Canine (presumed) glomerular proteinuria: Characterisation of urinary proteins, prognostic factors and response to treatment with angiotensin converting enzyme inhibitors

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Abstract

A urine protein creatinine ratio (UPC) of >2.0 has historically been taken to indicate glomerular disease in the dog. However, recent literature has questioned whether this cut-off remains appropriate. When glomerular disease is suspected, standard therapy (which usually includes an angiotensin converting enzyme inhibitor, ACEi) has been recommended to try to reduce the magnitude of the associated proteinuria. Despite these standard therapies being widely implemented, the proportion of dogs that respond is unknown. Additionally, whether response to standard treatment conveys significant survival benefit is undetermined.

This master's project was comprised of a retrospective and prospective study. The retrospective study aimed to assess the number of dogs that respond to treatment with ACEi and determine if response to treatment conveyed survival benefit. Additionally, the retrospective study assessed if the presence of baseline clinicopathological abnormalities impacted survival. The prospective study aimed to determine the proportion of dogs with a UPC of >2.0 that had tubular proteins present on urine protein electrophoresis (UPE) and determine the significance of the presence of such proteins. UPE patterns were also assessed to determine if dogs with and without an identifiable trigger for their proteinuria could be differentiated between. Urinary biomarkers of tubular damage (GGT and NGAL) were also measured to further evaluate for tubular involvement. Finally, we also aimed to mirror the retrospective study and screen for potential prognostic indicators.

The retrospective study demonstrated that although <50% of the population responded to treatment with an ACEi, response was associated with a survival benefit. Additionally, the presence of baseline azotaemia, hypoalbuminaemia and increasing magnitude of UPC were associated with a negative outcome. The prospective study documented that most dogs with a UPC >2.0 had evidence of tubular proteins on UPE and presence of such proteins may suggest more advanced disease. The presence of tubular damage was further demonstrated by measurement of NGAL which was elevated in nearly all dogs. We found that the UPE pattern could not be used to distinguish between dogs with and without a trigger for their proteinuria. Evaluation of clinicopathological variables from the prospective population of dogs mirrored the results of the retrospective study with regard to prognostic implications; additionally, dogs with a mixture of glomerular and tubular proteins present were found to have a worse prognosis than those with just glomerular proteins present.

The results of this master's project go towards filling previous knowledge gaps within the field of canine glomerular disease and further our understanding of this complex condition.

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AUTHOR'S DECLARATION

The work presented in this thesis was performed solely by the author. The only exception to this declaration is regarding the statistics for the retrospective paper; formal statistical analysis for this section was performed by Darren J. Shaw.

Emily Fulton, March 2023

LIST OF ABBREVIATIONS

1. ACEi – Angiotensin converting enzyme inhibitor
2. ADH – Antidiuretic hormone
3. AKI – Acute kidney injury
4. ARB – Angiotensin receptor blocker
5. BCA – Bicinchoninic acid
6. CI – Confidence interval
7. CKD – Chronic kidney disease
8. ELISA – Enzyme-linked immunosorbent assay
9. EM – Electron microscopy
10. FFAs – Free fatty acids
11. FSGS – Focal segmental glomerulosclerosis
12. GAG – Glycosaminoglycans
13. GBM – Glomerular basement membrane
14. GFR – Glomerular filtration rate
15. GGT – Gamma-glutamyl transferase
16. G-UPE - Glomerular proteins on UPE analysis
17. HPF – High power field
18. HMW – High molecular weight
19. ICGN – Immune complex mediated glomerulonephritis
20. IgA – Immunoglobulin A
21. IgG – Immunoglobulin G
22. IgM – Immunoglobulin M
23. IMHA – Immune mediated haemolytic anaemia
24. IRIS – International renal interest society
25. IVFT – Intravenous fluid therapy
26. kDa – Kilodaltons
27. LMW – Low molecular weight
28. MPGN – Membranoproliferative glomerulonephritis
29. M-UPE – Mixed (glomerular and tubular) proteins on UPE analysis
30. MCD – Minimal change disease
31. NAG – N-acetyl-β-(D)-glucosaminidase
32. NGAL – Neutrophil gelatinase-associated lipocalin
33. OD – Optical density
34. PI – Protease inhibitor

35. RAAS – Renin angiotensin aldosterone system
36. RBC – Red blood cells
37. RBP – Retinol binding protein
38. ROC – Receiver operating characteristic
39. SDMA – Symmetric dimethylarginine
40. SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
41. TKI – Tyrosine kinase inhibitor
42. TOD – Target organ damage
43. UPC – Urine protein creatinine ratio
44. UPE – Urine protein electrophoresis

PUBLICATIONS AND PRESENTATIONS

Some of the work contained within this thesis has been the subject of the following publications and presentations:

Conference Proceedings

'Response of dogs treated with angiotensin converting enzyme inhibitors (ACEi) for presumed glomerular proteinuria and the effect of a positive response on survival'. Fulton, E.A., McBrearty, A.R., Ridyard, A.E. Oral Abstract Presentation; Proceedings of the British Small Animal Veterinary Association (BSVA) Congress, Birmingham, UK, 2022

Publications

'Response of dogs treated with angiotensin converting enzyme inhibitors for presumed glomerular proteinuria and effect of a positive response on survival'. Fulton, E.A., McBrearty, A.R., Shaw, D.S., Ridyard. A.E. Submitted to the Journal of Veterinary Internal Medicine (JVIM); status: accepted pending revisions.

CHAPTER ONE

INTRODUCTION TO RENAL PHYSIOLOGY, PROTIENURIA AND GLOMERULAR DISEASE IN DOGS

1. Introduction

Glomerular disease represents a varied and complex group of diseases the hallmark of which is proteinuria of a high magnitude. Historically, it was thought that a urine protein creatinine ratio (UPC) of >2.0 was associated with glomerular disease (Centre et al., 1985, Centre et al., 1987, Cook et al., 1996) however, recent research challenges this statement and suggests such a definitive cut-off may not be appropriate (Hokamp et al., 2016, Hokamp et al., 2018, Schneider et al., 2013). The treatment of glomerular proteinuria involves use of angiotensin converting enzyme inhibitors (ACEi) regardless of the underlying aetiology of the proteinuria and it is suggested that reaching a UPC of <0.5 or achieving a ≥50% reduction in UPC constitutes treatment success (Brown et al., 2013). What is less well established is the number of dogs that reach this target and if reaching target conveys survival benefit. Despite recent advances in this field of veterinary medicine there are still significant gaps within our knowledge of glomerular proteinuria and this master's project aimed to address some of these gaps. A retrospective study was first performed to determine the percent of dogs responding to treatment with ACEi therapy and if response to treatment was associated with survival. A prospective study was then carried out to assess the proportion of dogs with presumed glomerular disease that also had evidence of tubular damage. The presence of tubular damage was also evaluated for association with disease progression or severity and survival. Further prognostic indicators, such as the presence of biochemical abnormalities, were also evaluated for. This introduction now presents the key concepts associated with these study aims and summarises the relevant pre-existing literature within this field.

1.1 Brief introduction to glomerular physiology

The focus of this master's project is glomerular proteinuria and therefore before proteinuria and glomerular dysfunction are discussed the normal glomerular physiology is introduced. The kidneys are responsible for filtration of blood amongst numerous other functions. Grossly, the kidney is contained within a capsule immediately beneath which is found the renal cortex, the renal medulla then sits between the cortex and the renal pelvis. The functional unit of the kidney, the nephron, consists of the glomerulus and its associated renal tubule. The glomerulus is responsible for formation of the filtrate which then passes into the proximal tubule at which point most of the filtered water and solutes are reabsorbed. Next, the filtrate passes through the loop of Henle with its thin descending portion and thick ascending limb before entering the distal convoluted tubule and collecting duct.

1.1.1 The glomerulus

The glomerulus is a vascular structure located between two arterioles; the afferent arteriole which leads into the glomerulus and the efferent which exits (Figure 1). The glomerulus sits within the Bowman's capsule which is the beginning of the tubular component of the nephron. At the glomerulus, the glomerular filtrate is formed; the filtrate is very similar to plasma regarding its composition of electrolytes and water. The glomerulus receives approximately 20-25% of the cardiac output (Littman, 2011, Verlander, 2020), and in health, the glomerulus should retain cellular components and medium to high weight molecular proteins within the blood whilst molecules such as glucose and amino acids pass freely through the glomerular filtration barrier and into the filtrate. The glomerulus provides a barrier that is composed of three layers: the fenestrated endothelium; the glomerular basement membrane (GBM) and the podocytes.

The fenestrated endothelium gets its name from the presence of numerous openings associated with the endothelial cells which are approximately 70-100nm in diameter (Tryggvason et al., 2006). The endothelial cells serve to function in the regulation of vasomotor tone amongst other roles (Ballermann, 2005). These endothelial cells have a glycocalyx (pericellular matrix) on their surface which contains a combination of sialoproteins and proteoglycans with a negative charge. A study looking at the glycosaminoglycans within the glycocalyx suggested, via both morphological and functional measurements, that it is this glycocalyx that forms an important part of the glomerular filtration barrier (Jeansson and Haraldsson, 2006).

The GBM itself measures 300-350nm in depth (Tryggvason et al., 2006). The membrane is made up of the lamina rara interna, lamina densa and lamina rara externa and is mainly composed of type IV collagen, laminin, proteoglycans and nidogen (Levidiotis and Power, 2005). The GBM is thought to be the main size barrier for the entrance of molecules into the filtrate. The GBM has an anionic charge mainly due to the lamina rara interna and externa; these layers include glycosaminoglycans (GAG) particularly heparan sulphate, which are negatively charged (Rabelink, 2015). Mice lacking the main anionic sites of the GBM have not been found to develop proteinuria suggesting that the charge of the GBM plays less of a role in preventing the filtration of macromolecules (Harvey et al., 2007, Goldberg et al., 2009, Rossi et al., 2003). However, in contrast, there is significant evidence to support the role of negatively charge heparan sulphates contributing to the charge-selective permeability of the GBM. For example, removal of GAGs in the GBM or administration of anti-heparan sulphate antibodies has been shown to lead to the development of albuminuria (Kanwar et al., 1980, van den Born et al., 1992). Additionally, in several important glomerular diseases

including systemic lupus erythematous, membranous glomerulopathy and diabetic nephropathy, a reduction in expression of heparin sulphate has been documented suggesting a role for this GAG in the development of proteinuria in such disease processes (van den Born et al., 1993, Shimomura and Spiro, 1987). Therefore, although further work is needed to confirm the full extent of the contribution of the anionic charge, it appears to at least in part contribute to the barrier properties of the GBM.

