of β protein sheets closed at each end with α helices (Fig. 1.2) (Flower 1996; Chakraborty et al. 2011). The fold’s purpose is ligand binding, with different lipocalins having different binding selectivity (Flower 1996).

Figure 1.2 The structure of the NGAL: the “lipocalin fold” comprises of eight β sheets (A-H) with the eighth connected to an α helix (α1). Diagram from Chakraborty et al. 2011.

NGAL expression has been identified in various cells and tissues including neutrophils, macrophages, lung, trachea, stomach, intestine, kidney, liver, bone marrow, thymus, prostate, uterus and adipose tissue (Flower 1996; Xu & Venge 2000; Chakraborty et al. 2011). Studies using ‘reporter mice’ have revealed that the origin of NGAL produced by the kidney is the epithelia of the thick ascending limb of Henle and collecting ducts; most of this renal NGAL is excreted in the urine (Haase et al. 2011b; Paragas et al. 2011). In the absence of AKI, the expression of NGAL by the distal nephron is small, however expression increases during tubular injury (Fig. 1.3) (Schmidt-Ott 2011). NGAL in the plasma may consist of some NGAL from non-renal origins (i.e. neutrophils and other organs), this NGAL is filtered by the glomerulus and then reabsorbed by the proximal tubules (thus only
entering the urine if there is failure of reabsorption) (Mori et al. 2005; Schmidt-Ott et al. 2007). It is known that different molecular forms of NGAL exist: the monodimer being of renal origin, and the homodimer form of neutrophil origin (Bangert et al. 2012; Lippi & Cervellin 2012).

**Figure 1.3** Renal transport of NGAL in (a) the normal kidney and (b) during AKI. (Diagram modified from Schmidt-Ott 2011).

Multiple roles have been suggested for NGAL with bacteriostatic abilities one of the most important. Neutrophil gelatinase-associated lipocalin has been shown to bind with strong affinity to siderophores (bacterial iron binding sites) thus depriving bacteria of an essential nutrient (Goetz et al. 2002; Yang et al. 2002; Mori et al. 2005; Chakraborty et al. 2011). Other suggested functions of NGAL include chemoattraction of neutrophils, inhibition of cellular oxidative stress, proliferation and synthesis of cartilage and proliferation of renal tubular epithelial cells (Chakraborty et al. 2011). It has been shown that introduction of systemic NGAL to mice prior to ischaemic kidney injury leads to improvement in subsequent kidney function with enhanced expression of protective enzymes, blunting of cell death and fewer abnormalities on renal histopathology when compared to mice that did not receive systemic NGAL (Mishra et al. 2004; Mori et al. 2005).
IDENTIFICATION OF THE INVOLVEMENT OF NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN IN KIDNEY INJURY

Neutrophil gelatinase-associated lipocalin has been identified as one of the most upregulated genes during renal I-R injury in a rodent model (Supavekin et al. 2003). Further investigations provide additional evidence of consistent up-regulation of NGAL within three hours of I-R injury, particularly in the proximal tubules (Supavekin et al. 2003). One study also assessed urine NGAL concentration (uNGAL) in mice and rats following renal ischaemia and found it to be easily detected in urine at the first sampling time of two hours after ischaemia. Furthermore, the appearance of increased uNGAL was associated with severity and duration of ischaemia (Mishra et al. 2003). These early rodent studies led to a plethora of human clinical trials to assess the usefulness of uNGAL as biomarker for the early diagnosis of AKI.

HUMAN CLINICAL STUDIES

A comparison between sCr and uNGAL or serum NGAL concentration (sNGAL) for the diagnosis of AKI following paediatric cardiopulmonary bypass was the first clinical study to be published (Mishra et al. 2005). A significant correlation was present between AKI (defined as a ≥ 50% increase in sCr from baseline) and increased uNGAL or sNGAL two hours post bypass. It should be noted that two hours was the first sampling time; therefore changes in NGAL concentration could have occurred earlier. Other similar studies have evaluated the use of uNGAL and/or sNGAL measurement in both adults and children in various settings, mainly during cardiopulmonary bypass or in the intensive care unit. An association between increased NGAL concentration measured as early as one hour post renal injury and the development of AKI has been found, with a relationship detected between NGAL concentration and severity and duration of AKI, length of hospital stay, requirement for renal replacement therapy and death in many studies (Dent et
al. 2007; Bennett et al. 2008; Nickolas et al. 2008; Haase et al. 2009; Devarajan 2010b; Macdonald et al. 2012). Unfortunately, many of these clinical studies have inherent limitations including being small, single-centre trials and the use of SCr for the definitive diagnosis of AKI rather than the gold standard, renal biopsy.

It should be stressed that unlike sCr, changes in NGAL concentration reflect renal tubular damage rather than changes in GFR. The difference between the role of sCr and uNGAL in AKI diagnosis has been described using a ‘forest fire’ analogy; where sCr is a measure of functional nephrons (‘trees’), and uNGAL the real-time extent of tubular lesions (the ‘fire’) (Mori & Nakao 2007). As change in uNGAL represents structural change to the kidney, it would not be expected to increase in purely pre-renal azotaemia (Schmidt-Ott 2011). Indeed, several clinical studies in human patients have confirmed an absence of a significant increase in uNGAL in patients with pre-renal azotaemia compared to those with intrinsic renal injury (Nickolas et al. 2008; Singer et al. 2011). These differences make uNGAL an earlier and more specific marker for acute tubular injury in comparison to sCr; and explain the apparent ability of uNGAL to predict severity of disease. In a recent meta-analysis of 2322 critically ill humans; 4.6% of patients with an increased NGAL concentration did not have a subsequent increase in sCr, with 2.5% of those patients going on to require renal replacement therapy (Haase et al. 2011a). This highlights that NGAL concentration may be better able to predict patients in the very early stages of AKI that may benefit from instigation of treatment at that time.

**Potential Limitations of Neutrophil Gelatinase-Associated Lipocalin Concentration in Acute Kidney Injury Diagnosis**

A limitation to the diagnostic value of uNGAL is the potential for changes in urine concentration and flow to alter uNGAL. To avoid this bias it has been suggested that correction for differences in urine concentration be performed when reporting
the concentration of urinary biomarkers. Normalisation of biomarker concentration to urine creatinine concentration may be used and involves measurement of urinary creatinine concentration and subsequent calculation of the uNGAL: urinary creatinine concentration (UNCR) (Goldstein 2010; Grenier et al. 2010). However, in order for urine creatinine concentration to be used for normalisation it is assumed that its excretion rate is constant both within an individual over time, and between individuals (Waikar et al. 2010; De Loor et al. 2013). During the early stages of AKI the GFR is likely to be changing rapidly leading to unstable urinary excretion of creatinine; in addition back-leak of creatinine across injured tubules may occur (Waikar et al. 2010). These changes are likely to result in an increased biomarker: creatinine ratio immediately after a reduction in GFR regardless of whether production of the biomarker itself is increased or not (Waikar et al. 2010; Ralib et al. 2012). A recent study comparing different methods of reporting urinary NGAL excretion in humans found that uNGAL performs best in AKI diagnosis, whereas UNCR is a better predictor of severity of disease (Ralib et al. 2012). Subsequently, it was suggested that uNGAL should be reported independently and as the UNCR in scientific reports investigating the use of urinary biomarkers in order to facilitate interpretation of results (Ralib et al. 2012). Calculation of urinary osmolality may also be used to correct for differences in urine concentration, however may be subject to similar limitations as UNCR during periods of low GFR (Tomonaga et al. 2012).

Concern has been raised as to the origin of the NGAL identified in urine and serum following renal injury, given that NGAL is not an organ-specific marker (Smertka & Chudek 2012). Elevated sNGAL has been noted in patients with lower respiratory tract infections, systemic inflammatory disease, thrombocytopenia, polycythaemia and neoplasia, while urinary tract infections may lead to elevated uNGAL (Smertka & Chudek 2012). Of particular concern has been potential
neutrophil origin for circulating NGAL rather than direct renal origin. To address this concern it has been postulated that uNGAL rather than sNGAL may correlate better with AKI. The NGAL excreted in the urine is believed to be of distal tubular origin (Fig. 1.3). In addition, the development and use of assays that are able to distinguish between the monodimer and homodimer forms of NGAL can allow resolution of this problem (Bangert et al. 2012).

Effects of pyuria and urinary tract infection (UTI) on uNGAL have recently been investigated in a canine study (Daure et al. 2013). Urinary NGAL concentration was measured in 80 dogs with UTI, and 19 healthy dogs with no evidence of UTI. Both uNGAL and UNCR were higher in dogs with UTI, however, only uNGAL varied significantly with the presence of pyuria. The authors were unable to determine whether the increased uNGAL was due to pyuria alone or to the disease process of a UTI. The assay used in the study did not differentiate between difficult molecular forms of NGAL.

A further concern regarding the use of NGAL as a diagnostic marker for AKI in humans is the wide variability in NGAL concentrations in patients without AKI (Smertka & Chudek 2012). This has made development of a reference interval difficult, and it has been shown that cut-off values for NGAL concentration to detect AKI are specific to each different patient population (Smertka & Chudek 2012).

**Neutrophil Gelatinase-Associated Lipocalin in Veterinary Literature**

Despite the availability of a canine specific NGAL enzyme-linked immunosorbent assay (ELISA), to date there are few veterinary papers published on the use of NGAL concentration as a diagnostic marker of renal disease. However, all
published canine clinical studies report a significantly increased uNGAL or UNCR in patients with AKI compared to patients without AKI.

The first published clinical study investigated changes in uNGAL following anaesthesia and surgery (Lee et al. 2012). Urine and serum samples were collected pre- and post-surgery from 39 dogs undergoing routine orthopaedic and soft tissue surgical procedures. Samples were collected every 12-24 hours from 12 hours post-surgery; however a sample was not collected from every dog at every time interval. Absolute NGAL concentration (rather than UNCR) was reported. Twelve of these dogs developed AKI as diagnosed by an increase in sCr of ≥ 26.5 μmol/L from baseline within 48 hours. The authors found that uNGAL at 12 hours post-surgery increased approximately 3.5 times from baseline in the dogs that were subsequently diagnosed with AKI, while there was no such change in uNGAL in the dogs that did not develop AKI. Interestingly sNGAL did not change in the dogs that developed AKI. Unfortunately, this study was limited in that it involved sample collection from only a small number of dogs, and there was no standardisation in surgical procedure, anaesthetic protocol or post-operative care. Due to the first sample time point being 12 hours post-surgery, the study was not designed to detect an early increase in NGAL concentration similar to that seen in human patients.

A small number of clinical canine studies have examined the use of NGAL concentration to diagnose AKI and CKD. One prospective study involved the collection of urine samples from 28 healthy dogs, 17 dogs with lower urinary tract disease, 20 dogs with CKD, 21 dogs with azotaemic AKI (IRIS stages 2-4) and 8 dogs with non-azotaemic AKI (IRIS stage 1) (Segev et al. 2013). Of the non-azotaemic dogs, seven developed AKI subsequent to heatstroke, and one developed AKI following general anaesthesia. Statistical analysis identified that UNCR values of the AKI groups were the highest of all groups, but there was no significant
difference between UNCR in dogs with azotaemic and non-azotaemic AKI. There was no correlation between UNCR and sCr. Receiver operating characteristics analysis of UNCR as a marker of azotaemic or non-azotaemic (IRIS Grade 1) AKI, when compared to non-AKI renal and urinary conditions, had an area under the curve of 0.94 and 0.96 respectively. Unfortunately, this study was limited by the inclusion of only a small number of dogs with AKI, particularly in the non-azotaemic group.

A single-centre clinical study investigated uNGAL and sNGAL in 17 dogs with AKI, 40 dogs with CKD and 12 healthy dogs (Hsu et al. 2014). Diagnosis of AKI was made on the basis of azotaemia being present for less than seven days and the absence of other clinical signs of CKD. Renal histopathology was not performed. Azotaemic dogs had increased sNGAL and uNGAL compared to control dogs. There was no association of sNGAL or uNGAL with 30 day mortality rate in dogs with AKI. However, sNGAL was found to be a better prognostic indicator for CKD survival than sCr. This study was again limited by a small sample size. There was large variation in the timing of sample collection in the disease process, and urine and serum samples were not always collected at the same time. Absolute NGAL concentrations rather than UNCR were reported. Without the use of histopathology, the method chosen to divide patients into AKI or CKD groups (duration of azotaemia and clinical history) may also have led to some patients with acute disease being classified as having CKD.