The final component of the membrane are the podocytes. Podocytes are highly specialised cells of the Bowman's capsule that enwrap the capillaries of the glomerulus. These cells are comprised of a cell body and processes extending outwards to form interdigitated foot processes which are attached to the GBM via cell membrane receptors. Between the foot processes of the podocytes, 25-40nm wide pores or 'slit diaphragms' exist. Ordinarily, minimal amounts of proteins with a molecular weight more than albumin (69 kilodaltons, kDa) are able to pass through into the glomerular filtrate; proteins with a negative charge are even less likely to cross the filtration barrier (Littman, 2011).

Defects or disease processes affecting any of these components of the glomerulus can contribute to glomerular protein loss which is further discussed shortly.

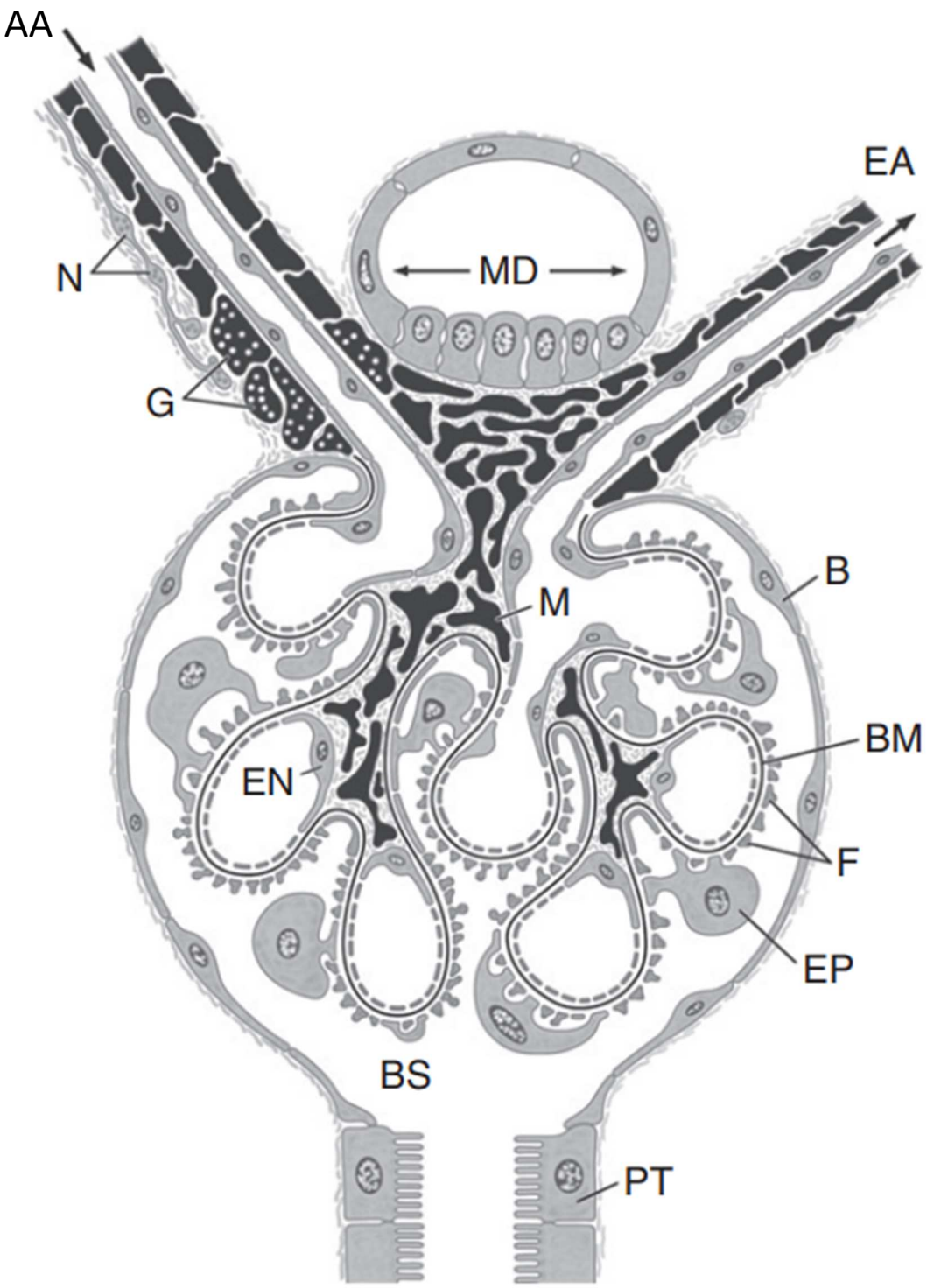


Figure 1: Schematic of the glomerulus

AA – afferent arteriole, EA – efferent arteriole, BS – Bowman space, PT – proximal tubule, B – Bowman capsule, G – granular cells (juxtaglomerular cells), N – sympathetic nerve terminals, M – mesangial cells, EN – fenestrated endothelial cells, EP – epithelial cells, F – Foot processes, BM – basement membrane, MD – macula densa. Adapted from (DiBartola, 2012).

1.1.2 Tubular reabsorption

After the glomerular filtrate has been formed it undergoes further processing to produce urine, for this to happen resorption and secretion must now occur. Tubular reabsorption is a complex and extensive process, the full description of which is beyond the scope of this introduction. Briefly, the proximal tubule is responsible for reabsorbing the majority of the ultrafiltrate formed at the glomerulus. Resorption of a substrate can occur via either primary or secondary active reabsorption or passive uptake. The level of protein in the ultrafiltrate should be low, however, any protein that is present is usually reabsorbed in the proximal tubule via receptor-mediated endocytosis. In humans two multi-ligand endocytic receptors, megalin and cubilin, have been shown to be largely responsible for the reabsorption of protein in the proximal tubules (Christensen and Birn, 2002).

1.1.3 The mesangium

The mesangium is another important structure to mention as it has several key roles. The mesangium functions to provide support to the glomerular tuft; it is a specialised type of connective tissue that is made up of mesangial cells embedded within an extracellular matrix that is produced by the mesangial cells themselves. The matrix is composed of collagens type IV and V alongside laminin A, B1 and B2; fibronectin and other polysaccharides and proteins (Scindia et al., 2010). The cells of the mesangium are thought to constitute 30-40% of the total human glomerular cell population (Olivetti et al., 1977). Two types of mesangial cells have been discovered; the majority (>90% of the population of human mesangial cells) are cells similar to vascular smooth muscle cells. These cells have either direct or indirect contact with the GBM and the juxtaglomerular apparatus (the juxtaglomerular apparatus is introduced later) and their contraction can lead to constriction of the afferent arteriole lumen and thus changes in blood flow to the glomerulus (Stockand and Sansom, 1998). The roles of the mesangial cells are numerous and include structural support and secretion of the previously mentioned extracellular matrix alongside modulation of glomerular filtration, phagocytosis of macromolecules and immune complexes and production and response to a number of cytokines (Cove-Smith and Hendry, 2008).

1.2 Classification of Proteinuria

In small animal medicine, the amount of protein in urine is standardly reported as the UPC with original studies performed in 1985 finding that healthy dogs had a UPC of <0.2 (Grauer et al., 1985, Center et al., 1985). It is still accepted that in normal dogs a small amount of protein within the urine will be present with a UPC of <0.2 being considered 'normal' and ratios between 0.2-0.5 now being defined as 'borderline proteinuric' (IRIS, 2019). These cut-offs are utilised for dogs with chronic kidney disease (CKD) whilst the ACVIM Consensus Guidelines for the Assessment and Management of Proteinuria proposes a simplified cut-off suggesting that a UPC of <0.5 represents a lack of significant proteinuria (Lees et al., 2005). Whilst the focus of this master's project is glomerular proteinuria, this is not the only category of proteinuria that can arise. Glomerular proteinuria falls within the broader category of 'renal proteinuria', and alongside renal proteinuria, pre-renal and post-renal proteinuria can also arise. These classifications of proteinuria are now further introduced.

1.2.1 Pre-renal Proteinuria

Pre-renal proteinuria occurs when there is normal permselective properties of the glomerulus, however, there is an abnormal amount of small proteins within the plasma (Lees et al., 2005). This abnormal circulating protein level can arise either due to the presence of normal proteins which are not normally freely present in the plasma or due to proteins that are themselves abnormal. As the circulating proteins are small, they are filtered at the glomerulus, however, as the levels within the plasma are abnormally high the resorptive capacity of the tubule is overwhelmed leading to proteinuria. An example of normal proteins which should not normally be detected within the plasma are haemoglobin or myoglobin. In human medicine, extracellular haemoglobin (due to haemolysis) is described as a trigger for several adverse clinical outcomes, one of which is renal impairment (Schaer et al., 2013). An example of abnormal proteins is provided via Bence Jones proteins which are most associated with multiple myeloma. In humans, it is reported that up to 49% of patients with myeloma will suffer with renal impairment during their disease with patients with a higher excretion of Bence Jones proteins more often experiencing renal failure (Knudsen et al., 1994). Proteinuria in combination with the presence of multiple myeloma is also reported in the canine population (Mayer et al., 2008, Giraudel et al., 2002).