Another clinical study involved collection of urine and plasma samples from 18 healthy dogs, 17 dogs with CKD and 48 dogs with AKI (Steinbach et al. 2014). The underlying causes of AKI were not disclosed. The samples were collected once for each patient, during the first 24 hours of presentation to the clinic or within 24 hours of development of AKI. Acute kidney injury was diagnosed on the basis of presence of renal azotaemia that persisted for at least 24 hours with a USG
< 1.025. If azotaemia resolved within 24 hours of fluid therapy it was assumed to be pre-renal and these dogs were excluded from the study. Urine NGAL concentration and plasma NGAL concentration, as well as UNCR, were higher in dogs diagnosed with AKI compared to healthy dogs. Plasma NGAL concentration, but not uNGAL was also higher in dogs with AKI when compared to those with CKD. Although this study investigated a larger cohort of dogs compared to previous studies, unfortunately 31 of the 48 dogs diagnosed with AKI were anuric, meaning that data for uNGAL and UNCR was only available for 17 dogs with AKI. Also, the exclusion of patients with ‘pre-renal’ azotaemia may have excluded some dogs with AKI given the previously discussed failings of sCr and urine specific gravity to accurately reflect tubular injury.

Finally, more recent studies have evaluated the value of NGAL measurement for the diagnosis of CKD in dogs. The use of various urinary biomarkers to diagnose and stage CKD in dogs with X-linked hereditary nephropathy was investigated in 25 related male mixed breed colony dogs that tested positive for the genetic mutation (Nabity et al. 2012). Urine NGAL concentration increased early in the disease process, however in the mid and later stages of CKD there was no further change; suggesting that uNGAL may be less useful to stage more advanced cases of CKD.

Serum NGAL concentration has been compared in 62 dogs diagnosed with CKD and eight normal dogs (Ahn & Hyun 2013). In dogs with IRIS stages 3 and 4 (moderate and severe) CKD, sNGAL was higher than in control dogs, and strongly correlated to sCr and BUN concentrations. However, sNGAL did not correlate well with severity of disease despite being significantly higher in dogs with IRIS stage 4 CKD. This inability to detect any correlation may have been due to the wide range of sNGAL concentrations in dogs with this late stage of CKD. The authors suggest that sNGAL’s prognostic performance was poor in the later stage of CKD in their
study because sNGAL only increases as a result of active injury, rather than due to changes in renal function (which are the parameters used to stage CKD).

**SUMMARY**

Acute kidney injury is an important disease in canine patients with a high mortality rate. Given the self-perpetuating nature of the renal injury prevention and early identification are essential. NGAL has been highlighted as a valuable diagnostic tool for AKI in human patients, and as such further investigation into its use in the veterinary field is warranted. As well as potential as a clinical diagnostic tool, with further validation canine NGAL may become useful as a research method to diagnose and potentially stage the development of AKI during studies into the pathophysiology, treatment and prevention of AKI during general anaesthesia and critical illness in veterinary patients. Despite the recent publication of clinical veterinary studies examining the use of NGAL concentration to diagnose AKI and chronic renal disease, no one has described early changes in uNGAL in veterinary species using a clinically relevant model. Only one study has investigated the magnitude of change from baseline in individual dogs at specific time periods during AKI (Lee et al. 2012). Furthermore, all canine studies investigating uNGAL to date have relied upon the finding of an increased sCr without clinical signs consistent with chronic renal disease to diagnose AKI; definitive diagnosis using renal biopsy has not been performed.
STUDY AIM AND HYPOTHESIS

The objective of this study was to describe the very early changes in uNGAL in dogs subjected to AKI induced by a model of I-R.

The first aim of the study was to develop a canine I-R model that would produce histological evidence of renal tubular damage. It was hypothesised that ischaemia induced by hypotension for more than 30 minutes, and reperfusion achieved by fluid resuscitation for more than 60 minutes, would be required to produce such damage.

The second aim was to optimise the use of a commercial dog NGAL ELISA for use in our laboratory. It was hypothesised that dilution of urine samples required in order to measure NGAL within the dynamic range of the assay would be higher than manufacturer’s recommendations.

The third aim was to use the model to determine the early temporal pattern of change in uNGAL following I-R. It was hypothesised that uNGAL would increase significantly from baseline within three hours following I-R due to acute haemorrhage and fluid resuscitation.
REFERENCES


CHAPTER 2 – ESTABLISHMENT OF A MODEL FOR THE ASSESSMENT OF BIOMARKERS OF ACUTE KIDNEY INJURY

INTRODUCTION

There has been recently been interest in the use of novel biomarkers for early diagnosis of AKI in dogs, due to limitations of current diagnostic methods. Of these, NGAL has received a lot of attention in the veterinary and medical literature. Several large and multi-centre clinical studies in humans have shown that uNGAL and sNGAL increase from as early as two hours following the initiation of I-R kidney injury subsequent to various aetiological factors (i.e. cardiopulmonary bypass, sepsis) (Smertka & Chudek 2012). A few small clinical studies investigating the use of uNGAL to diagnose I-R AKI in dogs have been reported. While these canine studies show elevated uNGAL in dogs from 12 hours of AKI initiation, no studies have so far investigated usefulness of the biomarker in the very early stage of the syndrome (Lee et al. 2012). The previous clinical veterinary studies were also limited by their small sample size and lack of standardisation including the method for confirming AKI and the stage of disease at which uNGAL was measured.

To date, most experimental investigations of novel biomarkers (such as NGAL) have used complete occlusion to flow by clamping renal vasculature for varying time periods to create I-R in rodent models (Mishra et al. 2003; Supavekin et al. 2003; Mishra et al. 2004; Mori et al. 2005). Whilst these models have been useful for identification of early biomarkers of AKI, they lack clinical relevance as reduction in renal perfusion rather than complete occlusion to flow is more likely to occur in veterinary and human patients (Li et al. 2012; Moore et al. 2012).
Pressure-guided haemorrhage models have previously been used to create haemorrhagic shock in dogs in order to study the cardiovascular effects of hypovolaemic shock, and to investigate possible treatments for kidney injury (Sabouni et al. 1988; Chintala & Jandhyala 1990; Tsukamoto & Pape 2009). The fixed pressure model, first suggested by Wiggers, involves reduction of MAP below a certain threshold for a set time period (Wiggers 1942). An alternative method is to use a set volume model, where a standardised volume is removed from all patients. However in the set volume model, variation in blood pressure can be observed due to differences in ability of patient to compensate for the volume deficit. Using a fixed pressure model reduces this variability, producing a more standardised reduction in blood pressure and thus organ perfusion. This type of canine model has not previously been used in research assessing AKI diagnosis; hence the duration and magnitude of haemorrhagic shock required to produce AKI is unknown. This must be determined before the model can be used to investigate novel biomarkers of I-R AKI.

To develop a robust model of disease for the validation of novel biomarkers; it is important that injury can be confirmed, even quantified, using the most accurate test possible, i.e. a ‘gold standard’. Currently the gold standard for confirming the presence of AKI in humans and veterinary species is histopathology. Many previously reported animal models of AKI have used histopathology to document the extent of renal damage induced. Unfortunately most of these studies extend the reperfusion stage to several days, and consequently renal histopathology changes are not well documented during the very early stages of AKI. In veterinary clinical practice renal biopsy is rarely utilised; and generally limited to the diagnosis of chronic renal disease. Hence there are limited data to suggest how early histopathology changes can be identified during AKI, and what severity of insult is necessary to produce such changes. Furthermore, it may be difficult to distinguish
acute tubular necrosis from autolysis unless histopathologic examination is performed by an experienced pathologist using a strict grading criterion (Kocovski & Duflou 2009).

A canine specific ELISA capable of determining NGAL concentration in urine, plasma or serum, tissue extracts and culture media is commercially available for research purposes (Dog NGAL ELISA Kit, Bioponto® Diagnostics, Denmark). The availability of such a test provides opportunity to explore the usefulness of this biomarker in canine patients. However it has been noted previously that canine urine samples often require dilution in order to obtain concentrations within the working range of the assay (Steinbach et al. 2014). Before such an assay can be established for use in a research laboratory, its operating procedure must be assessed to ensure good performance across the range of NGAL concentrations expected.

The objectives of this pilot investigation were to i) determine if MAP < 40 mmHg for 30 or 60 minutes, followed by 1 or 2 hours of reperfusion associated with fluid administration, was sufficient to produce histopathological evidence of acute tubular damage within two hours of reperfusion; ii) determine if increases in uNGAL and sNGAL can be observed within two hours of reperfusion and iii) determine the magnitude of increase in uNGAL and sNGAL at various durations of I-R and thus subsequent dilutions that may be required for measurement of NGAL in future studies.
METHODS

ANIMALS

Six male entire greyhound dogs (range: 30.5 – 36.5 kg body weight) were used for this pilot study (Table 2.1). Ethics approval for the study was obtained from the Murdoch University Ethics Committee (permit number R2389/11) and the dogs were cared for according to the “Australian code for the care and use of animals for scientific purposes”. Dogs were included in the study if deemed to be in good health based on a physical examination. Food was removed at least eight hours prior but ad lib access to water was provided up to premedication.

ANAESTHESIA

Premedication was provided with intramuscular methadone (0.3 mg kg\(^{-1}\)) 30 minutes prior to induction of general anaesthesia. Access to water was removed at time of premedication. A 20 gauge 1.16 inch cannula was placed in the cephalic vein. Anaesthesia was induced with alfaxalone (Alfaxan injection 10mg mL\(^{-1}\), Jurox, Australia) 1.3-2.7 mg kg\(^{-1}\) by slow intravenous (IV) injection, until sufficient depth of anaesthesia was obtained to provide suitable conditions for orotracheal intubation. An orotracheal tube was placed, and the dog positioned in left lateral recumbency. The orotracheal tube was connected to a circle rebreathing system and anaesthesia was maintained using isoflurane (I.S.O., VCA, Australia) delivered in 2 L minute\(^{-1}\) of up to 100% oxygen. The lungs were ventilated with a time-cycled volume control ventilator at an initial tidal volume of 20 mL kg\(^{-1}\) and respiratory rate of 10 breaths per minute. Tidal volume was adjusted to ensure normocapnia prior to the start of the study. Intravenous Hartmann’s solution (Compound Sodium Lactate, Baxter Healthcare Pty Ltd, Australia) was administered via the peripheral venous catheter at 10 mL kg\(^{-1}\) hour\(^{-1}\). Dogs were warmed with a forced warm air blanket (Bair Hugger warming unit Model 505, Critical Assist, Australia) placed
over the dog and heating mat placed under the dog. Routine anaesthetic monitoring was performed at five minute intervals using a multi-parameter monitor (Surgivet V9203; Smiths Medical, USA) and included heart rate, respiratory rate, end-tidal carbon dioxide, pulse oximetry, core temperature, three-lead electrocardiography and non-invasive blood pressure measurement.

INSTRUMENTATION

Instrumentation was performed within the first 30 minutes of induction and included cannulation of the femoral artery, and placement of a urinary catheter. The left femoral artery was cannulised to facilitate measurement of arterial blood pressure and removal of blood to generate experimental haemorrhage. Following regional anaesthesia of the femoral nerve with bupivacaine 0.5%, the femoral artery was surgically exposed and a 14 gauge 3 inch cannula inserted and secured in place. For measurement of arterial blood pressure, the femoral artery catheter was connected by fluid filled non-distensible tubing to an electronic pressure transducer (DTX Plus, Argon Critical Care Systems, Singapore) placed at the level of the right atrium and attached to the multi-parameter monitor which displayed and recorded the arterial pressure waveform continuously as well as systolic, diastolic and mean blood pressure. The transducer was zeroed to atmospheric pressure before use, then flushed with heparinised saline prior to each measurement, and opened to the atmosphere at the end of each study to confirm the absence of baseline drift. An 8 French 55 centimetre Foley urinary catheter was placed for collection of urine samples. After instrumentation, the dog was repositioned in dorsal recumbency and allowed to stabilise before starting baseline measurements.
ISCHAEMIA - REPERFUSION MODEL

Following instrumentation, each dog was subjected to varying duration of I-R (Table 2.1). Ischaemia was generated via experimental blood loss by removing blood from the femoral arterial catheter. Sufficient volume was removed to maintain mean arterial blood pressure (MAP) below 40 mmHg. The reduced MAP was maintained for 30 minutes in Dog 2 and 60 minutes in the other five dogs (Table 2.1). Two to three standard units (32 - 48 mL kg$^{-1}$) of blood was collected over the first 20-30 minutes and then increments were removed as required to maintain MAP < 40 mmHg. The total volume of blood removed from each dog was recorded. After haemorrhage, reperfusion was produced by administration of fluid to increase and maintain MAP > 60 mmHg. Each dog received synthetic colloid +/- Hartmann’s solution (Compound Sodium Lactate, Baxter Healthcare Pty Ltd, Australia) 40 mL kg$^{-1}$ hour$^{-1}$ IV to maintain MAP > 60mmHg during the reperfusion period (Table 1). As synthetic colloids are excreted in urine to a variable extent, two colloids, commonly used clinically, were chosen to screen for possible effects of colloid molecule on measurement of uNGAL: either succinylated gelatine solution 4% (Gelofusine®, B Braun Australia Pty Ltd, Australia) or 6% hydroxyethyl starch 130/0.4 (Voluven®, Fresenius Kabi Australia Pty Ltd, Australia). Duration of reperfusion was 60 or 120 minutes (Table 2.1). At the end of the reperfusion period the dogs were euthanised by rapid intravenous administration of pentobarbitone 150 mg kg$^{-1}$ (Lethabarb Euthanasia Injection, Virbac, Australia).