1.2.3 Renal Proteinuria

Renal proteinuria has been defined as arising due to abnormal handling of normal plasma proteins (Lees et al., 2005) and can be either functional or pathological with the latter being further split into glomerular, tubular and interstitial (Figure 2). Functional renal proteinuria is reported to occur due to strenuous exercise, seizure, fever, stress or exposure to extreme temperatures. In companion animals, minimal studies have been conducted investigating functional proteinuria, however, it should be a transient finding only and resolution should be seen with removal of the inciting cause. In humans, orthostatic proteinuria is also reported as a form of functional proteinuria, occurring when the person is standing and subsequently resolving if they are lying down (Uehara et al., 2014). It is most common in children with a prevalence of 2-5% reported in adolescents (Mazzoni et al., 2011). There are no renal lesions associated with orthostatic proteinuria and it is instead thought that this condition is likely mediated by glomerular haemodynamic changes.

In contrast to functional renal proteinuria, pathological renal protein occurs when structural or functional lesions are present within the kidneys associated with either the glomerulus, the tubules or the interstitium (or possibly a combination thereof). Glomerular proteinuria arises due to alterations of the glomerular filtration barrier and loss of the glomerular selectivity introduced previously, whilst tubular proteinuria is thought to occur due to an abnormality in the tubular reabsorption process meaning that proteins normally filtered at the glomerular level and later reabsorbed by the tubules are instead lost into the urine. Finally, for interstitial renal proteinuria to arise inflammation must be present within the interstitial/parenchymal tissue which causes protein to leak from the peritubular capillaries into the urine. Historically, it has been proposed that initial indications as to the origin of renal proteinuria can be acquired via interpretation of the magnitude of the UPC with tubular proteinuria generally being associated with low-level proteinuria and higher magnitude UPCs attributed to glomerular disease. Early research performed by Di Bartola *et al.* (1980) showed that 24-hour urine protein excretion was significantly different between dogs with either amyloidosis, glomerulonephritis or glomerular atrophy (which was considered an end stage change and not a primary glomerular disease) suggesting that dogs with glomerular disease had higher levels of proteinuria. However, this study did not report UPC values and instead urinary protein loss was expressed as mg/kg/day. Centre *et al.* (1985) then later went on to indicate that a UPC of >2.0 always indicated abnormal urinary protein loss and that this degree of proteinuria was mainly noted in dogs with glomerulonephritis rather than those with chronic interstitial nephritis. The work of Cook *et al.* (1996) further supported this

initial suggestion that dogs with glomerular proteinuria have increased magnitude of UPC by reporting dogs with amyloidosis to have a UPC of 20.4±12.2 and those with idiopathic glomerulonephritis a UPC of 12.5±8.0 whilst Centre *et al.* (1987) provided further evidence by reporting the UPC of dogs with glomerulonephritis to be >2.0 in 86.4% of cases. Studies evaluating non-glomerular disease and proteinuria have also lent support to this theory; for example, tubulointerstitial fibrosis is the leading cause of CKD in cats (DiBartola et al., 1987) and if proteinuria is present in these cases it is usually of low magnitude, for example 90% of cats with CKD were reported to have a UPC of <1.0 (Syme et al., 2006).

These early studies led to it being commonly accepted that a UPC of >2.0 is indicative of glomerular disease and this cut-off has been widely implemented in clinical practice. Indeed, the current ACVIM Consensus Guidelines for the Assessment and Management of Proteinuria states that a UPC of >2.0 in dogs is sufficiently high enough to support the presence of pathological, renal glomerular proteinuria (Lees et al., 2005).

However, more recent research has questioned whether it is correct to be so definitive with the use of a UPC of >2.0 to indicate glomerular disease whilst considering a UPC of below this threshold to indicate tubular disease. There is now evidence to suggest that some dogs with a UPC <2.0 may also have primary glomerular pathology (Hokamp et al., 2016, Hokamp et al., 2018), whilst it has also been proven that dogs with primary tubulointerstitial disease can have a UPC of >2.0 (Hokamp et al., 2016, Schneider et al., 2013). Furthermore, recent studies have also suggested that dogs can have concurrent glomerular and tubular disease and it is not necessarily one or the other that is present, for example, Ferlizza *et al.* showed that in seven dogs with CKD and a UPC of >2.0 both high and low molecular weight protein bands were present suggesting concomitant glomerular and tubular damage (Ferlizza et al., 2020). Hence, these recent studies have questioned the use of the cut-off of >2.0 to indicate only glomerular disease and whilst this emerging evidence suggests this pre-existing cut-off may not be ideal, additional research is required to further characterise the urinary proteins present in a wider population of dogs with higher magnitude UPCs to help understand the location of protein loss and to ascertain whether the type of proteins lost (i.e. glomerular or tubular or mixed) has particular significance, for example if there is a prognostic implication.

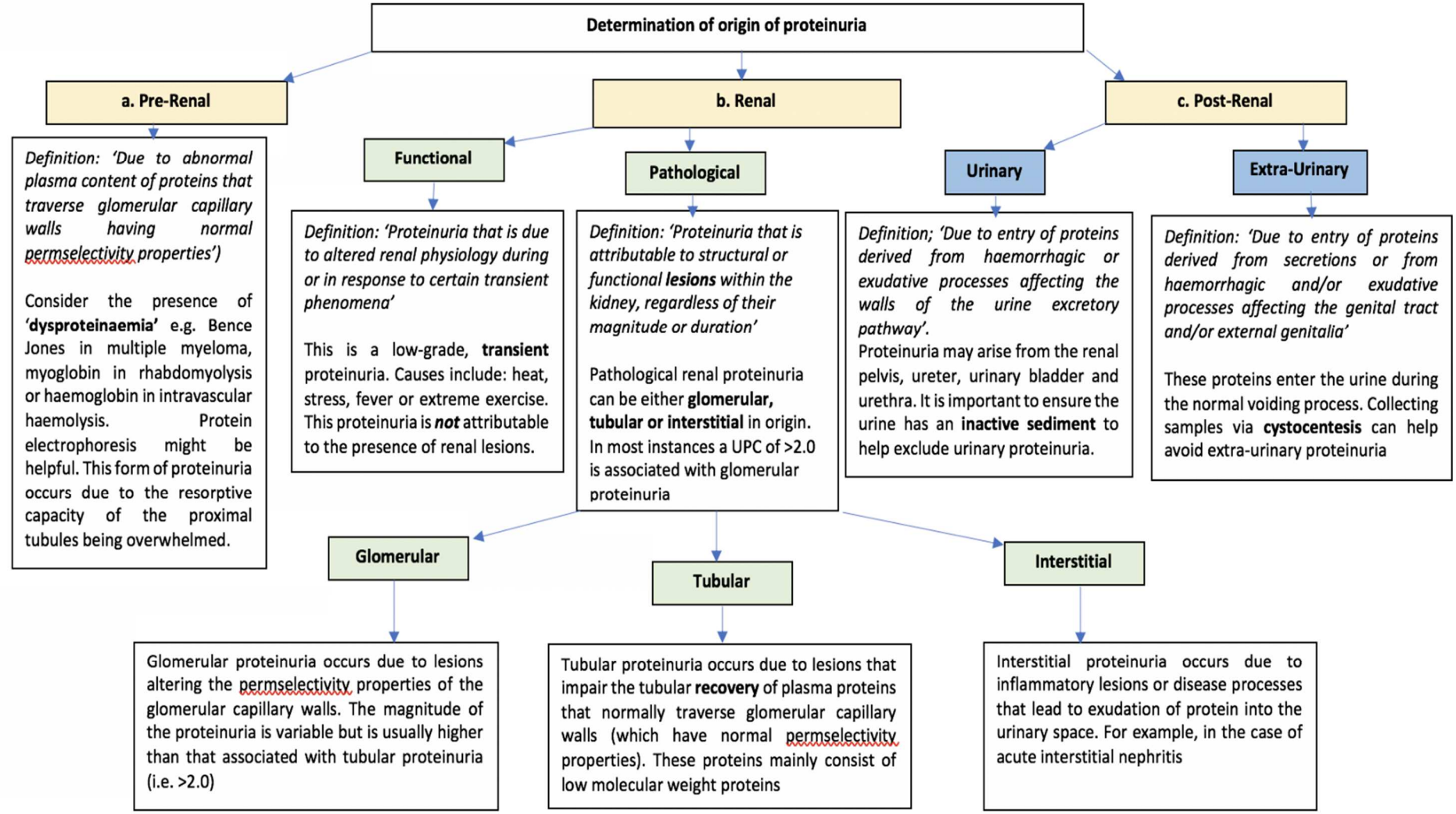


Figure 2: Determination of origin of proteinuria. Adapted from (Lees et al., 2005)

1.2.3 Post renal proteinuria

Post renal proteinuria occurs when there is a disease process, such as inflammation, infection, urolithiasis or neoplasia affecting any part of the urinary tract from the renal pelvis to the urethra. Post renal proteinuria can further be sub-categorised into urinary and extra-urinary. Urinary post-renal proteinuria is defined by the AVCIM Consensus Statement for the Assessment and Management of Proteinuria as being due to entrance of proteins arising from haemorrhagic or exudative processes affecting the urinary tract whilst extra-urinary post-renal proteinuria occurs due to processes affecting the genital tract and/or external genitalia (Lees et al., 2005).

Interpretation of the UPC can be complicated by concurrent sediment changes such as haematuria or pyuria that may arise due to post-renal causes but may also possibly mask renal proteinuria. Pre-existing literature does not appear to reach a common conclusion regarding the impact that various sediment changes can have on UPC and the presence/absence of proteinuria. *In vitro* studies suggest that haematuria must be of a magnitude to allow for visible contamination of the urine to cause samples to be proteinuric (Bagley et al., 1991, Vientos-Plotts et al., 2018, Vaden et al., 2004); increasing red blood cells (RBC) within a urine sample that does not lead to visible contamination appears not to be associated with the development of proteinuria (Jillings et al., 2019, Vientos-Plotts et al., 2018). A few studies have been performed on *in vivo* samples; Vaden *et al* (2004) found that there was no significant difference in UPC in samples with and without haematuria in a population of dogs with pyuria whilst another study reported that although most dogs with experimentally induced bladder injury in which haematuria, pyuria and bacteriuria developed became proteinuric, there was no correlation between the level of RBCs present and UPC (Vaden et al., 2004, Bagley et al., 1991). It appears that the presence of pyuria in urine samples may not contribute greatly to an increase in UPC; previous studies of pyuric samples have demonstrated 81% to have a UPC<0.4 (Vaden et al., 2004). Additionally, in the study mentioned above in dogs with experimentally induced bladder injury there was no correlation between the extent of pyuria and the UPC (Bagley et al., 1991). It is thought that further work is required to determine the impact of various sediment changes on the UPC and as a result, for the studies reported in this thesis only dogs with an inactive urinary sediment (defined later) were included.