DATA COLLECTION

Collection of urine and blood samples was performed approximately 60 minutes after induction of anaesthesia prior to haemorrhage (T0), immediately following
the haemorrhage (ischaemic) phase (T1); and then every 30 - 60 minutes during reperfusion. Urine samples were collected for all six dogs, but blood was collected only in dogs 2, 4, 5 and 6. Urine was collected from the urinary catheter and stored on ice for up to two hours. All urine in the bladder was removed at each sample time. Samples were then centrifuged for 5 minutes at 250 g to remove sediment. Blood was collected from the jugular vein and placed in serum tubes, allowed to clot, and centrifuged for 10 minutes at 500 g. Aliquots of both urine and serum were stored at -18 to -20°C for up to 24 hours prior to storage at -80°C for later measurement of NGAL concentration.

Immediately following euthanasia, both kidneys were harvested from five of the dogs (dogs 2 – 6) and 5 – 7 mm thickness cortico-medullary sections taken from the centre of each kidney were placed in 10% formalin. Samples were processed routinely, embedded in paraffin, sectioned at 3µm thickness and stained with haematoxylin and eosin and periodic acid-Schiff. One veterinary pathologist, unaware of the duration of I-R, examined cortex and medulla of the sections by light microscopy. In order to quantify tubular damage, sixty cortical fields (10X) from each kidney were chosen at random and then assessed for the presence of tubular damage. Tubular damage was defined as the presence of single cell necrosis / apoptosis, or the presence of epithelial cells undergoing vacuolar degeneration. The average number of damaged tubules per fields across both kidneys was calculated for each dog.

**Neutrophil Gelatinase-Associated Lipocalin Assay**

Neutrophil gelatinase-associated lipocalin concentrations in urine and serum were determined using a commercially available canine NGAL ELISA (Dog NGAL ELISA Kit, BioPorto Diagnostics, Denmark). Initially the ELISA was performed using the manufacturer’s recommended sample dilution of 1 in 100 (Plate 1). All
samples of serum or urine collected from the six dogs were included with each sample measured in duplicate. A subsequent plate (Plate 2) was used to investigate different dilutions when results fell out with the measureable range of the assay (4 – 400 pg mL\(^{-1}\)).

The assay was performed according to the manufacturer’s instruction (Dog NGAL ELISA Kit, BioPorto Diagnostics, Denmark). The assay is a sandwich ELISA performed in microwells coated with a mouse monoclonal antibody against canine NGAL. Bound NGAL is detected with a mouse monoclonal antibody labelled with biotin and the assay is developed with horseradish peroxidase—conjugated streptavidin and a colour forming substrate. The limit of detection of the assay was 0.56 pg mL\(^{-1}\), with an intra-assay coefficient of variation (CV) of 1.5% for urine and 2.5% for plasma.

Seven calibrator NGAL standards of concentrations 1, 4, 10, 20, 40, 100, 200 and 400 pg mL\(^{-1}\) were provided and used. Samples were diluted using the sample diluent provided, and mixed by moderate vortexing. 100µL of calibrator or diluted sample was added to each well, the plate was then incubated for one hour at room temperature on a shaking platform set at 200 revolutions per minute. The plate was washed with the wash buffer provided for three wash cycles using an automatic washer, then 100µL of biotinylated dog NGAL antibody added to each well. The plate was again incubated for one hour at room temperature on a shaking platform before three wash cycles to remove unbound detection antibody. 100µL of horseradish peroxidase-streptavidin was then added to each well, and the plate incubated for one hour at room temperature before three wash cycles were applied to remove unbound conjugate. Finally, 100µL of tetramethylbenzidine was added to each well and incubated in the dark for ten minutes before 100µL of dilute sulphuric acid was added to stop the colour change reaction. The optical density (absorbance) of each well was read at 450nm using an ELISA plate reader.
(iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, USA). The results of this plate demonstrated several measurements of NGAL concentration that lay out with the measureable range thus analysis of a second plate was performed.

The second NGAL ELISA plate contained serial dilutions of the four urine samples with the highest NGAL concentration and four urine samples with the lowest NGAL concentration, as measured in plate one. Urine containing a high NGAL concentration was diluted to 1 in 100, 1 in 200, 1 in 400, 1 in 800 and 1 in 1600. Urine with a low NGAL concentration was diluted to 1 in 25, 1 in 50, 1 in 75 and 1 in 100; an undiluted sample was also included. The ELISA procedure was then performed exactly as described above, with all samples included in duplicate.

CONTROL DOG

To investigate the effects of autolysis alone on renal histology, one additional greyhound dog (“control dog”) underwent direct euthanasia, rather than general anaesthesia and haemorrhage. Following placement of a cephalic intravenous cannula, intravenous pentobarbitone 150 mg kg⁻¹ was administered. Upon confirmation of death both kidneys were harvested in a method identical to that of the other six dogs, and stored in formalin. The same renal pathologist, blinded to the identity of the dog, then examined tissue sections stained with haematoxylin and eosin and periodic acid-Schiff from both kidneys.

STATISTICAL ANALYSIS

For each ELISA plate, a calibration curve was created using a trend line generated by an ELISA software program (MPM6 Microplate Manager Software; BioRad Laboratories Inc, CA, USA) using a linear y-axis (absorbance) and log x-axis (concentrations of standards) fitting a 4-parameter logistic curve (Fig. 2.1). The NGAL concentration (pg mL⁻¹) could then be calculated as proportional to the absorbance value. Coefficient of variation was calculated for all duplicates of
samples within a single ELISA plate. The mean of duplicated sample measurements was recorded as the sample concentration, however if the coefficient of variance between the two results was more than 15% the result was discarded. For ELISA plate two; the CV was also calculated between subsequent dilutions of the same urine sample. Data were reported as mean (confidence interval, CI) if normally distributed, or median (interquartile range, IQR) if not normally distributed.

RESULTS

Target MAP was achieved in five dogs; however difficulty was experienced maintaining the MAP of Dog 5 below 40 mmHg for 60 minutes consistently due to the development of pulsus alternans with complete absence of every second pulse and associated increase in the pressure generated by remaining pulse (Table 2.1, Fig. 2.2). It is presumed that the slower mechanical activity allowed improved ventricular filling during diastole and thereby increased cardiac output and MAP. The mean (95% CI) volume of blood required to be withdrawn to achieve MAP <40 mmHg for the period of haemorrhage was 50.0 mL kg\(^{-1}\) (range: 43.2 – 56.8).

Urine output was sufficient to obtain urine samples by 30-minute reperfusion in only three dogs (Dogs 1, 4 and 5); UOP was sufficient in the remaining three dogs by 60-minute reperfusion.

Average number of damaged renal tubules for Dogs 2 - 6 were around 4 times higher overall than those of the control dog (Table 2.2). Mean (95% CI) average number of damaged tubules over 60 fields (10X) for Dogs 2 – 6 was 0.127 (0.083 – 0.017). The lowest average number of damage tubules (other than that of the control dog) was observed in Dog 5 (the dog that maintained a higher MAP during the ischaemia phase).
For each urine sample on ELISA plate one, CV was < 15% between the two duplicate measurements; mean (95% CI) CV was 2.98% (2.28% – 3.69%) for duplicates. Of the 40 samples included in plate one, two returned no results, and their values were assumed to be out with the measureable range of the assay. Both of these samples were collected from Dog 4; one at 90 minutes following reperfusion and the other at 120 minutes following reperfusion. Nine samples measured < 4 pg mL$^{-1}$ and so were below the measureable range of the assay (Table 2.3). Six samples measured > 400 pg mL$^{-1}$ and so were above the measureable range of the assay (Table 2.3).

When serial dilutions were analysed on Plate 2 it was found that two samples required a 1 in 400 dilution, and two required a 1 in 600 dilution, in order to obtain an NGAL concentration measurement of < 400 pg mL$^{-1}$. There was large variation in NGAL concentration measurements between subsequent dilutions of the same urine sample until a concentration of < 400 pg mL$^{-1}$ was reached. When NGAL concentration of a diluted sample was > 400 pg mL$^{-1}$, mean (95% CI) CV between subsequent dilutions of the same sample was 18.88% (12.29 – 25.47). When NGAL concentration of a diluted sample was < 400 pg mL$^{-1}$, mean (95% CI) CV between subsequent dilutions of the same sample was 4.73% (3.26 – 6.18). Of the four urine samples containing low NGAL concentrations that were analysed on plate two, dilutions of 1 in 25 or less produced samples containing NGAL concentrations within the assay’s measureable range of 4 – 400 pg mL$^{-1}$. At these lesser dilutions, regardless of dilution there was wide variation in NGAL concentration measured between subsequent dilutions of the same urine sample, with mean (95% CI) CV between subsequent dilutions 34.25 (15.31 – 44.66).

Taking into account uNGAL reported from Plate 1 and those measurements repeated at more appropriate dilutions on Plate 2, uNGAL appeared to be elevated with respect to baseline in four dogs during reperfusion (Table 2.3). Median (IQR)
uNGAL at baseline was 5.0 (4.0 – 9.5) ng mL⁻¹. In three dogs uNGAL appeared to be elevated compared to baseline by 60-minute reperfusion (Dogs 1, 2 and 4), and in another dog uNGAL appeared elevated by 90-minute reperfusion (Dog 3). Urine NGAL concentration did not appear to be different when compared to baseline in the remaining two dogs (Dogs 5 and 6) (Table 2.3). Median (IQR) sNGAL at baseline was 29.3 (23.8 – 31.2) ng mL⁻¹ and sNGAL was not elevated from baseline at either 60-minute or 120-minute reperfusion (Table 2.4).

**DISCUSSION**

Histopathological evidence of renal tubular damage was successfully demonstrated in all dogs subjected to 30 - 60 minutes of ischaemia and up to 120 minutes of reperfusion. Furthermore, calculation of the average number of damaged tubules across a set number of 10X fields allowed correct identification of was able to correctly identify true ischaemic tubular damage in the six dogs that underwent haemorrhage from changes associated only with autolysis in the control dog. The results indicated that the optimal model of I-R required to produce AKI to use for subsequent studies was maintenance of MAP of < 40 mmHg for 60 minutes followed by at least 120 minutes of reperfusion.

As mentioned, most experimental animal studies investigating the pathophysiology, diagnosis, treatment and prevention of I-R AKI have utilised occlusion of renal vasculature to produce ischaemia. However, while a reduction in renal blood flow contributes to the development of I-R AKI during clinical situations such as anaesthetic-related hypotension and haemorrhagic shock, renal complete vascular occlusion is uncommon during veterinary surgery; thus the clinical relevance of these models is limited. Studies have shown that as well as tissue ischaemia subsequent to reduced renal perfusion, there are many other mechanisms involved in the development of tubular injury in these clinical
situations. Vascular congestion in the kidney during renal hypoperfusion has been found to produce more significant tubule injury than complete renal vascular occlusion (Moore et al. 2012). During shock, the activation of the sympathetic nervous system and altered plasma levels of hormones and cytokines exacerbate tubular injury (Chintala & Jandhyala 1990). The current model of haemorrhage induced renal hypoperfusion and subsequent I-R injury might therefore be expected to be more clinically relevant than the previous vascular occlusion models. The results of this pilot study support the use of this model in future studies.

The ELISA had good performance in our laboratory as evidenced by well-fitting calibration curves and low CV values for duplicates. Validation and quality control data from the manufacturer states that the assay is reliable in detecting NGAL concentrations within a range of 4 – 400 pg mL\(^{-1}\). In order to stay within this range, several urine samples containing a high concentration of NGAL required dilution of a much higher magnitude than the 1:100 suggested by the manufacturer. The observation that variance between calculated NGAL concentration in different dilutions of the same urine sample was reduced to < 10% once measured NGAL was below 400 pg mL\(^{-1}\) supports this value being the upper limit of reliable measurement of this assay. Our results suggest that, to obtain accurate results, urine samples containing high NGAL concentrations in subsequent studies should be diluted to at least 1 in 800. High variance was noted between different dilutions of the same urine sample when low NGAL concentration was present. This is not unexpected as any operator error will be amplified for samples containing low levels of a measured analyte.

Urine NGAL concentration was elevated in four of the six dogs in which AKI was demonstrated by histological evidence of tubular damage. This elevation was evident by 60-minute reperfusion in three of the four dogs and by 90-minute reperfusion in the remaining dog. Urine was difficult to obtain in 50% of the dogs
at 30-minute reperfusion due to inadequate UOP. However, as none of the 30-minute reperfusion urine samples showed any increase in uNGAL from baseline there is possibly no value in attempting to obtain samples at this early stage.

Surprisingly, Dogs 5 and 6 showed a small decline in uNGAL between 60 and 120 minutes of reperfusion. Dog 5 underwent less severe hypotension and also received the lowest tubular injury score, perhaps indicating that only mild renal injury was produced. However, Dog 6 underwent a full 60 minutes of MAP < 40mmHg with 120 minutes of reperfusion, and had a higher average number of damaged tubules, refuting an explanation that the decrease in uNGAL in these dogs was due to milder AKI. A similarity between Dogs 5 and 6 was that HES, rather than gelatin, was used for intravenous fluid therapy during reperfusion. Of the four dogs (Dogs 1 - 4) that showed an increase in uNGAL over time, three received gelatin and one hydroxyethyl starch. It was noted that urine samples obtained from all dogs during reperfusion became viscous and ‘sticky’, presumably due to high concentrations of these colloid-based solutions within the urine. This leads us to query a possible matrix effect of one or both of these colloid solutions, which could potentially explain variation in the magnitude of change in uNGAL between the dogs. Matrix effect refers to interference by constituents of the sample other than the analyte being measured on the response measured by the plate reader (NATA 2012). It is possible that the presence of either colloid solution could cross react with a stage of the immunoassay itself, or that they may affect the optical density of the ELISA plate wells during the plate reading process.

Another explanation for the variation in uNGAL noted between the dogs following haemorrhage could be that values were not corrected for urine creatinine concentration. Several human clinical studies of uNGAL as a biomarker of AKI suggest that the UNCR should be used rather than absolute uNGAL in order to account for variation in urine dilution (Grenier et al. 2010; Delanaye et al. 2011).
However, it is also suggested that during times of rapidly changing glomerular filtration rate (i.e. acute hypotension followed by diuresis subsequent to reperfusion) urine creatinine concentration may not be an accurate marker of urine dilution (Ralib et al. 2012). The best solution may be to present both absolute NGAL concentration and UNCR in subsequent studies.

A further explanation for variation in measured uNGAL between dogs could be the method of sample storage prior to analysis. Samples were stored on ice for up to two hours prior to centrifugation, and aliquots were then stored at -18°C to -20°C for up to 24 hours prior to storage at -80°C. To date there are no published studies investigating the stability of canine NGAL in urine at different storage temperatures. However, the effects of various storage temperatures on the stability of NGAL in human urine have been investigated. It was found that human NGAL is sufficiently stable in urine stored at 4°C for up to seven days, with <2% change in NGAL concentration between days one and seven (Grenier et al. 2010). For longer term storage (months) however human urine samples are required to be stored at -75°C as those stored at -20°C showed significant decrease in their NGAL concentration (Bennett et al. 2008). Until data reporting the stability of NGAL in canine urine is presented, it may be advisable to store urine samples at temperatures of -75°C or below for a maximum of 12 months.

Serum NGAL concentration failed to increase over time in the four dogs in which it was measured. This is not particularly surprising, as it mirrors another study investigating NGAL as a biomarker of AKI in dogs (Lee et al. 2012). This finding may be related to the different in origin of sNGAL as compared to the NGAL that appears in the urine. Serum NGAL may be partly or wholly derived from non-renal origins (i.e. neutrophils and other organs), and so some authors have suggested that uNGAL may correlate better with AKI than sNGAL (Haase et al. 2011; Paragas et al. 2011). The lack of increase in sNGAL also suggests that systemic effects of
shock did not cause increases in NGAL of other sources and were also unlikely to contribute to changes in urinary NGAL.

This pilot study provided encouragement that AKI in dogs can be achieved by acute haemorrhage to maintain MAP below 40mmHg for one hour followed by at least two hours of reperfusion. The renal tubular damage induced was clearly identifiable on histopathological examination as different to normal autolysis. The commercially available canine NGAL ELISA performed well within its recommended levels of measurement; however urine samples containing high NGAL concentrations required a much higher degree of dilution than suggested by the manufacturer. A significant increase in uNGAL was evident within 90 minutes of injury in 67% of dogs studied, although an increase in sNGAL was not observed. Inability to detect increases in all dogs may be due to potential matrix effects of colloid-base intravenous fluid solutions and this should be investigated as a potential bias during measurement of uNGAL before further animal studies are conducted. In addition, in future animal studies, all samples should be processed and frozen immediately to prevent degradation that might occur due to delayed sample processing.
REFERENCES


NATA Technical note 17: Guidelines for the validation and verification of quantitative and qualitative test methods, National Association of Testing Authorities (NATA).


Figures

Figure 2.1 Calibration curves (4-parameter logistic curve) obtained from (a) ELISA Plate 1; \( r^2 0.9996 \) (b) ELISA Plate 2; \( r^2 0.9993 \).
Figure 2.2 Mean arterial pressure (mmHg) recorded every five minutes in six individual greyhound dogs undergoing an ischaemia-reperfusion model of acute kidney injury. Zero to 60 minutes: instrumentation, 60 – 120 minutes: ischaemic phase, 120 – 240 minutes: reperfusion phase. Dogs 1, 2, and 6 underwent 60 minutes of reperfusion only.
Table 2.1  Signalment and anaesthetic data for six greyhound dogs that underwent haemorrhagic shock and fluid resuscitation during pilot investigations into a canine model of ischaemia-reperfusion acute kidney injury. Mean arterial blood pressure (MAP) measurements were made every five minutes during the haemorrhage phase. [SD; standard deviation].

<table>
<thead>
<tr>
<th>Dog</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Duration of haemorrhage phase (minutes)</th>
<th>Volume of blood removed during haemorrhage (mL)</th>
<th>Mean (SD) MAP during haemorrhage phase (mmHg)</th>
<th>Duration of reperfusion prior to euthanasia (minutes)</th>
<th>Type of colloid solution administered</th>
<th>Hartmann’s 40 mL kg⁻¹ IV during reperfusion (yes / no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>32</td>
<td>60</td>
<td>1825</td>
<td>33.2 (8.7)</td>
<td>60</td>
<td>Gelatin</td>
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</tr>
<tr>
<td>2</td>
<td>3</td>
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<td>30</td>
<td>1350</td>
<td>23.0 (7.4)</td>
<td>60</td>
<td>Gelatin</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>35</td>
<td>60</td>
<td>1350</td>
<td>26.9 (6.9)</td>
<td>120</td>
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<td>yes</td>
</tr>
<tr>
<td>4</td>
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<td>30.5</td>
<td>60</td>
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</tr>
<tr>
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<td>31</td>
<td>60</td>
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<td>120</td>
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<td>32.5</td>
<td>60</td>
<td>2003</td>
<td>34.5 (4.5)</td>
<td>120</td>
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Table 2.2 Average number of damaged tubules per 60 cortical fields (10X) for six greyhound dogs that underwent haemorrhagic shock and fluid resuscitation during pilot investigations into a canine model of ischaemia-reperfusion acute kidney injury, and of one normal control greyhound dog that had not undergone the acute kidney injury model

<table>
<thead>
<tr>
<th>Dog #</th>
<th>Average number of damaged tubules - right kidney</th>
<th>Average number of damaged tubules - left kidney</th>
<th>Average number of damaged tubules - Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>0.05</td>
<td>0.125</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>0.2</td>
<td>0.22</td>
<td>0.21</td>
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<tr>
<td>5</td>
<td>0.12</td>
<td>0.05</td>
<td>0.085</td>
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<tr>
<td>6</td>
<td>0.05</td>
<td>0.2</td>
<td>0.125</td>
</tr>
<tr>
<td>Control</td>
<td>0.03</td>
<td>0.03</td>
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**Table 2.3** Urine NGAL concentration (uNGAL) and tubular injury scores from greyhound dogs that underwent haemorrhagic shock and fluid resuscitation during pilot investigations into a canine model of I-R AKI. - No sample obtained due to poor urine output. Where measured NGAL concentration of a diluted sample from ELISA Plate 1 (1:100 dilution) was >400pg mL\(^{-1}\) or <4.0 pg mL\(^{-1}\), the concentration of the sample is reported as > 40 ng mL\(^{-1}\) or <4.0 ng mL\(^{-1}\). † Urine NGAL concentration measured when sample was diluted 1 in 1600 (ELISA Plate 2). ‡ Urine NGAL concentration measured when sample was undiluted (ELISA Plate 2).

<table>
<thead>
<tr>
<th>Dog #</th>
<th>uNGAL Pre-anaesthesia (ng mL(^{-1}))</th>
<th>uNGAL 30-minute reperfusion (ng mL(^{-1}))</th>
<th>uNGAL 60-minute reperfusion (ng mL(^{-1}))</th>
<th>uNGAL 90-minute reperfusion (ng mL(^{-1}))</th>
<th>uNGAL 120-minute reperfusion (ng mL(^{-1}))</th>
<th>Tubular injury score</th>
</tr>
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<td>&lt; 4.0</td>
<td>&gt; 40</td>
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<td>-</td>
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<td>12.83</td>
<td>-</td>
<td>&gt; 40</td>
<td>177.96†</td>
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<td>0.125</td>
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<td>433.17†</td>
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<tr>
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<td>-</td>
<td>4.35</td>
<td></td>
<td>&lt; 4.0</td>
<td>0.125</td>
</tr>
</tbody>
</table>


Table 2.4 Serum NGAL concentration (sNGAL) from greyhound dogs that underwent haemorrhagic shock and fluid resuscitation during pilot investigations into a canine model of I-R AKI.

<table>
<thead>
<tr>
<th>Dog #</th>
<th>sNGAL Pre-anaesthesia (ng mL⁻¹)</th>
<th>sNGAL 60-minute reperfusion (ng mL⁻¹)</th>
<th>sNGAL 120-minute reperfusion (ng mL⁻¹)</th>
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<tbody>
<tr>
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<td>27.78</td>
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<td>31.47</td>
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<td>32.30</td>
<td>20.01</td>
<td>17.18</td>
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<tr>
<td>6</td>
<td>11.73</td>
<td>17.83</td>
<td>12.01</td>
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CHAPTER 3 - INVESTIGATION OF INTERFERENCE FROM SYNTHETIC COLLOIDS ON THE PERFORMANCE OF A CANINE NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN IMMUNOASSAY

INTRODUCTION

Measurement of NGAL concentration in canine urine has recently gained interest as part of the search for an early biomarker of AKI (De Looor et al. 2013). Veterinary studies using a commercially available canine NGAL ELISA show that uNGAL is increased in canine patients with AKI when compared to those without renal disease (Segev et al. 2013; Steinbach et al. 2014).

As described in Chapter Two, the authors encountered a large degree of variation in measured urine NGAL concentration between individual dogs undergoing a haemorrhage-based model of I-R AKI. The type of fluid administered in order to produce reperfusion was not standardized between dogs and included an isotonic crystalloid solution, a gelatin-based colloid solution or a hydroxyethyl starch. A subjective increase in viscosity was noted for the urine of dogs that had received the colloid solutions.

Fluid therapy is one of the mainstay treatments for AKI in both veterinary and human patients, both as specific treatment (i.e. restoration of tissue perfusion subsequent to ischaemic AKI) and supportive in order to maintain appropriate hydration, acid-base and electrolyte status (Ross 2011). Fluid therapy may involve the use of synthetic colloid solutions, especially for volume expansion during the very early stages of AKI. Synthetic colloids are polydisperse macro-molecular solutions including gelatin-based solutions, starches and dextrans. These solutions
effectively increase intravascular volume as their high molecular weight allows them to both retain and draw water into the vascular space (Mensack 2008).

Synthetic colloids are eliminated from the body in the urine. Gelatin-based colloids contain molecules of a lower molecular weight that can be filtered by the glomerulus, whereas starch based solutions tend to contain larger molecules that must first undergo hydrolysis by plasma enzymes prior to renal elimination (Jungheinrich et al. 2002).

Given that the synthetic colloids are commonly used in the management of veterinary patients with AKI; it is highly likely that many urine samples obtained from animals with, or at risk for, AKI will contain some colloid within their matrix. It has been shown that as long as some urine production is present, patients with renal failure are still capable of renal excretion of the synthetic colloid, hydroxyethyl starch (Jungheinrich et al. 2002).

When establishing an immunoassay for use in a specific setting it is important to rule out any pre-analytical or analytical error, due to the presence of interfering substances within the sample. Interference may be described as any factor causing bias in an assay result, other than the presence of a true cross-reacting substance (Davies 2013). Interfering substances that may be found in the sample matrix are either endogenous (i.e. naturally occurring substances such as lipids, proteins and haemoglobin) or exogenous (i.e. drugs, toxins, storage effects and sample additives) (Dimeski 2008). These “matrix effects” can affect the performance of an immunoassay in the following ways: alteration of the effective analyte concentration, interference with antibody binding, interference with the binding of the capture antibody to the solid phase, interference in the separation stage, or interference with the spectrophotometric measurement (Davies 2013). Interference results in a falsely high or low measurement of analyte concentration. The magnitude of this effect usually depends on the concentration of the interfering
substance (Tate & Ward 2004). Immunoassays exposed to interference also may fail to display linearity when serial dilutions are performed (Systems; Tate & Ward 2004). Commercially available ELISAs undergo rigorous testing by the manufacturer to ensure there is no interference from many of the commonly recognized endogenous culprits; however some exogenous substances unique to a particular clinical setting or situation may not previously have been investigated. Hence, it is important to rule out any interference from the presence of synthetic colloids on sample matrix prior to the use of immunoassays to investigate the usefulness of potential biomarkers for AKI.