1.3 Investigation into an abnormal UPC

The presence of an elevated UPC warrants further investigation; when an abnormal UPC is first detected repeat sampling is advised for two different reasons depending on the initial magnitude of the UPC value. When the initial magnitude of proteinuria is low, for example, the UPC ratio is <2.0, then repeated sampling is recommended to ensure that the proteinuria is persistent (Lees et al., 2005). It is recommended that persistent proteinuria is documented via serial sampling on at least three or more occasions performed on samples collected two or more weeks apart (Lees et al., 2005). If the initial reading is higher the likelihood of it being attributable to glomerular disease is increased; and thus repeat sampling to ensure persistent proteinuria is no longer as crucial as it can usually be presumed that disease is present (Littman et al., 2013). However, repeat UPC measurements are usually still recommended even when the initial value is high to increase the accuracy of the baseline reading for the patient. For example, a recent study has advised that an average of up to 4-5 measurements are taken with UPC ratios of >8.0 to obtain a reliable estimate of the real UPC value (Nabity et al., 2007). However, the study recognised that obtaining 5 measurements might be impractical in the clinical setting and suggests that even by averaging two measurements the accuracy of the UPC reading can be increased. As will be discussed later, the establishment of an accurate baseline UPC is important if treatment is initiated as this initial UPC is utilised to help to determine response to therapy. When UPCs are being serially tracked to monitor treatment success, in order to distinguish between normal day to day variation and actual significant difference in serial UPC readings it is reported that the UPC needed to change by a minimum of at least 35% at high UPC readings (near 12) whereas a change of 80% was required at lower values (0.5) (Nabity et al., 2007). Performing repeat UPCs can be an expensive process for the client; it has been proven that pooling urine samples has an acceptable accuracy compared to performing separate serial samples (Shropshire et al., 2018, LeVine et al., 2010). In clinical practice, the normal procedure for monitoring UPC of dogs receiving treatment would be analysis of a 3-day pooled urine sample (i.e. urine from three consecutive days is collected by the owner and an aliquot from each sample taken and pooled to make the final sample submitted).

1.3.1 Further investigations into suspected glomerular proteinuria

Once glomerular proteinuria has been confidently identified, further testing is required to determine the underlying aetiology. Investigations indicated in the work-up of a case with

suspected glomerular proteinuria are outlined in the ACVIM Consensus Statement for Diagnostic Investigations of Dogs with Suspected Glomerular Disease (Littman et al., 2013). The Consensus advises that dogs are placed into tiers based on the presence of clinical manifestations of disease (Table 1).

Tier I		Persistent renal proteinuria. Normal albumin and creatinine
	A	Persistent subclinical renal proteinuria not accompanied by any obvious renal related signs of sequalae
	B	Persistent renal proteinuria with hypertension the only evidence of sequalae from renal disease (target organ damage may or may not be present)
Tier II		Renal proteinuria associated with hypoalbuminaemia, but azotaemia is not present
	A	Persistent renal proteinuria with hypoalbuminaemia with or without any of its associated complications but without hypertension or azotaemia
	B	Persistent renal proteinuria with hypoalbuminaemia, with or without any of its associated complications with hypertension but without azotaemia
Tier III		Renal proteinuria with renal azotaemia
	A	Renal proteinuria with renal azotaemia but normal albumin and no hypertension
	B	Renal proteinuria with renal azotaemia and hypertension but without hypoalbuminaemia
	C	Renal proteinuria with renal azotaemia and hypoalbuminaemia with or without any of its associated complications which is often (but not always) associated with hypertension

Table 1: Description of tiers recommended for grouping of dogs with glomerular disease to assist in determination of required work-up. Adapted from Littman et al. (2013)

Where the patient falls within this tier system will govern the extent of the recommended work-up. A standard work-up for cases with suspected glomerular disease includes routine serum biochemistry, haematology and urinalysis as well as blood pressure measurement and any appropriate testing for infectious disease for dogs living in endemic areas. The rationale for the initial testing outlined by the Consensus is to screen for conditions that may promote secondary glomerular injury that could be treated. For example, clinicians should be assessing for the presence of infectious disease, inflammatory or vascular conditions, certain endocrinopathies (particularly hyperadrenocorticism and diabetes mellitus) as well as

neoplasia and immune mediated disorders. If an appropriate work-up is performed and no secondary disease process detected, renal biopsies should be considered to fully characterise the origin of the proteinuria.

1.3.2 Specific systemic conditions associated with proteinuria

As above, the purpose of the initial diagnostics performed when glomerular proteinuria is suspected is to screen for systemic conditions that may be contributing to the presence of proteinuria. The identification of any underlying systemic condition is important as treatment of any such condition is likely to be a key component of the patient's overall proteinuria treatment plan. An association between certain endocrinopathies and neoplasia and the presence of proteinuria have been reported, therefore, the associations between proteinuria and diabetes mellitus, hyperadrenocorticism and neoplasia are now briefly discussed.

1.3.2.1 Diabetes Mellitus and Proteinuria

In human medicine, diabetic nephropathy is defined as a 'syndrome characterised by the presence of pathological quantities of urine albumin excretion, diabetic glomerular lesions and loss of glomerular filtration rate (GFR)' (Lim, 2014). In human patients, diabetes associated nephropathy (possibly in combination with hypertensive nephropathy) is the most common cause of end stage renal disease (Ghaderian et al., 2015). In human medicine, diabetes mellitus, increased blood pressure, proteinuria and nephropathy are often linked, however, whether hypertension is a cause or consequence of nephropathy or proteinuria is not fully determined (Mazzi et al., 2008). Systemic hypertension is reported to potentially play a key role in the development and progression of diabetic nephropathy (Patel et al., 2007); one proposed mechanism for the occurrence of systemic hypertension in diabetic human patients is due to increased peripheral vascular resistance (Cruickshank et al., 2002). The lesions that are typically associated with diabetic nephropathy in humans appear to be related to the occurrence of two main alterations; firstly, the glomerular basement membrane becomes thickened and secondly there is expansion of the tubular basement membrane and mesangium mainly due to the development of increased mesangial matrix (Dalla Vestra et al., 2000, Shaw, 2005).

In dogs, diabetic nephropathy is less clearly defined and reported. The percentage of diabetic dogs reported to be proteinuric is between 20-60% (Struble et al., 1998, Herring et al., 2014).

Histopathological lesions consistent with diabetic nephropathy have been documented in dogs with experimentally induced diabetes mellitus (Gaber et al., 1994) but further research is required to further understand the association between diabetes mellitus and proteinuria in dogs.

1.3.2.2 Hyperadrenocorticism and Proteinuria

Hyperadrenocorticism occurs due to the excessive production of endogenous glucocorticoids. Hyperadrenocorticism is linked to proteinuria in the dog; 46% of dogs with untreated pituitary dependent hyperadrenocorticism have been shown to have an elevated UPC (Ortega et al., 1996). The exact mechanism by which hyperadrenocorticism leads to proteinuria is unknown with hypotheses including an alteration in renal haemodynamics or concurrent hypertension (Goy-Thollot et al., 2002). Increased circulating levels of free fatty acids (FFAs) are also reported in hyperadrenocorticoid patients due to altered lipid metabolism (Kubota, 2001); in human diabetic patients increased FFAs have been linked to an increase in albuminuria despite normal renal selectivity (Hayashi et al., 1990). Therefore, the FFA status in hyperadrenocorticoid patients may explain to some extent the proteinuria noted. Although achieving clinical control of hyperadrenocorticism has been associated with a reduction in UPC (Ortega et al., 1996), this is not the case for all dogs and in some, the proteinuria proves to be persistent (Hurley and Vaden, 1998).

Although the exact mechanism of damage is not completely understood, administration of exogenous glucocorticoid therapy is also suggested to promote proteinuria and glomerular damage (Waters et al., 1997, Schellenberg et al., 2008).

1.3.2.3 Neoplasia and Proteinuria

Neoplasia is a known cause of proteinuria in the dog (Prudic et al., 2018). Again, the exact aetiology for the development of proteinuria is unknown, however, there are several proposed theories.

In humans, paraneoplastic glomerular diseases associated with solid or blood tumours are thought most likely to be related to the abnormal products of the neoplastic cells (Jhaveri et al., 2013). The most common solid tumours associated with glomerulonephropathy are bronchial and gastric carcinomas (Bacchetta et al., 2009) with membranous glomerulonephropathy being the most frequently reported glomerulopathy in human patients with solid tumours (a definition and introduction to the different types of glomerular disease, such as membranous glomerulopathy, is provided later). In contrast, blood neoplasms such

as Hodgkin's and other forms of lymphoma are typically associated with minimal change disease (MCD) in people; MCD is reported to occur in approximately 1% of Hodgkin's patients (Jhaveri et al., 2013). In humans with newly diagnosed non-Hodgkin's lymphoma, Pedersen *et al.* (2003) documented an association between urinary albumin excretion and levels of serum C-reactive protein, interleukin 6 and tumour necrosis factor-alpha suggesting a possible inflammatory aetiology in cases of neoplasia and glomerular loss of albumin.

Proteinuria is also observed frequently in dogs with neoplasia: in a study of 60 client owned dogs with confirmed neoplasia, proteinuria was detected in 51% of cases, although only two dogs had a UPC of >2.0 (Prudic et al., 2018). In dogs it has been shown that those with lymphoma are more likely to be proteinuric compared to age matched controls, however, only 8 out of 32 dogs had a UPC value of 0.5 or higher (Di Bella et al., 2013). A similar finding has been suggested in dogs with mammary carcinoma who have also been shown to have significantly higher UPC ratios when compared to age matched controls, however, again the magnitude of this proteinuria was low with no dog having a UPC of >2.5 (Crivellenti et al., 2016). Proteinuria has been reported to be associated with a worse prognosis in dogs diagnosed with osteosarcoma (Saam et al., 2011). However, dipstick analysis was the main methodology used to determine proteinuria with UPCs being performed in only two dogs (UPC 2.2 and 4.0); the authors suggest that the documented proteinuria may have been attributable to renal metastasis or potentially paraneoplastic glomerulopathy (Saam et al., 2011).