The aim of the present study was to investigate effects of two different synthetic colloid solutions; 4% succinylated bovine gelatin and 6% hydroxyethyl starch for potential interference with a commercially available dog NGAL ELISA. Following the variation noted in Chapter 2 it was theorized that the presence of synthetic colloid in the sample matrix could interfere with the performance of the assay. Effect of synthetic colloid on the assay could take the form of interference of signal measurement or direct/indirect interference with antibody-antigen binding.

**METHODS**

**PART (I) ASSESSMENT OF INTERFERENCE WITH LIGHT ABSORBANCE DURING SPECTROPHOTOMETRIC MEASUREMENT**

The effect of various concentrations of each colloid solution on mean absorbance measured via spectrophotometry was investigated. Samples tested included A) an undiluted sample of normal canine urine and the following solutions consisting of varying dilutions of urine with colloid; B) 1:3 urine: 4% succinylated bovine gelatin solution (Gelofusine®, B Braun Australia Pty Ltd, Australia [GEL]), C) 1:1 urine: GEL, D) 3:1 urine: GEL, E) 1:3 urine: 6% hydroxyethyl starch (Voluven®, Fresenius Kabi Australia Pty Ltd, Australia [HES]), F) 1:1 urine: HES, G) 3:1
urine: HES. Each of the six solutions, and the un-spiked urine (solution A), were then diluted to 1 in 100 using sample diluent. 200 μL of each of the seven solutions was then pipetted into 12 different wells of a clean 96-well ELISA plate. Solutions were allocated to wells in a manner such that each solution was measured at both the top, bottom, left and right of the plate in order to account for any drift during measurement. The optical density (absorbance) of each well was read at 450 nm using an ELISA plate reader (iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, USA). The mean absorbance of each solution was calculated as the average absorbance of each well containing that solution. One-way analysis of variance was used to determine differences in absorbance between the seven solutions. Where there was a significant effect at p < 0.05 post hoc testing using Tukey’s multiple comparisons test was used to determine which solutions differed. An adjusted p value of < 0.05 was considered significant.

(II) ASSESSMENT OF THE EFFECT OF LINEAR DILUTION

To assess for interference with the performance of the Bioporto® Dog NGAL ELISA due to synthetic colloid presence in the sample matrix, a standard solution of NGAL was linearly diluted with various colloid solutions. Expected and observed concentrations were compared. Standards were prepared as follows: 200 μL of the 400 pg mL⁻¹ NGAL ELISA kit calibrator solution was added to either 200 μL of kit sample diluent (Bioporto® Dog NGAL Kit sample diluent solution; prepared as instructed by manufacturer) to produce solution A, or 200 μL of each of six colloid solutions to create solutions B - G containing 200 pg mL⁻¹ of NGAL (i.e. a 1:2 solution). The six colloid solutions were: B) 1:3 sample diluent: GEL, G) 1:1 sample diluent: GEL, C) 3:1 sample diluent: GEL, E) 1:3 sample diluent: HES, F) 1:1 sample diluent: HES, B) 3:1 sample diluent: HES. The seven NGAL spiked solutions were then serially diluted using the same (un-spiked) solution as diluent, to 1:4, 1:8, 1:16 and 1:32. The dog NGAL ELISA was then performed as described.
previously (Chapter 2). The absorbance values of the kit calibrators were used to create a calibration curve using linear x and y axes with 4-parameter logistic curve fitting. All standards and samples were run in duplicate, and samples with a high duplicate error (CV >15%) were rejected. Linearity was then assessed for each serial dilution by calculating recovery where % recovery = [observed value / (value of previous dilution * dilution factor)] *100. Dilutions were assessed as being linear where recoveries fell between 80 – 120%.

RESULTS

PART (I)
The mean (±SD) absorbance values for each of the seven solutions were: solution A 0.116 (±0.022), solution B 0.097 (±0.028), solution C 0.094 (±0.020), solution D 0.095 (±0.015), solution E 0.088 (±0.019), solution F 0.090 (±0.013) and solution G 0.097 (±0.024). Mean absorbance values for solution A were significantly higher than those for solution E (p = 0.0137) and solution F (p = 0.0448). Mean absorbance between all other solutions were similar.

PART (II)
Recovery values ranged from 90.6 – 113.8% for all solutions at dilutions of 1:8 or less (Table 3.1). For solutions of 1:16 dilution recovery was < 80% for all GEL solutions, and for solutions E (1:3 sample diluent: HES) and G (3:1 sample diluent: HES). For 1:32 dilutions, all solutions returned recoveries of < 80% (Table 3.1).

DISCUSSION
The findings of this study do not support the presence of significant interference from the synthetic colloid 4% succinylated bovine gelatin on the dog NGAL ELISA investigated. A difference in mean absorbance measured in Part (I) for
urine compared to the two most concentrated HES solutions suggests that there may be a small amount of interference from 6% hydroxyethyl starch during spectrophotometry. Spectrophotometry is a method of quantifying colourimetric change by measuring the amount of light absorbed by a solution at a specific wavelength. For a ‘normal’ solution, Beer-Lambert’s law reveals a direct correlation between light absorbance of a molecule and its concentration, provided path length remains constant (Upstone 2000). Deviation from Beer-Lambert’s law or a change in path length could lead to erroneous concentration measurement. It is possible, though unlikely, that were any colloid incompletely washed from the ELISA microplate prior to spectrophotometry it could cause such a deviation.

Potentially, the addition of GEL or HES to the solution contained in a well of the microplate could lead to variation in path length, for instance if foaming tendency were higher with the colloid due to altered surface tension or due to alteration of the shape of the meniscus. Additionally, any alteration to the pH of a solvent can result in changes to molecular electron distribution and hence change that molecule’s absorbance spectrum (Upstone 2000). The GEL solution used in our study has a pH of 7.4 ± 1.3, whereas the HES solution has pH 4.0-5.5. It is possible that the lower pH of HES was responsible for the different mean absorbance observed with that solution in this experiment. The significance of the difference in mean absorbance witnessed in Part (I) on the performance of an ELISA is unknown; in this experiment the wells contained only sample and therefore a high concentration of diluent and colloid. In reality an ELISA plate would undergo several wash stages following addition of sample by the time spectrophotometry were performed, hence the concentration of colloid remaining in the wells would be much lower than that tested here and so the impact of HES on the mean absorbance measurement would be expected to be insignificant.
Potential methods for synthetic colloid solutions interfering with the antibody binding stage of the immunoassay include direct interference with antibody: antigen binding, or indirect interference. Direct interference is unlikely given that the proteins present in the colloid solutions are not binding proteins, reactive antibodies or autoantibodies, and do not structurally resemble NGAL. However, possible mechanisms by which synthetic colloids could interfere with antibody binding include: physical masking of the antibody coating of the plate, or alteration of antibody binding site conformation though changes in ionic strength or pH of the reaction medium (Davies 2013). Potential methods of indirect interference by synthetic colloids include: incomplete washing leading to sample carry-over, increased risk of pipetting error, or interruption of proper mixing of solutions. Presence of synthetic colloid makes sample matrix more viscous; this can lead to pipetting error due to an increased foaming tendency (i.e. tiny bubbles in the pipette tip lead to a smaller than anticipated volume being dispensed) or due to failure of all liquid to leave the pipette tip (Koivisto 2009). In assessment of linearity during serial dilution experiments recovery of 80-120% is considered acceptable. Recovery was within this acceptable range for all solutions at the 1:2, 1:4 and 1:8 dilutions. However at 1:16 all GEL solutions and two HES solutions gave recoveries <80%. This could suggest some interference from the synthetic colloids at these lower NGAL concentrations, however it is difficult to explain why recovery remained >80% for the 1:1 HES solution at this dilution if this were the case. At 1:32 solution recovery was very poor across all solutions, including kit sample diluent. The most likely explanation is that at this low dilution some of the NGAL concentrations measured were below 4 pg mL\(^{-1}\) which is the low end of the measurable range of the assay. Pipetting error also becomes more important at low dilutions, so the poor recovery could indicate problems with pipetting technique unrelated to the presence of synthetic colloid.
The concentration of colloid molecules in the urine of dogs following fluid resuscitation with synthetic colloid fluids in Chapter 2 could not be determined. Thus, it is not possible to know if the concentrations of the colloid solutions used in the *in vitro* experiments were an accurate representation of the urine concentrations. It is anticipated that concentration of colloid molecules was high as the urine became visibly viscous following administration of 20 mL kg\(^{-1}\) of each colloid intravenously. As the concentration was not known, testing for interference was performed on a range of concentrations, in attempt to capture the likely concentration within the urine.

A limitation to this study is that a full spiking-recovery study was not performed. It has been recommended that in addition to assessment of linearity during serial solutions, recovery studies are also performed whereby low, medium and high concentrations of the analyte to be measured are spiked into samples containing the matrix being examined for interference (Tate & Ward 2004). To improve the validity of our study it would have been optimal to spike multiple different samples containing each synthetic colloid with three different known concentrations of NGAL.

To conclude, this study found no evidence of significant interference on the performance of a commercially available canine NGAL ELISA by a 4% succinylated bovine gelatin solution. A 6% hydroxyethyl starch solution produced some variation in mean absorbance during spectrophotometry; however the relevance of this during performance of the ELISA is expected to be minimal. Recovery from all solutions containing 6% hydroxyethyl starch was poor at dilutions of more than 1:16 onwards, however this is likely to be a result of poor assay performance due to diluted samples containing NGAL concentrations below the measurable range of the assay. Based on these results, gelofusine may be the
preferred fluid for resuscitation in future studies investigating changes in uNGAL in a canine I-R model.
REFERENCES


**Table 3.1.** Assessment of linearity of neutrophil gelatinase-associated lipocalin (NGAL) concentration measured using a commercial dog NGAL enzyme-linked immunoassay during serial dilutions of a canine urine sample with solutions containing varying concentrations of synthetic colloids; 4% gelatin (GEL) or 6% hydroxyethyl starch (HES). † recovery falls outwith acceptable range of 80 – 100%.

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CHAPTER 4 – INVESTIGATION OF CANINE URINARY NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN CONCENTRATION DURING AN ISCHAEMIA-REPERFUSION MODEL OF ACUTE KIDNEY INJURY

INTRODUCTION

Acute kidney injury, a common syndrome in human and veterinary medicine, can be caused by I-R injury subsequent to renal hypoperfusion during general anaesthesia (Moore et al. 2012; Behrend et al. 1996; Mugford et al. 2013). Four sequential stages of AKI have been identified: initiation, extension, maintenance and recovery (Mugford et al. 2013; Devarajan 2006; Basile et al. 2012). Animal studies have shown that therapeutic interventions to reverse or prevent further progression of AKI must occur during the initiation or extension phases (Devarajan 2006). Unfortunately, AKI diagnosis and staging to date has relied upon indicators of glomerular filtration deficiency (e.g. sCr, UOP) that do not change significantly until the maintenance phase, two to three days following initiation of injury (Basile et al. 2012). Late diagnosis and delayed intervention contribute to the high mortality and morbidity associated with AKI (Moore et al. 2012; Basile et al. 2012).

Early diagnosis of AKI may be possible by measuring the urine concentration of NGAL, a biomarker of renal tubular injury. Urine NGAL concentrations are increased two hours following initiation of I-R AKI in rodent models using uni- or bi-lateral renal occlusion (Mishra et al. 2003; Mori et al. 2005). Clinical studies in people placed on cardiopulmonary bypass demonstrate increases in uNGAL within
two hours of renal I-R (Bennett et al. 2008; Mishra et al. 2005). While clinical studies in dogs report increases in uNGAL 12 hours after injury (Lee et al. 2010; Steinbach et al. 2014), whether or not uNGAL can detect tubular injury in dogs at earlier time points is unknown.

The aim of the current study was to document uNGAL changes in anaesthetised dogs using a model of renal I-R produced by experimental haemorrhage and fluid resuscitation. We hypothesised that the model would produce histological evidence of renal tubular damage and that uNGAL would increase from baseline within three hours of renal hypoperfusion.

**METHODS**

**ANIMALS AND SELECTION CRITERIA**

Seven donated adult entire male greyhound dogs, median (range) body weight 33 (30-35) kg, scheduled to be used as terminal blood donors were included in the study. Physical examination, renal ultrasonography, urinalysis, complete blood count, and sCr, BUN and serum albumin concentrations for all dogs were within reference intervals previously reported for healthy adult greyhounds (Zaldivar-Lopez et al. 2011). Ethics approval was granted by the Murdoch University Animal Ethics Committee (permit number R2586/13) and the dogs cared for according to the ‘Australian code for the care and use of animals for scientific purposes’.

**ANAESTHESIA**

Dogs were fasted for at least eight hours prior to the procedure. *Ad lib* water access was provided until premedication. Intramuscular methadone 0.3 mg kg$^{-1}$ (Ilium Methadone 10 mg mL$^{-1}$, Troy Laboratories Australia) was administered 30 minutes prior to induction of general anaesthesia using IV alfaxalone (Alfaxan injection 10 mg mL$^{-1}$, Jurox, Australia) 1.7 - 2.2 mg kg$^{-1}$. Following orotracheal intubation, dogs were positioned in left lateral recumbency. Anaesthesia was
maintained with isoflurane (I.S.O., VCA, Australia) delivered in an oxygen and medical air mix (30% inspired fraction of oxygen), via a circle system. End tidal isoflurane was maintained at 1.3 – 1.4% (1 x MAC) (Steffy & Howland 1977; Valverde et al. 2003). Hartmann’s solution (Compound Sodium Lactate, Baxter Healthcare, Australia) 10 mL kg⁻¹ hour⁻¹ IV and fentanyl (Fentanyl injection 50 µg mL⁻¹, AstraZeneca, NSW, Australia) 2 µg kg⁻¹ hour⁻¹ IV were administered throughout anaesthesia. The fentanyl infusion provided additional analgesia, particularly at end of study when the effects of the methadone premedication and femoral nerve might begin to wane. Mechanical ventilation was performed to maintain normocapnia (arterial carbon dioxide tension 35-45 mmHg) using a volume-controlled time-cycled veterinary anaesthesia ventilator (Model TH-1, Beijing Read Eagle Technology Co Ltd, China). Active warming maintained oesophageal temperature between 36.5 – 37.5°C.

INSTRUMENTATION

A 12 gauge 13 cm cannula (Angiocath; Becton Dickinson Infusion Therapy Systems Inc.; USA) was inserted into the cranial vena cava via the right jugular vein for collection of blood samples and injection of lithium chloride. The left femoral artery was cannulated (20 gauge 3 cm Angiocath; Becton Dickinson Infusion Therapy Systems Inc.; USA) to facilitate measurement of mean arterial pressure (MAP), measurement of lithium chloride voltage for cardiac output (Qt) calculation and removal of blood to generate experimental haemorrhage. A nerve stimulation-guided (Innervator 272; Fisher & Paykel Health Care; New Zealand) femoral nerve block was performed at the femoral triangle with bupivacaine (0.1 mL kg⁻¹; Bupivacaine hydrochloride 50 mg mL⁻¹; Pfizer, NSW, Australia), before the femoral artery was surgically exposed and a 14 gauge 9 cm cannula (Angiocath; Becton Dickinson Infusion Therapy Systems Inc.; USA) inserted. The femoral artery cannula was connected by heparinised saline filled non-distensible
tubing to a pressure transducer (DTX Plus, Argon Critical Care Systems, Singapore) interfaced with a multi-parameter monitor (Surgivet V9203; Smiths Medical, USA). Transducer accuracy was verified by calibration with a mercury manometer at 40, 80 and 120 mmHg prior to use in each animal. After instrumentation, the transducer was positioned at the level of the right atrium (level with the manubrium of the sternum) and zeroed to atmospheric pressure. A rapid flush test was performed to assess the damping factor subjectively. At the end of each study the transducer was opened to the atmosphere to confirm absence of baseline drift. An 8Fr 55cm urinary catheter (Foley urinary catheter; Smiths Medical, USA) was inserted into the bladder for collection of urine samples and measurement of UOP. After instrumentation, which occurred within the first 30 minutes of anaesthesia, the dog was repositioned in dorsal recumbency.

**EXPERIMENTAL DESIGN**

Ischaemia was produced by removing sufficient blood volume via the femoral arterial catheter to maintain MAP ≤ 40 mmHg for 60 minutes. Total blood volume removed from each dog was recorded. Reperfusion was produced by maintaining MAP ≥ 60 mmHg for three hours via administration of IV succinylated gelatine solution 4% (Gelofusine, B Braun, Australia). Initially 20 mL kg⁻¹ was administered at 1200 mL h⁻¹, and additional colloid administered as required. Total volume of colloid administered was recorded. Following the reperfusion period, dogs were euthanised using pentobarbitone (Lethabarb Euthanasia Injection 300 mg ml⁻¹, Virbac, Australia) 150 mg kg⁻¹ IV.

**DATA COLLECTION**

Data collection was performed at five time points: T0 (baseline) = 60 minutes after induction of anaesthesia and before haemorrhage; T1 = 60 minutes from the time
MAP reached $\leq 40$ mmHg; T2 = at one hour, T3 = at two hours and T4 = at three hours after fluid resuscitation achieved a MAP > 60 mmHg.

At each time point, 5 consecutive measurements of MAP were recorded and the mean MAP was calculated. Arterial and central venous (cranial vena cava) blood was collected into commercial pre-heparinised syringes (PICO syringe; Radiometer, Denmark) and processed immediately for arterial and central venous oxygen tension ($\text{PaO}_2$ and $\text{PcvO}_2$ respectively), sodium concentration ($\text{Na}^+$), haemoglobin concentration (Hb) and arterial and central venous oxygen saturation, using an in-house blood gas analyser and co-oximeter (ABL 725, Radiometer, Denmark).

Cardiac output was measured by lithium dilution technique (Mason et al. 2001). For each Qt measurement at T0 and T1, 0.15 mmol (1 mL) lithium chloride was administered. At T2 – T4, 0.3 mmol (2 mL) lithium chloride was administered for accurate measurement during the high Qt state observed during reperfusion. Two Qt measurements were performed within 5 minutes at each time point. Duplicate measurements were repeated if the two measurements were disparate by >10%

Blood was obtained from the jugular vein catheter at each time point and serum stored at -80°C for later SCr measurement (AU 480; Beckman Coulter; USA).

All urine was collected, and UOP recorded at each time point. Urine was immediately centrifuged at 250 g for 5 minutes. Cytospin preparations of urine sediment were air-dried and stained with Wright’s stain before examination using light microscopy at low (x100) and high (x400) power. Urine sediment examination was performed on a minimum of 10 high power fields. Urine creatinine concentration was measured for each sample. Remaining urine supernatant was divided into aliquots for storage at -80°C.

Kidneys were removed from all dogs and 5 – 7 mm thickness cortico-medullary sections taken from the centre of each kidney were placed in 10% formalin within 10 minutes after euthanasia. Samples were processed routinely, embedded in
paraffin, sectioned at 3µm thickness and stained with haematoxylin and eosin and periodic acid-Schiff. One veterinary pathologist, unaware of the results of the biomarker analysis, examined the sections by light microscopy. Twenty cortical fields (20X) from each kidney were chosen at random (by moving the microscope to a new field while not observing the section through the oculars) and then assessed for the presence of tubular damage. Tubular damage was defined as presence of epithelial cells undergoing vacuolar degeneration or tubules with intraluminal detached necrotic cells. The average number of damaged tubules per fields across both kidneys was calculated for each dog.

Urine NGAL concentration was determined using a commercially available enzyme linked immunoassay (ELISA) validated for use in the dog (Dog NGAL ELISA Kit, BioPorto Diagnostics, Denmark) performed according to the manufacturer’s instructions. Sample diluent provided was used to create a 1:1000 dilution of each urine sample. Each sample was analysed in duplicate and the mean of the two measurements recorded. If the coefficient of variation of the mean absorbance of two duplicates was >15%, the result was discarded and the sample re-analysed in duplicate. The measureable range of the assay as reported by the manufacturer is 4 – 400 pg mL⁻¹. Concentrations greater or less than this range were assigned 4 pg mL⁻¹ or 400 pg mL⁻¹, respectively.

**Statistical Analysis**

To determine presence and then resolution of shock present after haemorrhage and following reperfusion, OER was calculated. This first required calculation of body surface area, cardiac index (CI), oxygen delivery (DO₂I), and oxygen consumption (VO₂I) (Appendix 1). Severe shock was defined as OER > 0.5 (Gutierrez et al. 1986; Samsel et al. 1988; Cilley et al. 1989). In addition urine NGAL:creatinine concentration ratio (UNCR) were calculated (Appendix 1). Haemodynamic and
uNGAL data were tested for normality using a Shapiro-Wilk normality test and parametric data described as mean and 95% confidence interval (CI). Where the calculation of the 95% CI resulted in a negative lower confidence limit, the limit was reported as zero. Repeated measures one-way ANOVA was used to determine change in OER, sCr, uNGAL and UNCR over time. Where there was a significant effect at $p < 0.05$, post hoc testing using Dunnett’s test for multiple comparisons to baseline was performed with an adjusted $p < 0.05$ considered significant. The fold change from baseline at T3 and T4 was calculated by dividing the difference between uNGAL (or UNCR) at T3 or T4 by the uNGAL (or UNCR) at T0. The 95% CI of the fold change was also calculated.

**RESULTS**

Mean (95% CI) volume of blood withdrawn to achieve MAP $\leq 40$ mmHg for 60 minutes was $53 (48–57)$ mL kg$^{-1}$ (Fig. 1). Oxygen extraction ratio after 60 minutes of hypotension (T1) was significantly elevated from T0 ($p < 0.0001$), however OER was $> 0.5$ in only 6 dogs, and 0.44 in the remaining dog (Table 1). Mean (95% CI) volume of succinylated gelatin solution required to maintain MAP $\geq 60$ mmHg was $23 (20–26)$ mL kg$^{-1}$. Administration of gelofusine was accompanied by a decrease in OER at T2 and T3 to values not significantly different from baseline OER.

Serum creatinine concentration was increased when compared to baseline (T0) after 60 minutes of hypotension (T1) ($p < 0.0001$) and remained elevated at T2 ($p < 0.0001$), T3 ($p < 0.0001$) and T4 ($p = 0.005$) (Table 1).

Urine output of $< 1$ mL kg$^{-1}$ hour$^{-1}$ (oliguria) occurred at T1 and T2, with small increases in UOP at T3 and T4 (Table 1). Urine sediment examination of one dog
revealed presence of degenerate neutrophils at T1 – T4. Neutrophils were not present in urine of any other dog at any time point.

The NGAL concentration for all 1:1000 dilutions fell below the upper end of the measurable range of the ELISA. The NGAL concentration for 7 samples (two at T0 and five at T1) was below the lower end of the measurable range of the ELISA and recorded as 4 pg mL$^{-1}$.

Urine NGAL concentration was significantly elevated compared to baseline (T0) at T3 ($p = 0.016$) and T4 ($p = 0.046$) (Table 1). Mean (95% CI) uNGAL fold increase was 24.2 (7.3 – 41.0) at T3 and 18.1 (4.7 – 31.5) at T4. Urine NGAL:creatinine ratio was significantly elevated compared to baseline (T0) at T3 only ($p = 0.005$) (Table 1). Mean (95% CI) fold increase in UNCR was 41.1 (10.8 – 71.4) at T3 and 24.5 (3.7 – 45.2) at T4.

Urine NGAL concentrations at T0 and T1 from one dog (Dog F) were considerably higher (10x) than of other dogs. Statistical analysis was performed with and without data from this dog (Table 2).

When dog F was excluded from data analysis, uNGAL was significantly elevated compared to baseline (T0) at T3 ($p = 0.021$) and T4 ($p = 0.039$) (Table 2). Mean (95% CI) uNGAL fold increase was 27.9 (10.4 – 45.5) at T3 and 21.0 (6.7 – 35.3) at T4. Urine NGAL: creatinine ratio was significantly elevated compared to baseline (T0) at T3 only ($p = 0.015$) (Table 2). Mean (95% CI) fold increase in UNCR was 47.3 (14.7 – 80.0) at T3 and 28.3 (5.2 – 51.4) at T4.

Renal tubular damage was evident in both kidneys from all dogs on histologic examination (Fig. 2). Lesions were identified in the proximal tubule, predominantly at the tubulo-glomerular junction and included vacuolated cells, intra-tubular detached necrotic cells or cellular debris and attenuated epithelial cells. The average number of damaged tubules within a 20x magnified high power field for all dogs ranged from 1 to 20 (median 3). Histopathology scores for all
dogs are displayed alongside fold change in uNGAL and UNCR at T3 and T4 in Table 3.

**DISCUSSION**

This study determined uNGAL and UNCR changes during an I-R model designed to simulate renal hypoperfusion and reperfusion secondary to intra-operative haemorrhage and subsequent fluid resuscitation. Histology confirmed that the model successfully produced acute tubular damage (Devarajan 2010). The results confirmed increases in uNGAL and UNCR from baseline within two hours of reperfusion by 28-fold and 47-fold, respectively.