As well as neoplasia being directly associated with proteinuria, tyrosine kinase inhibitors (TKIs) are used to treat a variety of neoplasms and this class of drug has been linked to the development of canine proteinuria. For example, recent retrospective studies have documented between 18-24% of dogs treated with toceranib developed proteinuria whilst on treatment (Piscoya et al., 2018, Kuijlaars et al., 2021). The pathophysiology behind the development of proteinuria with TKIs is uncertain. TKIs are reported to have both anti-tumour and anti-angiogenic activity which leads to the inhibition of vascular endothelial growth factor receptor and platelet derived growth factor receptor (London et al., 2003). Nephrin (Neph1) is a crucial protein for the integrity of the slit diaphragm of the glomerulus with inactivation of this protein in mice leading to high levels of proteinuria (Kawachi et al., 2009). Recently, inhibition of vascular endothelial growth factor receptor has also been demonstrated to inhibit nephrin production, hence providing a possible methodology via which TKIs may lead to proteinuria (Sugimoto et al., 2003). Hypertension has also been reported with the use of TKIs and it is possible that the development of hypertension secondary to TKIs may contribute to the presence of proteinuria (Kandula and Agarwal,

2011). In humans, the use of TKIs have been associated with the development of minimal change disease (MCD) and focal segmental glomerulosclerosis (FGGS) (these conditions are outlined later) (Betton et al,. 2018, Furuto et al., 2018, Ihara et al., 2021). In dogs, histopathology is rarely performed in cases developing proteinuria following TKI therapy, however, there is one case report of a dog developing MCD after treatment with masitinib (Sum et al., 2010).

It should be added that in the addition to the presence of certain endocrine diseases and neoplasia, proteinuria can also be associated with inflammation. For example, proteinuria has been reported in dogs with systemic inflammatory response syndrome due to a variety of underlying aetiologies including pyometra and peritonitis (Schaefer et al., 2011). Finally, there is now evidence to suggest that the presence of hyperlipidaemia can contribute to proteinuria. Hypertriglyceridemia in humans is associated with an increased risk of the development of proteinuria (Tozawa et al., 2002). In rats, high triglyceride levels have been reported to lead to progressive FSGS (Kamanna and Kirschenbaum, 1993, Joles et al., 2000). In the canine population, most work on the assessment of hypertriglyceridemia has been done with the Miniature Schnauzer. It has been shown that Miniature Schnauzers have a strong positive correlation between their triglyceride concentrations and magnitude of their UPC (Furrow et al., 2016). On histopathology, 20 out of 27 proteinuric Miniature Schnauzers have ultrastructural evidence of lipid deposition within their glomerular tufts (Furrow et al., 2017).

Whilst the association between the presence of proteinuria and various systemic conditions is well recognised, it is not yet fully understood as to what extent these conditions may have. It is currently unknown whether dogs with and without a trigger for proteinuria lose different proteins whilst it is also not fully known to what extent the urinary proteins may change in response to controlling the underlying disease. For example, in dogs with hyperadrenocorticism treated with trilostane; if the UPC reduces following initiation of trilostane therapy it is uncertain if this is due to alterations in the type of proteins lost into the urine or simply a reduction in the quantity of the original proteins being lost. Further research is required to explore the urinary proteins lost in dogs with and without triggers for their proteinuria to determine if any patterns can be established.

1.4 Renal biopsies and glomerular disease

As previously discussed, the work up of cases with suspected glomerular proteinuria first involves screening as much as possible for a systemic underlying cause for the proteinuria (such as those introduced above). If no such cause is found, or if the proteinuria fails to resolve with treatment of an underlying cause, then a renal biopsy is indicated. Renal histopathology assists in allowing a definitive diagnosis to be made allowing the most appropriate treatment protocol to be determined and prognostic information to be gained. Currently, renal biopsy is the only way to achieve this information, in the ideal world, the use of urinary biomarkers would allow us to predict the renal pathology present in a less invasive way. As yet, specific biomarkers associated with a given glomerular disease have not been identified but this represents an area of great possibility and research.

Renal biopsies can be obtained by several methods including percutaneous ultrasound guided, laparoscopic or surgical wedge biopsy. Reported complications of renal biopsy include haemorrhage, formation of an arteriovenous fistula, development of hydronephrosis, infarction/thrombosis, infection and scar formation/fibrosis (Vaden, 2005). A retrospective study of 283 dogs undergoing renal biopsy reported a complication rate of 13.4% with severe haemorrhage being the most frequently reported whilst hydronephrosis and death were uncommon (Vaden et al., 2005).

In general, veterinary literature tends to split glomerular diseases based on whether they are associated with an underlying immune mediated aetiology or not. The JVIM Consensus Statement on Pathological Renal Biopsies classifies glomerular disease as immune complex mediated glomerulonephritis (ICGN); non-immune mediated or amyloidosis (Cianciolo et al., 2013). In dogs with suspected immune mediated disease, a further classification can be made regarding the nature of the immune complexes; glomerular disease can either arise from the deposition of circulating soluble immune complexes within the glomeruli or can be due to the development of antibodies directed against antigens in the glomerular basement membrane itself. ICGN are thought to be one of the leading causes of proteinuria in dogs with a prevalence of 48% documented in Northern America and 50% in Europe (Schneider et al., 2013, Aresu et al., 2017), although geographical variations are likely to exist; with a recent study performed by Vessieres *et al.* (2019) documenting ICGN as the cause of glomerular proteinuria in just 27% of dogs in the UK.

The main glomerular diseases are now introduced and are split into immune complex mediated disease versus non-immune complex mediated disease.

1.4.1 Immune Complex Mediated Glomerulonephritis

Of the ICGN there are three main classifications: membranous ICGN; proliferative ICGN and membranoproliferative ICGN.

1.4.1.1 Membranous glomerulonephritis

Membranous glomerulonephritis is associated with glomerular damage mediated by complement dependent mechanisms and immune complexes deposition. In this glomerulonephropathy, the immune complexes are located on the subepithelial (i.e. the podocyte) side of the glomerular basement membrane away from the capillaries.

To make an accurate diagnosis of membranous glomerulonephritis, electron microscopy (EM) must be performed. In a recent study of canine renal biopsies submitted to the European Veterinary Renal Pathology Service, 28 dogs were initially diagnosed with membranous glomerulonephritis, however, this diagnosis was revised in 58.1% of cases following EM evaluation demonstrating the importance of EM evaluation in these cases (Aresu et al., 2017).

In humans, primary membranous glomerulonephritis is reported and there is evidence to support the role of formation of immune complexes in situ with one of the nephritogenic antibodies associated with human membranous glomerulopathy being recently identified (Debiec et al., 2002). Eighty percent of cases of membranous glomerulonephritis in people are reported to be primary with just 20% being due to a systemic cause; the systemic (or secondary) causes associated with membranous glomerulonephritis include human immunodeficiency virus, neoplasia and auto-immune diseases such as thyroiditis (Couser, 2017). Conditions that have been associated with secondary membranous nephropathy in the dog include systemic viral and parasitic infections, neoplasia and several drug or heavy metal toxicities (Jaenke and Allen, 1986). In dogs, membranous glomerulonephritis appears to be progressive, although, in some cases such progression is slow enough to enable dogs to continue to live relatively normal lives. However, reported survival times are very variable with a range of 4 days to over 3 years (Jaenke and Allen, 1986).

1.4.1.2 Proliferative glomerulonephritis

Proliferative glomerulonephritis occurs when there is either endocapillary or mesangial proliferation. Mesangial proliferative glomerulonephritis reveals mesangial cell hyperplasia

on histopathologic examination whereas endocapillary proliferative glomerulonephritis is characterised by a proliferation of endothelial cells with or without increased mesangial cellularity. Immunofluorescence allows the detection of deposits of immunoglobulin G (IgG) or immunoglobulin M (IgM) or both in the glomerular basement membrane and mesangium. The condition often occurs secondary to systemic disease and therefore, immune complexes are often located within the mesangium or occasionally within the capillary walls.

In human medicine, a pathologic diagnosis of proliferative glomerulonephritis is made based on both a morphological description (e.g. mesangial proliferative glomerulonephritis) and a specific disease designation (e.g. Immunoglobulin A (IgA) nephropathy or lupus glomerulonephritis). It is reported to be a common complication following bacterial infection in people, especially skin infection with streptococcus bacteria but also after streptococcal pharyngitis (in which case it is referred to as poststreptococcal glomerulonephritis) (Rodriguez-Iturbe and Haas, 2016).

Proliferative glomerulonephritis seems rare in the dog with a recent study reviewing pathological features of 89 biopsies not detecting any cases (Cianciolo et al., 2013). Two older studies have reported a prevalence of 6-16% in dogs undergoing renal biopsy (Koeman et al., 1987, Vilafranca et al., 1994). Due to the rarity of this condition, the prognosis for dogs has not been thoroughly researched.

1.4.1.3 Membranoproliferative glomerulonephritis

Membranoproliferative glomerulonephritis (MPGN) occurs when immune complexes are found in the glomerular mesangium and thickening of the GBM arises. Histopathological diagnosis of MPGN is made when there is both thickening of the capillary loops and mesangial hypercellularity. The glomerulus may take on an enlarged, segmented, or lobular appearance. When immunofluorescence is performed, immune complex deposits are seen as granular deposits of C3 in combination with IgG, IgM and IgA. EM can be further used to identify the immune deposits.