Changes in uNGAL produced by this clinically relevant model of acute ischaemic tubular injury are consistent with those from rodent vascular occlusion models and human clinical studies. Increased uNGAL within two hours of acute tubular ischaemia occurred in experimental studies using renal vascular clamping models of ischaemic AKI in rodents (Mishra et al. 2003). Increased uNGAL and UNCR were also reported within one hour of ischaemic insult in human patients who developed AKI subsequent to cardiac surgery. Following cardiopulmonary bypass, uNGAL increased by almost 100-fold in children within two hours, and by 40-fold in adults within one hour (Mishra et al. 2005; Wagener et al. 2006).

The current study reports changes in both uNGAL and UNCR. It is suggested that measuring UNCR avoids variation in uNGAL caused by altered urine concentration in subjects with a steady state glomerular filtration rate (Goldstein 2010; Grenier et al. 2010). However, normalisation to urine creatinine concentration assumes constant urinary creatinine excretion in an individual over time, and between individuals, a requirement unlikely to be met during AKI.
Due to controversy in this area, it was decided to report both uNGAL and UNCR in the current study.

Of note, uNGAL in one dog (Dog F) was initially higher compared with the other dogs. This finding created difficulty in analysis since it substantially skewed data at early time points. With a small sample size, it cannot be known if this dog represents normal variation or a spurious finding. It is unlikely that pre-existing renal disease was present given a normal pre-anaesthetic assessment and lack of changes consistent with chronic renal disease on histology. The UNCR was also higher at baseline for this dog; meaning more concentrated urine was unlikely to have produced the observed increased uNGAL. Wide variation in baseline uNGAL for healthy people has created difficulty in determining a reference interval leading to suggestion that magnitude of uNGAL change from baseline is more appropriate than a single measurement (Smertka & Chudek 2012; Clerico et al. 2012).

Statistical analysis with and without data from this dog was still able to detect increases in NGAL during our study, supporting the usefulness of the biomarker for detecting AKI when baseline measurements were available. However, further work is required to determine usefulness in clinical practice where a baseline sample is frequently not available.

Our study also revealed variation in magnitude of uNGAL and UNCR increase from baseline following I-R injury. Variation in uNGAL and UNCR measurements between individual dogs with AKI in other studies is similarly wide (Steinbach et al. 2014; Segev et al. 2013). Potential explanations include: differing severity of tubular injury between dogs, or presence of neutrophil origin NGAL. In humans, correlation between magnitude of uNGAL increase and mortality suggests that greater increases are associated with more severe injury (Haase et al. 2009). Histology revealed variation in the degree of tubular damage induced between dogs, and this may account for some variation in the magnitude of uNGAL change.
between individual dogs. The reason for differing severity of tubular damage between dogs was unclear. Possibly, despite attempts to produce a standardised I-R model, degree of shock induced in each dog differed and may have resulted in differences in renal hypoperfusion. Severe shock (the point at which anaerobic metabolism occurs due to mismatch between oxygen delivery and consumption) has been shown to occur at OER > 0.5 during various types of shock in anaesthetised dogs (Cilley et al. 1989; Samsel et al. 1988; Gutierrez et al. 1986). In the current study, six dogs had an OER > 0.5 after haemorrhage, while one had an OER of 0.44. Furthermore, it is possible the severity of shock was not accurately quantified in this study due to measurement of central venous oxygen rather than mixed venous oxygen. It is also important to point out that OER is measure of global oxygen delivery and without measuring renal blood flow we cannot determine whether changes in uNGAL and tubular damage were associated with variable changes in renal oxygen delivery.

Another potential source of variation may be an extra–renal source of NGAL such as neutrophils (Smertka & Chudek 2012; Mori & Nakao 2007; Lippi & Cervellin 2012). Elevated uNGAL has been detected in non-azotaemic dogs with pyuria, presumably due to increased presence of neutrophils in the urinary tract (Daure et al. 2013). Human neutrophils produce a different molecular form of NGAL compared to the renal form (Lippi & Cervellin 2012; Daure et al. 2013; Cai et al. 2010). Although ELISAs that distinguish between different molecular forms of human NGAL are available, canine NGAL ELISAs are currently unable to make this discrimination (Bangert et al. 2012). Pyuria is unlikely to explain uNGAL variation in the current study, as only one dog had neutrophils in its urine and uNGAL was not higher than the other dogs.

The current study demonstrated a small increase in SCr from baseline after hypotension and reperfusion. The International Renal Interest Society AKI grading
system defines AKI Grade I and Grade II as a non-azotaemic (<140 µmol L\(^{-1}\)) or mildly azotemic (141-220 µmol L\(^{-1}\)) patient with progressive increase in SCr of >26.4 umol L\(^{-1}\) within 48 hours (Cowgill & Langston 2011). Using this system, all dogs would have been assigned as Grade II from T1 onwards. However, this grading system defines azotaemia as SCr > 140 µmol L\(^{-1}\), and the reference interval for SCr is higher in greyhounds than other canine breeds, thus IRIS may not be an appropriate grading system for this breed. Indeed, peak SCr during the study was above the normal reference interval for greyhounds in only three dogs (Zaldivar-Lopez et al. 2011). Significant SCr increases are generally not observed until the maintenance phase of AKI, when functional changes such as altered glomerular filtration rate become apparent, so it is more likely that the small increases in SCr in our dogs occurred due to pre-renal changes (Basile et al. 2012). This is suggested by the finding that increased SCr was evident immediately following hypotension (T1) and did not increase further during reperfusion. As change in uNGAL represents structural change to the kidney, it would not be expected to increase in purely pre-renal AKI, potentially making it a more sensitive marker of kidney damage than SCr.

A limitation of our study is that uNGAL was measured for only three hours following I-R, so we were unable to report whether the dogs would have progressed to the maintenance phase of AKI. The fold increase in uNGAL observed in our study was similar to that seen within a few hours of ischaemic injury in adult humans who progressed to the maintenance stage of AKI (Wagener et al. 2006). The decrease in mean uNGAL at T4 following its initial peak at T2 and T3 might suggest that the AKI induced by our model was minor and reversible, although it could represent a similar trend observed in human studies whereby an early rise in uNGAL is followed by a lesser yet sustained increase (Mishra et al. 2005). Canine clinical studies performed over a longer timeframe are required to clarify the relevance of the temporal change observed in the current study.
The finding that uNGAL was significantly elevated from baseline within two hours of reperfusion in a clinically relevant model of renal I-R injury provides encouragement for the use of uNGAL as an early biomarker for AKI in dogs. However, given the potential for baseline variation and differing magnitude of change between individual dogs, further work is required to clarify the usefulness of this marker in clinical practice.
REFERENCES


**Figure 4.1** Mean arterial pressure recorded every five minutes for seven individual greyhound dogs that underwent a haemorrhagic shock model of ischaemia-reperfusion acute kidney injury. Study design: instrumentation, 0 – 60 minutes; ischaemic phase, 60 – 120 minutes; reperfusion phase, 120 – 300 minutes.
### TABLES

**Table 4.1** Formulae for calculated variables determined in seven greyhounds undergoing a haemorrhagic shock model of renal ischaemia and reperfusion.

Body surface area calculation has previously been published for use in dogs (Rosenthal 1995).

<table>
<thead>
<tr>
<th>Calculated variable</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (m²)</td>
<td>10.1 x (bodyweight in grams(^{1/3}) x 10(^{-4})</td>
</tr>
<tr>
<td>Cardiac index (L minute(^{-1}) (m²)(^{-1}))</td>
<td>Qt ÷ BSA</td>
</tr>
<tr>
<td>CaO(_2) (mL L(^{-1}))</td>
<td>(1.36 x SaO(_2) x Hb) + (0.003 x PaO(_2)) x 10</td>
</tr>
<tr>
<td>DO(_2)I (mLO(_2) minute(^{-1}) (m²)(^{-1}))</td>
<td>Cardiac index x CaO(_2)</td>
</tr>
<tr>
<td>CcvO(_2) (mL L(^{-1}))</td>
<td>(1.36 x ScvO(_2) x Hb) + (0.003 x PcvO(_2)) x 10</td>
</tr>
<tr>
<td>VO(_2)I (mLO(_2) minute(^{-1}) (m²)(^{-1}))</td>
<td>Cardiac index x (CaO(_2) – CcvO(_2))</td>
</tr>
<tr>
<td>OER</td>
<td>VO(_2)I ÷ DO(_2)I</td>
</tr>
<tr>
<td>UNCR</td>
<td>uNGAL (ng mL(^{-1})) ÷ urine creatinine concentration (g/L)</td>
</tr>
</tbody>
</table>

BSA, body surface area; Qt, cardiac output; CaO\(_2\), arterial oxygen content; DO\(_2\)I, oxygen delivery index; CcvO\(_2\), central venous oxygen content; OER, oxygen extraction ratio; VO\(_2\)I, oxygen consumption index; uNGAL, urine neutrophil gelatinase-associated lipocalin concentration; UNCR, uNGAL: urine creatinine ratio
Table 4.2 Indices of cardiovascular and urinary function in seven anaesthetised greyhound dogs before haemorrhage (T0), after 60 minutes of haemorrhagic (ischaemic phase) 40 mmHg (T1) and 1 hour (T2), 2 hours (T3) and 3 hours (T4) after fluid resuscitation (reperfusion phase). At T0 and T1, urine neutrophil gelatinase-associated lipocalin concentration (uNGAL) from one dog was considerably higher than of other dogs, consequently statistical analysis was performed with all seven dogs, and also without data from this dog. Values are mean (95% confidence interval). *Significantly different from T0 ($p < 0.05$)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
</tr>
<tr>
<td>Mean arterial pressure (mmHg)</td>
<td>65 (60 – 69)</td>
</tr>
<tr>
<td>Oxygen extraction ratio</td>
<td>0.15 (0.10 – 0.19)</td>
</tr>
<tr>
<td>Urine output (mL kg$^{-1}$ hour$^{-1}$)</td>
<td>0.2 (0.2 – 0.3)</td>
</tr>
<tr>
<td>Serum creatinine concentration (μmol L$^{-1}$)</td>
<td>134 (116 – 153)</td>
</tr>
<tr>
<td>uNGAL (ng mL$^{-1}$)</td>
<td>12.1 (0 – 30.6)</td>
</tr>
<tr>
<td>uNGAL (ng mL$^{-1}$) (n=6)</td>
<td>4.6 (3.9 – 5.2)</td>
</tr>
<tr>
<td>UNCR</td>
<td>4.84 (0 – 11.01)</td>
</tr>
<tr>
<td>UNCR (n=6)</td>
<td>2.38 (0 – 5.51)</td>
</tr>
</tbody>
</table>
Table 4.3 Average number of injured renal tubules per 20X field alongside fold increase of urine neutrophil gelatinase-associated lipocalin concentration (uNGAL) and urine NGAL:creatinine ratio (UNCR) for seven greyhound dogs anaesthetized with isoflurane and subjected to 60 minutes of haemorrhage shock (ischaemic phase) and 3 hours of reperfusion. Dogs are listed in order of descending severity of tubular injury. T3, 2 hours of reperfusion; T4, 3 hours of reperfusion

<table>
<thead>
<tr>
<th>Dog</th>
<th>Average number of injured tubules per 20X field</th>
<th>uNGAL fold change</th>
<th>UNCR fold change</th>
<th>uNGAL fold change</th>
<th>UNCR fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>3.95</td>
<td>29.2</td>
<td>82.6</td>
<td>23.2</td>
<td>51.2</td>
</tr>
<tr>
<td>D</td>
<td>3.85</td>
<td>32.9</td>
<td>66.1</td>
<td>33.4</td>
<td>52.5</td>
</tr>
<tr>
<td>G</td>
<td>3.65</td>
<td>16.4</td>
<td>22.8</td>
<td>8.7</td>
<td>7.0</td>
</tr>
<tr>
<td>A</td>
<td>3.00</td>
<td>57.4</td>
<td>78.9</td>
<td>39.2</td>
<td>38.6</td>
</tr>
<tr>
<td>C</td>
<td>1.55</td>
<td>9.9</td>
<td>32.0</td>
<td>4.7</td>
<td>17.2</td>
</tr>
<tr>
<td>E</td>
<td>1.30</td>
<td>21.1</td>
<td>6.4</td>
<td>16.5</td>
<td>3.2</td>
</tr>
<tr>
<td>F</td>
<td>1.05</td>
<td>1.7</td>
<td>3.9</td>
<td>1.2</td>
<td>1.6</td>
</tr>
</tbody>
</table>
CHAPTER 5 - GENERAL DISCUSSION

This series of studies developed a clinically relevant model of I-R using experimental haemorrhage and fluid resuscitation, which was subsequently used to investigate the temporal changes in uNGAL during early AKI. A pilot study confirmed that when mean arterial pressure was maintained below 40 mm Hg for 60 minutes, the model successfully produced acute renal tubular injury within two hours of reperfusion, as confirmed by histology. The pilot study also allowed determination of appropriate urine sample dilution when using a commercially available canine NGAL ELISA, and ruled out potential assay interference by synthetic colloid fluid administration during reperfusion. Subsequently the model was used to investigate early changes in uNGAL and UNCR associated with I-R AKI. The results of the final component of the study confirmed significant increases in uNGAL and UNCR from baseline within two hours of reperfusion.

To determine the changes in uNGAL during the early stages of I-R tubular injury in dogs, a standardised clinically relevant model of renal I-R was first developed (Chapter 2). Previously, most experimental animal studies investigating I-R AKI have utilised occlusion of renal vasculature to produce ischaemia (Mishra et al. 2003; Mori et al. 2005). However, complete renal vascular occlusion is uncommon during veterinary surgery and so the clinical relevance of these models is limited (Moore et al. 2012). As well as tissue ischaemia subsequent to reduced renal perfusion, there are many other mechanisms involved in the development of tubular injury during hypoperfusion-related I-R. Altered renal vascular resistance and pro-inflammatory effects subsequent to activation of the sympathetic nervous system, and altered plasma levels of hormones and cytokines, exacerbate tubular damage during I-R AKI (Chintala & Jandhyala 1990; Li et al. 2012). The model of I-R AKI developed in the pilot study was designed to simulate renal hypoperfusion and
reperfusion secondary to severe intra-operative haemorrhage and subsequent fluid resuscitation. Based on work from the pilot study it was identified that maintaining MAP below 40 mmHg for 60 minutes followed by two hours reperfusion was sufficient to cause tubular damage which was confirmed histologically. The pilot study also confirms that the histological changes associated with the I-R are easily distinguished from changes associated only with autolysis. The histological changes seen following I-R in this pilot study were consistent with those identified in other species (Devarajan 2010), supporting the ability of the model to produce early tubular injury.

The pilot study indicated the magnitude of change in uNGAL to be expected following I-R, and thus allowed determination of appropriate sample dilutions in order to obtain uNGAL within the dynamic range of the ELISA. Initial measurement using the manufacturer’s recommended dilution of 1 in 100 for urine samples was found to be insufficient to produce concentrations within the dynamic range of the assay. Subsequent measurement revealed that a 1 in 1000 dilution was required to accurately determine the uNGAL due to the high uNGAL witnessed during reperfusion.

Serum NGAL concentration measured following I-R was not different to baseline measurements in the pilot study. Findings of veterinary clinical studies investigating the use of serum or plasma NGAL concentration to diagnose AKI are mixed. One study found that while uNGAL increases within 12 hours of anaesthesia in dogs that develop AKI post-operatively, sNGAL does not change (Lee et al. 2012). However, plasma NGAL concentration has been shown to become elevated in dogs diagnosed with AKI compared to controls, and furthermore it is suggested that plasma NGAL concentration may be helpful to differentiate AKI from chronic kidney disease (Steinbach et al. 2014). Further work is required to establish the use of sNGAL for
diagnosis of AKI in dogs. Given the finding that sNGAL did not increase following I-R during our pilot study, we limited further investigation to uNGAL only.

During the performance of the pilot study, it was observed that there was large variation in measured uNGAL between individual dogs. In two dogs, uNGAL remained below the dynamic range of the assay during reperfusion, despite histopathological evidence of AKI. One possible reason for this may have been the presence of interfering substances within the urine. When establishing an immunoassay for use in a scientific study it is important to rule out any measurement bias that may be introduced by the presence of interfering substances within the sample to be tested. Interfering substances found in the sample matrix can affect the performance of an immunoassay resulting in falsely high or low measurements of analyte concentration (Tate & Ward 2004). Commercially available ELISAs, such as the one used in this study, undergo rigorous testing by the manufacturer to ensure there is no interference from many commonly recognized endogenous matrix substances; however some exogenous substances unique to a particular clinical setting or situation may not previously have been investigated. During performance of the pilot study, the urine was observed to become increasingly viscous after administration of the synthetic colloids. The authors hypothesised that the small molecular weight components of the synthetic colloid solutions used during fluid resuscitation were being excreted in large quantities during reperfusion and the presence of these molecules in the urine may have interfered with the performance of the ELISA. In order to test this hypothesis, difference in spectrophotometry measurement of mean absorbance between urine, colloid solutions and empty well was investigated, and linearity of the assay at serial dilutions of a standard solution containing a known concentration of NGAL was assessed. No evidence of significant interference on the performance of the assay was found by 4% succinylated gelatin. The 6% hydroxyethyl starch produced some variation in
spectrophotometric measurement; however this is not expected to have significant impact on assay performance. Subsequently (Chapter 4) the use of synthetic colloids during reperfusion was standardised to 4% succinylated gelatin at a total volume administered of 20 – 30 mL kg\(^{-1}\).

As the colloid solutions were not found to be a likely cause of assay interference, the reason for variation in uNGAL during Chapter 2 is not clear. It was postulated that degradation of samples during storage may have contributed to variable results, as some samples were stored at room temperature for up to one hour prior to processing. Previous work has shown that human NGAL is sufficiently stable in urine stored at room temperature for up to 24 hours, and also at 4°C for up to seven days with < 2% change in NGAL concentration between days one and seven (Han et al. 2009; Grenier et al. 2010). For longer term storage (months) human urine samples are required to be stored at -75°C as those stored at -20°C showed significant decrease in their NGAL concentration (Bennett et al. 2008; Han et al. 2009). To date, there are no published studies investigating the stability of canine uNGAL at different storage temperatures. A standardised storage method was subsequently used in the final study whereby urine samples were immediately centrifuged and aliquots of supernatant placed on ice for a maximum of four hours prior to storage at -80°C. When this standardised processing was performed (Chapter 4), all dogs showed increases in uNGAL. This suggests that handling of the samples may be important. Further studies comparing effects of storage at room temperature and refrigeration on canine uNGAL are required to determine appropriate sample handling for accurate determination of uNGAL and concentrations of other urinary biomarkers in dogs, particularly if these biomarkers are to become clinically useful.

Results of Chapter 4 confirmed that during I-R AKI, uNGAL and UNCR increase from baseline within two hours of reperfusion, by an average of 28-fold and 47-fold, respectively. These findings are consistent with those from rodent vascular occlusion
models and human clinical studies. Increased uNGAL within two hours of acute tubular ischaemia occurs in experimental studies using renal vascular clamping models of ischaemic AKI in rodents (Mishra et al. 2003). Following cardiopulmonary bypass, uNGAL increased by almost 100-fold in children within two hours, and by 40-fold in adults within one hour of surgery (Mishra et al. 2005; Wagener et al. 2006). In this study, variation in baseline uNGAL was encountered, with uNGAL in one dog more than tenfold higher than that of all other dogs. Wide variation in baseline uNGAL for healthy people has created difficulty in determining a reference interval (Clerico et al. 2012; Smertka & Chudek 2012). Many veterinary patients diagnosed with AKI present to a veterinarian after injury has occurred (e.g. snake envenomation, trauma, urinary tract obstruction) and so it is not possible to obtain baseline measurements. Despite statistically significance differences in mean (or median) uNGAL between dogs with AKI and controls in previous veterinary studies, when the spread of data is taken into account many of the individual uNGAL measurements from dogs with AKI and controls dogs appear to overlap (Lee et al. 2012; Hsu et al. 2014; Steinbach et al. 2014). When the variation in baseline uNGAL measurements detected in Chapter 4 are taken into account, these findings indicate that use of a single uNGAL measurement, after injury has occurred, may be limited for the diagnosis of AKI in dogs.

Less variation between individual dogs at baseline was encountered when UNCR was calculated; this may indicate that some variation can be accounted for by differing urine concentration between individual dogs. It has previously been suggested that the UNCR, rather than total uNGAL, should be reported in studies investigating the use of NGAL as a biomarker for AKI (Grenier et al. 2010). The rationale for this is that by normalising to urine creatinine concentration bias that might be introduced by changes in urine concentration and flow during steady state GFR are removed (Goldstein 2010). The findings of this study suggest that during
periods of stable GFR (e.g. baseline), UNCR may a more appropriate measurement than uNGAL in order to reduce bias introduced by differing hydration status and urine flow rate between patients. However, use of UNCR rather than uNGAL during I-R may be less appropriate because normalisation to urine creatinine assumes a constant creatinine excretion rate within an individual over time (Waikar et al. 2010; De Loor et al. 2013). This was supported by similar variability in UNCR and uNGAL measured after haemorrhage and reperfusion. Regardless, both uNGAL and UNCR were significantly increased from baseline by two hours of reperfusion, indicating that at this time interval the two could be used interchangeably. By three hours of reperfusion (T4) the increase in UNCR was no longer significant, while the increase in uNGAL remained significant. A reduction in biomarker: creatinine ratio is likely during the very early stages of AKI regardless of whether concentration of the biomarker itself is elevated, simply due to increased urinary excretion of creatinine (Waikar et al. 2010; Ralib et al. 2012). During the early stages of AKI, GFR changes rapidly leading to unstable urinary excretion of creatinine; in addition back-leak of creatinine across injured tubules may occur (Waikar et al. 2010). Given the findings of the current study it is tempting to agree with authors who suggest that, given the large fold changes in uNGAL that have been shown to occur during AKI, it is unnecessary, and potentially misleading, to normalise to urine creatinine in these circumstances (Mori & Nakao 2007).

The wide variation in the magnitude of change of uNGAL and UNCR between individual dogs following I-R may be explained by different levels of induced tubular injury, despite use of a standardised model, or may represent different physiological responses of individual dogs during AKI. However, this study was not designed to investigate baseline variability in uNGAL, or to investigate whether differing severity of renal I-R injury correlated with the magnitude of change to uNGAL, UNCR or histopathology changes. Further study in these areas is required
to establish the usefulness in these biomarkers for predicting severity of injury and possible outcome in dogs suffering AKI.

This study identified a slight reduction in uNGAL at the final sample time (T4), following a peak at T3. This finding might suggest that the AKI induced by this model was minor and reversible, alternatively it could represent a similar trend to that observed in human studies whereby an early rise in uNGAL is followed by a lesser yet sustained increase (Mishra et al. 2005). The fold increase in uNGAL observed in our study was similar to that seen within a few hours of ischaemic injury in adult humans who progressed to the maintenance stage of AKI (Wagener et al. 2006). The studies outlined in this thesis were limited to the investigation of uNGAL during only the very early stages of I-R AKI, and investigations into longer term change in concentration of the biomarker in dogs are required in order to clarify the relevance of the temporal change observed.

**CONCLUSIONS**

The results of this study show that the clinically relevant model of I-R developed in Chapter 2 consistently produced acute tubular injury. Using this model we confirmed that uNGAL is significantly elevated from baseline within two hours of reperfusion. This provides encouragement for its use as a biomarker for AKI in dogs. However, given the potential for baseline variation and differing magnitude of change between individual dogs; there is scope for further investigation. Further work using a larger cohort of dogs is required to determine if a reference range can be established for NGAL. In addition, the effects of storage at different temperatures, and for varying duration, on the stability of NGAL in canine urine should be investigated.
Given the variation in baseline measurements and magnitude of change of uNGAL during a standardised model of I-R AKI, it may be suggested that this biomarker alone is not capable of fulfilling all the required qualities of an ‘ideal’ biomarker for AKI (Cobrin et al. 2013). Whilst NGAL is likely to play an important role in the diagnosis of AKI both in the research and clinical setting in the future, a panel of urinary and serum biomarkers such as NGAL, with or without traditional markers such as sCr, may become the most useful and reliable method of AKI diagnosis and grading (Lisowska-Myjak 2010; Cobrin et al. 2013).
REFERENCES


APPENDIX – RENAL HISTOPATHOLOGY IMAGES

Figure A.1 Examples of vacuolar degeneration and attenuated tubular epithelium in photomicrographs of kidney obtained from greyhounds that underwent a haemorrhagic shock model of ischaemia (60 minutes) and reperfusion (three hours). 

(a) Dog D; vacuolar degeneration of proximal tubular epithelial cells (arrows), an adjacent tubule (*) is lined by markedly attenuated epithelium. 

(b) Dog F; vacuolar degeneration of proximal tubular epithelial cells (arrows), the adjacent glomerulus (*) is within normal limits. Periodic acid-Schiff staining, magnification 400x.
Figure A.2 Examples of detached necrotic tubular cells in photomicrographs of kidney obtained from greyhounds that underwent a haemorrhagic shock model of ischaemia (60 minutes) and reperfusion (three hours). Proximal tubules contain sloughed epithelial cells (arrows). (a) Dog B (b) Dog D (c) Dog F. Periodic acid-Schiff staining, magnification 400x