MPGN is one of the most common glomerular diseases reported in the dog with the mean age of diagnosis being 10.5 years (Vilafranca et al., 1994). In the Bernese Mountain Dog, MPGN is reported as a familial disease. An older study reported that between 1988 and 1992, nephropathies were frequently diagnosed in the Bernese Mountain dog. Twenty animals presented during this period and a morphologic diagnosis of MPGN with concomitant interstitial nephritis was made in all cases. A pedigree analysis was performed and indicated

that the MPGN was likely of hereditary genesis (Minkus et al., 1994). MPGN has also been associated with *Borrelia burgdorferi* infection in dogs (Lyme nephritis) (Dambach et al., 1997).

In humans, MPGN is reported to be a slowly progressive disease. Although, detailed prognostic information is not available due to lack of research in dogs, the WSAVA study looking at the classification of glomerular disease in dogs found that MPGN was associated with the most severe collection of clinical signs; dogs with MPGN had amongst the highest UPC as well as highest serum creatinine and lowest serum albumin and were also more likely to be hypertensive suggesting their prognosis may be poor (Cianciolo et al., 2013). In people negative prognostic indicators are reported to be the presence of nephrotic syndrome, systemic hypertension and the presence of sclerosis or crescents on histopathology (Cameron et al., 1983, Pedersen, 1995).

1.4.2 Non-immune complex mediated glomerular disease

In addition to ICGN there are several other glomerular diseases that have been recognized and are of importance in the dog. These are non-immune complex mediated diseases.

1.4.2.1 Amyloidosis

Amyloid deposits can occur locally; be limited to within one organ or be generalized affecting any tissue or organ. In human medicine there are three distinct systemic forms of amyloidosis that are recognized: immunoglobulin associated (primary); reactive (secondary) and senile (heredofamilial) with each being associated with a particular protein precursor of amyloid (Moyssakis et al., 1999). In canine patients, amyloidosis is most often considered to be reactive or secondary (Gruys, 2004). With the exception of the Shar Pei the amyloid is most often deposited within the glomeruli and deposits are made up of the protein amyloid A which is an amino terminal fragment of serum amyloid A (DiBartola et al., 1989). In the Shar Pei, amyloid is instead most commonly found deposited within the renal medulla with 64% of the breed having concurrent glomerular involvement (DiBartola et al., 1990). Reactive amyloidosis can be idiopathic but is often associated with chronic inflammation, infection, or neoplasia (Brunger et al., 2020).

Amyloidosis accounts for approximately 15% of all glomerular lesions in the dog (Schneider et al., 2013). In a retrospective review of 91 dogs comparing renal amyloidosis in Shar Peis

with other breeds it was shown that Shar Peis were significantly younger at the time of diagnosis with a median age of 4.8 years (range 3.6-17) versus a median of 9 years (range 2.4-11.1) in other breeds (Segev et al., 2012). Hypoalbuminaemia was the most reported biochemical abnormality and was seen more frequently in the non-Shar-Pei dogs (100%) compared to the Shar Peis (64%) (Segev et al., 2012).

Prognosis is reported to be poor with a median survival time of just 5 days being reported by one study and another reporting that 58% of dogs with amyloidosis died or were euthanized at the time of diagnosis with the remaining cases surviving between 2 and 20 months whilst survival times of >12 months were reported in only 8.5% of cases (Segev et al., 2012, DiBartola et al., 1989).

1.4.2.2 Glomerulosclerosis

Glomerulosclerosis occurs following a persistent insult to the glomerulus leading to accumulation of extracellular matrix within the glomerulus. Glomerulosclerosis can be focal or global; focal glomerulosclerosis occurs when sclerosis is present within small sections of an initially limited number of glomeruli. In humans most of the literature refers to FSGS of which there are three categories: genetic, primary and secondary. Secondary forms are further split into maladaptive, viral and drug induced. Primary FSGS is a disease that is assumed to be caused by a circulating factor, such as a cytokine, which leads to generalized injury to the podocytes. Secondary maladaptive FSGS occurs due to a reduction in the number of nephrons that are functioning or from a normal nephron population subjected to an abnormal level of stress (De Vriese et al., 2018). For example, secondary forms can occur in cases of hypertension, obesity, and diabetes mellitus (Tervaert et al., 2010, D'Agati et al., 2004). Drug induced FSGS has been reported in people in association with lithium and pamidronate (De Vriese et al., 2018). A critical review published in America has reported ‘convincing’ evidence that the cytomegalovirus has links with FSGS in humans and there is also evidence for a pathogenic role of the Epstein-Barr and parvo virus (Chandra and Kopp, 2013). Genetic causes of FSGS can have a range of inheritance patterns (autosomal dominant or recessive, X-linked or mitochondrial). The age of onset of the genetic FSGS conditions is usually early in childhood.

In humans, immunomodulatory therapy is only used for primary FSGS. Secondary maladaptive is usually treated with inhibition of the renin angiotensin aldosterone system. Globally sclerotic glomeruli are thought to be due to normal ‘wear and tear’ in human

literature and there is not a specific disease mechanism in most cases. In people under the age of 40 it is possible for up to 10% of the glomeruli to be sclerotic. The extent of the sclerosis will increase with age and is usually thought to reach 30% by 80 years of age (Weinstein and Anderson, 2010).

In dogs there are a variety of explanations for the pathogenesis of FSGS including both genetic and acquired factors. In a study of 501 dogs that underwent biopsy for suspected glomerular disease 20% were found to have glomerulosclerosis making it the most common non-ICGN (Schneider et al., 2013). Within this category a range of focal and global glomerulosclerosis was detected. This study also highlights the importance of performing full histopathological assessment (i.e. inclusion of EM and immunohistochemistry) on renal biopsies as initially 5% of the dogs with glomerulosclerosis were misdiagnosed as having an ICGN based on light microscopy alone (Schneider et al., 2013). FSGS in the dog may progress to diffuse and global glomerulosclerosis, eventually leading to end-stage renal disease and thus a guarded prognosis.

1.4.2.3 Minimal change disease

MCD is commonly seen in humans, especially in children. In humans the cause of the disease is unknown, and it is thought that several different subclassifications may exist each with slightly variable pathophysiology, however, immune dysfunction and modification of the podocytes are thought to act synergistically to alter the integrity of the GBM leading to the development of proteinuria (Vivarelli et al., 2017). In humans, especially younger children/adolescents the disease is usually idiopathic although secondary disease also occurs (Vivarelli et al., 2017). In MCD the glomerular capillary wall loses its anionic charge and leading to collapse of the podocyte food processes. The loss of charge of the glomerular capillary wall also leads to proteinuria which is usually high in magnitude. On histopathological examination there are no lesions identified under light microscopy (hence the name). Immunoglobulin deposition is absent when evaluated by immunofluorescent microscopy; however, there may be an increase in staining for vimentin.. EM is required for definitive diagnosis; EM shows effacement of the foot processes of the podocytes.

MCD is uncommonly described in the dog with only a few case reports. MCD has been reported following administration of masitinib (Sum et al., 2010) and following experimentally-induced *Ehrlichia canis* infection (Codner et al., 1992) whilst there have also been reports of two ‘idiopathic’ cases (Vilafranca et al., 1993, Travail et al., 2022). The

treatment for idiopathic MCD in humans is corticosteroids with an expected response rate of 80-90% (Vivarelli et al., 2017). Relapses in human patients are relatively common reported in about two thirds of patients, however, progression of disease is rarely documented (Lee et al., 2016). In veterinary literature little is reported regarding the expected prognosis. A recent case report has been published documenting successful treatment of a dog with MCD receiving combination therapy (mycophenolate mofetil and telmisartan); further research is required before this protocol is recommended (Travail et al., 2022).

1.5 Treatment of Glomerular Disease

1.5.1 Why is it necessary to treat glomerular proteinuria?

It is now clear that glomerular proteinuria can arise due to numerous conditions but what has not yet been addressed is why proteinuria is of concern? In human literature, numerous studies have demonstrated persistent proteinuria to be correlated with progression of renal disease in both diabetic and non-diabetic patients, often documented by faster decrease in GFR or earlier development of end stage renal disease (Ruggenenti et al., 1997b, Wright et al., 2002, Keane et al., 2006, Atkins et al., 2005, Jafar et al., 2001, Walls, 2001, Tryggvason and Pettersson, 2003). Fewer studies have been performed in the veterinary literature but it is thought that proteinuria is also a risk factor for renal disease progression in dogs with persistent proteinuria. For example, a study assessing dogs with proteinuric CKD found that the risk of adverse outcome was approximately 1.5 times higher for each unit increase in UPC (Jacob et al., 2005). Additionally, glomerular disease is also thought to be an important cause of CKD in dogs accounting for up to 50% of cases (Macdougall et al., 1986).

Persistent proteinuria is suggested to precipitate ongoing renal damage via numerous different mechanisms, for example, it can lead to protein accumulation within the glomerulus stimulating mesangial cell proliferation and increased production of the mesangial matrix (Jerums et al., 1997). Additionally, with ongoing and/or high magnitude protein loss tubular damage can arise. For example, the intracellular pathways of the tubular cells that usually handle filtered albumin can become overloaded, the tubular response to which includes production of cytokines leading to the recruitment of inflammatory cells (Ruggenenti et al., 2003b, Liu et al., 2015). Tubulointerstitial injury is further contributed to by the fact that proteinuria is thought to have a profibrotic effect leading to activation of transcription factors and up-regulation of pro-inflammatory genes. It has been proposed that glomerular proteins,

such as albumin, may have a directly toxic effect on the tubular epithelial cells, in addition, it is suggested that albumin in combination with free fatty acids such as oleic/linoleic acid can also have a negative effect on tubular cells (Thomas et al., 2002, Jia et al., 2019, Kamijo et al., 2002). An alternative mechanism for tubular damage in the face of glomerular disease is the hypothesis that the tubular cells are damaged by molecules similar in size to albumin which are also lost across the GBM in the face of disease, such molecules may include cytokines, growth factors or components of complement (Nangaku et al., 2002). Ongoing tubulointerstitial damage can lead to loss of the tubule which would then lead to death of the nephron and hence a reduced nephron mass present within the kidney and reduced GFR and a move towards azotaemic renal failure.

As well as contributing to progression of renal disease, persistent proteinuria can also lead to a range of other systemic complications including development of a hypercoagulable state, increases in serum cholesterol and decreases in serum albumin which may be associated with the development of nephrotic syndrome, ascites and altered amino acid profiles (Lennon et al., 2013, Donahue et al., 2011, Parker et al., 2019, Klosterman et al., 2011).

Treatment of glomerular disease can be divided into specific therapy, implemented when a histopathological diagnosis has been made, and standard therapy which is applicable to all cases of glomerular proteinuria and is targeted at reduction of proteinuria. Standard therapy will be discussed first before specific therapies are introduced.

1.5.2 Standard therapy

Regardless of the underlying aetiology for glomerular proteinuria, ‘standard’ therapy is nearly always recommended. Standard therapy is primarily aimed at reducing proteinuria via suppression of the renin angiotensin aldosterone system (RAAS). Before standard therapy is discussed further, the juxtaglomerular apparatus (which is a key component in the RAAS) and the RAAS itself will be briefly introduced so that therapeutic targets are understood.

1.5.2.1 The juxtaglomerular apparatus

The juxtaglomerular apparatus is composed of the tubular component, termed the macula densa, and the juxtaglomerular cells located within the terminal part of the afferent arteriole of the glomerulus (Figure 1). The cells of the macula densa are positioned in a way such as their apical membrane is in contact with tubular fluid and their basilar membrane is in contact

with the cells of the mesangium and juxtaglomerular cells (Peti-Peterdi and Harris, 2010). The cells of the macula densa are responsible for detecting alterations in the electrolyte balance of the tubular fluid and transmitting signals to the juxtaglomerular apparatus which is responsible for alterations in renal blood flow via two mechanisms; the release of renin and tubuloglomerular feedback (Bell et al., 2003). The cells of the juxtaglomerular apparatus function as stretch receptors within the arteriolar wall, releasing renin in response to decreased renal perfusion, of which one of the most common cause is systemic hypotension.

1.5.2.2 Introduction to the Renin Angiotensin Aldosterone System

The RAAS plays an important role in the regulation of GFR and renal blood flow as well as systemic blood pressure. The enzyme renin is made as preprorenin by the granular extraglomerular mesangial cells (specialised juxtaglomerular epithelioid cells). Preprorenin is processed to prorenin and released in this form or is further cleaved to form active renin which is then released in granules. Baroreceptors located in the in the afferent arterioles can detect a drop in renal perfusion, usually arising because of systemic hypotension, when such a drop is detected the release of renin is stimulated. Figure 3 briefly outlines the interaction between the different components of the RAAS.

The schematic in Figure 3 is somewhat simplified. Renin can also be released in response to sympathetic nerve stimulation of the beta1-adrenoceptors located on the juxtaglomerular cells. Furthermore, a decrease in the level of sodium chloride in the tubular fluid will also stimulate the release of renin. The concentration of sodium chloride is monitored by macula densa cells located in the distal tubule.

Additionally, angiotensin II acts on the posterior pituitary to release anti-diuretic hormone (ADH). ADH exerts an effect by binding to principal cells in the distal tubule and collecting ducts. ADH leads to the insertion of aquaporin-2 (water) channels in the apical membrane which allows water to passively move into the cells i.e. water reabsorption is increased. ADH also leads to contraction of vascular smooth muscle leading to increase in systemic blood pressure.

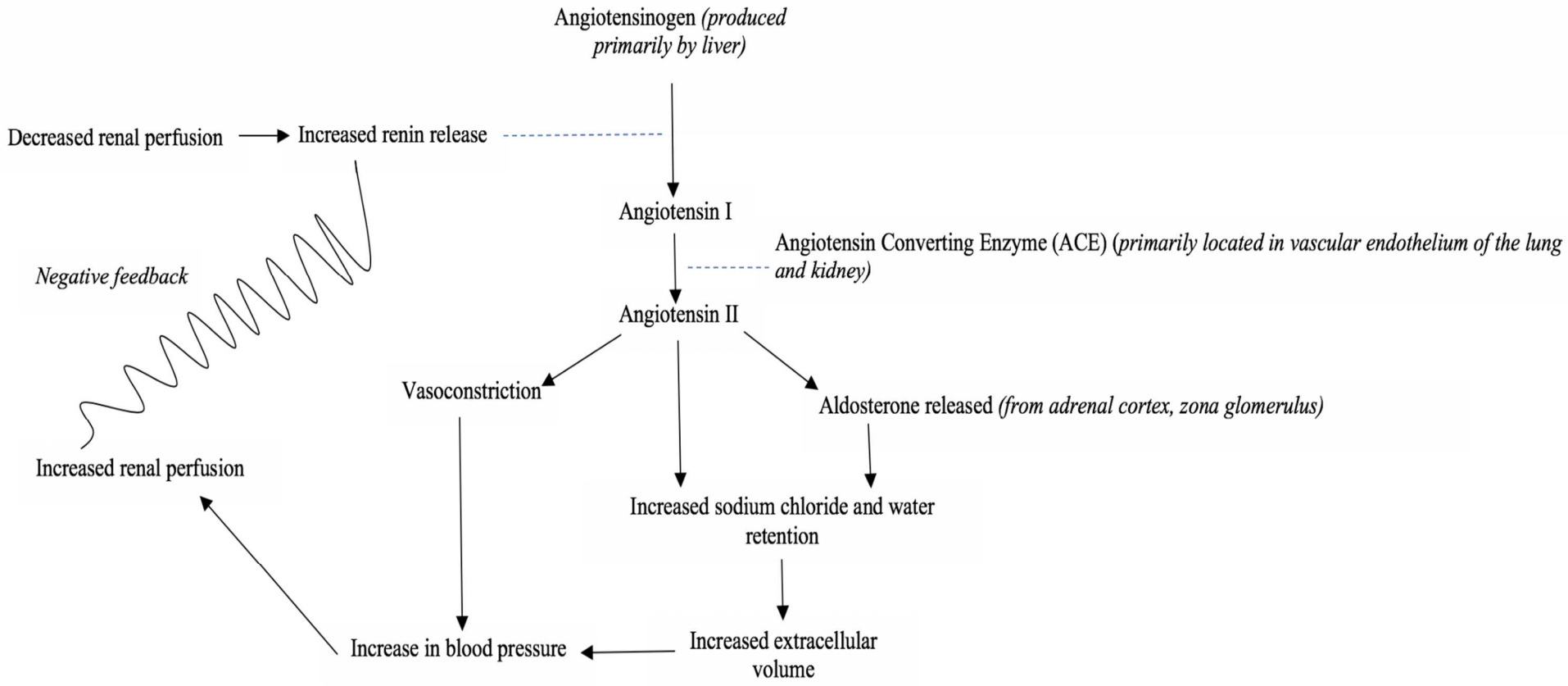


Figure 3: Schematic of the renin-angiotensin-aldosterone system. Dotted lines represent enzyme catalysation and wavy line represents negative feedback. Modified from (Verlander, 2020).

1.5.2.3 Inhibition of the renin angiotensin aldosterone system

Inhibition of the RAAS is the mainstay of treatment for glomerular proteinuria and this can be achieved by employing several different classes of drugs as discussed below.

1.5.2.3.1 Angiotensin converting enzyme inhibitors (ACEi)

The RAAS is the main target for therapies aimed at reducing proteinuria. Angiotensin II has been implicated in the pathophysiology of proteinuric renal disease for numerous reasons. For example, angiotensin II causes vasoconstriction of the glomerular arterioles having a greater impact on the efferent in comparison with the afferent and thus leading to an increase in the intraglomerular pressure (Yamada et al., 1990). Angiotensin II can also affect the glomerular mesangium causing it to contract. Contraction of the glomerular mesangium can lead to several events, including alteration of the size of the mesangial channels which impacts on the movement of macromolecules through this region (Raij and Keane, 1985). Additionally, experimentally, it has been found that angiotensin II can increase the production of vasoactive substances, such as transforming growth factor β which has been implicated with the development of glomerulosclerosis (Kagami et al., 1994). Hence, reduction/blockage of angiotensin II is thought to be of benefit in the treatment of glomerular proteinuria. ACEi have many proposed mechanisms of action, for example, they are thought to lead to changes in the glomerular transcapillary hydraulic pressure (Anderson et al., 1986, Brown et al., 1993). Additionally, ACEi can prevent the loss of heparan sulphate which can occur with glomerular disease, reduce the size of the glomerular capillary endothelial cell pores and slow mesangial cell growth (Kagami et al., 1994, Erley et al., 1992, Reddi et al., 1991, Morelli et al., 1990, Raij and Keane, 1985, Wiegmann et al., 1992). In human medicine, there are extensive studies documenting the use of ACEi and their benefits including reduction of proteinuria and delaying progression of renal disease (Jafar et al., 2001, Palmer et al., 2015, Ihle et al., 1996, Maschio et al., 1996, Gansevoort et al., 1993, Lewis et al., 1993, Ruggenenti et al., 1997a). In veterinary medicine, there are fewer studies assessing the effect of ACEi on delaying disease progression but the outcome of the existing studies appears to agree with human literature (Grauer et al., 2000, Brown et al., 1993, Grodecki et al., 1997). Interference with the RAAS, especially with ACEi is considered standard of care for dogs with glomerular disease (Brown et al., 2013). Benazepril and enalapril are two commonly used ACEi in veterinary medicine, enalapril is renally excreted whilst benazepril is mainly excreted via the biliary system meaning that in dogs with renal compromise lower doses of

enalapril may be required (Lefebvre et al., 1999). Benazepril is a readily available ACEi in the UK and the recommended starting dose is 0.5mg/kg/day with dose increases in increments of 0.5mg/kg/day being made as required to a maximal dose of 2mg/kg/day. Dogs need to be monitored closely whilst receiving ACEi to ensure that worsening azotaemia does not occur. Although it has been shown that in humans with severe renal failure, ACEi still slowed progression of disease, the use of such therapy in dogs with International Renal Interest Society (IRIS) CKD stage IV is cautioned (Ryan and Tuttle, 2008).

1.5.2.3.2 Angiotensin receptor blockers (ARB)

ARBs represent an alternative to ACEi therapy targeting the RAAS at a different point. ARBs block the angiotensin II type I receptor. Telmisartan is the most commonly used ARB, the starting dose is 1.0mg/kg/day with dose increases of 0.5mg/kg/once daily recommended to a maximum of 2mg/kg/day. In people, ARBs including losartan, telmisartan and irbesartan are commonly used and have been shown to have good effect for example, it has been shown that following the use of irbesartan for each 50% drop in proteinuria in the initial 12-months the chances of a negative renal outcome (defined as doubling of baseline serum creatinine or development of end-stage renal disease) were reduced by more than half (Atkins et al., 2005). Although there are no studies assessing a population of dogs with only glomerular proteinuria treated with telmisartan, there are several studies assessing slightly different populations of proteinuric dogs. Lecavalier *et al.* (2021) assessed the use of telmisartan in dogs that were not azotaemic with a UPC ≥2 and those that were azotaemic with a UPC≥0.5 and found an 80% response rate at 6 months. Telmisartan has also been proven to reduce proteinuria in dogs with proteinuric CKD and lead to an improved response in dogs that do not respond as hoped to ACEi (Miyagawa et al., 2020, Bugbee et al., 2014, Lourenco et al., 2020). Defining response to treatment with ACEi or ARB is discussed later.

1.5.2.3.3 Combined therapy

Complete blockade of the RAAS is not achieved with either ACEi or ARBs. ARB therapy can potentially actually lead to an increase in renin activity whilst one proposed mechanism for dogs failing to respond to ACEi is non-ACE pathways which still allow for the formation of angiotensin II (Campbell, 2009, Li et al., 2004, Barreras and Gurk-Turner, 2003). It has been suggested treating with an ACEi and ARB simultaneously may allow for the drugs to act synergistically (Linas, 2008). In humans, there is debate as to whether combination

therapy is advisable with some conflicting evidence in the literature. Combination therapy is reported to have a beneficial effect on reduction of proteinuria and is thought to be beneficial and safe based on meta-analysis of several small trials (Jennings et al., 2007, Kunz et al., 2008, MacKinnon et al., 2006). However, other studies have suggested that combination therapy is associated with an increased risk of adverse events, such as hyperkalaemia (Mann et al., 2008, McAlister et al., 2011, Zhao et al, 2021). Currently, clinical studies in dogs treated with combination therapy are lacking and hence whilst it remains an option dogs should be carefully monitored if started on both ACEi and ARB therapy simultaneously.

1.3.4.3.4 Aldosterone receptor blocker

In human patients, it has been shown that aldosterone levels can increase in people undergoing long-term suppression of the RAAS; a phenomenon known as aldosterone breakthrough (Moranne et al., 2013). In experimental models using murine kidneys, aldosterone has been suggested to promote not only proteinuria but also glomerulosclerosis, fibrosis and vascular changes (Nishiyama et al., 2004, Takeda, 2009). Therefore, the use of aldosterone receptor blockers has been trialled in people alongside ACEi/ARB therapy and this has led to a reduction in proteinuria (Bianchi et al., 2006, Saklayen et al., 2008). A recent study reported between 35 and 59% of dogs with proteinuric CKD treated with RAAS inhibition showed aldosterone breakthrough (Ames et al., 2022). However, there is limited evidence on the use of aldosterone receptor blockers in the dog for the management of proteinuria and it is currently thought that their use would only be beneficial in dogs with increased aldosterone concentration (Brown et al., 2013). This represents an area for further research and improved understanding regarding the number of dogs treated with RAAS inhibition that develop aldosterone breakthrough and if such breakthrough impacts on their response to treatment and/or prognosis. Additionally, it remains to be seen if the use of aldosterone receptor blockers such as spironolactone would be of benefit in these cases.

1.5.2.4 Treatment aims

Treatment aims for the use of ACEi are outlined in the Consensus Recommendations for Standard Therapy for Glomerular Disease which advises that the ideal target is a UPC of <0.5, however, a reduction of ≥50% from baseline is also considered acceptable as a therapeutic target (Brown et al., 2013). These recommendations appear to stem from research performed by Grauer *et al.* (2000) who suggested that a ≥50% reduction in UPC with a stable serum creatinine defined an improvement in dogs with idiopathic glomerulonephritis. What is currently unknown is the proportion of dogs that reach these outlined targets and whether achieving such therapeutic targets is associated with an improved survival benefit. This knowledge would have significant clinical benefit; currently a substantial amount of time and cost is required in cases with glomerular proteinuria for monitoring and performing dose escalations to ensure target is achieved and confirmation of whether this is valid is warranted.

1.5.2.5 Other supportive care

1.5.2.5.1 Anti-thrombotic medications

It has been suggested that dogs with glomerular proteinuria are hypercoagulable with thrombotic disease being detected in 25% of cases at post-mortem in one study (Cook and Cowgill, 1996). The CURATIVE review describes three prospective studies in dogs with protein losing nephropathy all of which support the presence of a hypercoagulable state within this population (Donahue et al., 2011, Lennon et al., 2013, White et al., 2016). The CURATIVE guidelines also report thromboembolic complications in 6-42% of dogs with protein losing nephropathy and hence recommend anti-thrombotic therapy in this group of dogs (deLaforcade et al., 2019). Anti-thrombotic therapy is also recommended by the ACVIM Consensus for Standard Therapy for Glomerular Disease with no preference over aspirin versus clopidogrel reported (Brown et al., 2013).

1.5.2.5.2 Dietary therapy

In models of canine CKD and in dogs with spontaneously occurring disease, diet has been shown to modify the extent of proteinuria present (Polzin et al., 1984, Zatelli et al., 2016). Omega 3 supplementation may also be beneficial in proteinuric dogs as it has been demonstrated in humans with glomerular disease that supplementation with

eicosapentaenoic acid and docosahexaenoic acid can reduce the magnitude of proteinuria (Decaterina et al., 1993). In canine patients with glomerular disease, supplementation with n-3 polyunsaturated acids (which include eicosapentaenoic acid and docosahexaenoic acid) has been shown to be renoprotective (Brown et al., 1998). If dietary supplementation with n-3 polyunsaturated acids is administered the recommended dose is 0.25-0.5g/kg (Brown et al., 2013).

1.5.2.5.3 Anti-hypertensive medication

The prevalence of hypertension in dogs with glomerular disease is not known, however the reported prevalence of hypertension in association with CKD varies from 9-93% (Anderson and Fisher, 1968, Buranakarl et al., 2007, Cortadellas et al., 2006, Bodey and Michell, 1996). The presence of hypertension has been associated with both proteinuria and renal damage (Jacob et al., 2003, Cortadellas et al., 2006, Bacic et al., 2010); in dogs with azotaemic CKD, blood pressure has not only been associated with proteinuria but also shortened survival times (Jacob et al., 2003, Wehner et al., 2008). Guidelines for dogs with CKD are outlined by the International Renal Interest Society (IRIS) guidelines which suggest that the requirement for anti-hypertensive medication be made on an individual basis with blood pressure being categorised based on the risk of development of target organ damage (TOD) (IRIS, 2019). Similarly, the ACVIM Consensus for Standard Therapy of Glomerular Disease in Dogs suggests that the decision to implement treatment for hypertension in dogs with glomerular proteinuria be made based on all the clinical information available and several, repeat blood pressure measurements with the goal of treatment being to reduce the risk of future TOD as much as possible (Brown et al., 2013).

1.5.3 Specific therapy

As discussed above, in cases when a definitive diagnosis has been achieved then specific therapy may be implemented. Some specific therapies are now introduced.

1.5.3.1 Immunosuppressive therapy

One of the key purposes of performing a renal biopsy is to assist in determining if there is an immune-mediated component to the glomerular disease. When renal biopsies are collected it is strongly advised that they are submitted for comprehensive analysis including

electron and immunofluorescent microscopy, this is to try to avoid misclassification as discussed previously. The strongest evidence for immune-mediated glomerular disease on histopathology is reported to be achieved via EM (demonstrating electron-dense immune deposits located in the subendothelium, subepithelium, intramembranous or mesangium) or on immunofluorescent microscopy (with detection of immunoglobulins, light chains and/or complement) (Segev et al., 2013a). Currently, there is limited literature regarding the best immunosuppressive protocol in dogs determined to have active immune mediated disease. There is just one study in the literature assessing the efficacy of immunosuppression with cyclosporine for the treatment of glomerulonephritis which concluded that cyclosporin did not significantly reduce proteinuria nor did its use impact on outcome (Vaden et al., 1995).

In the absence of extensive literature, guidelines have been proposed by Segev *et al.* (2013a) in the 'Consensus Recommendations for Immunosuppressive Treatment of Dogs with Glomerular Disease Based on Established Pathology' and it is advised that the immunosuppressive protocol be determined on the expected speed of progression of disease. The guidelines suggest that dogs are split into those with peracute or rapidly progressive disease versus those with stable or slowly progressive disease; the former being treated with mycophenolate or cyclophosphamide +/- prednisolone whilst it is suggested that the latter should be treated with either mycophenolate, chlorambucil, cyclophosphamide or cyclosporine (Segev et al., 2013a). Before any immunosuppressive therapy is started it is strongly advised to ensure there is no infectious trigger for glomerular pathology via polymerase chain reaction and serology-based testing as appropriate.

It is appreciated that renal biopsies are not possible in all situations. In cases whereby a renal biopsy is not an option and the dog has failed to significantly respond to standard therapy, a trial of immunosuppressive therapy could be considered (assuming there are no contraindications). Trial immunosuppressive therapy is especially recommended in dogs with a serum creatinine of >265µmol/L or where the azotaemia is progressive or in dogs with severe hypoalbuminaemia i.e. <20g/L (Pressler et al., 2013).

1.5.3.2 Treatment for serology positive glomerular disease

Numerous infectious diseases can lead to glomerulonephritis but perhaps the most frequently associated are *Borrelia burgdorferi* and Leishmania. The presence of evidence of an active infection in combination with proteinuria often indicates a causal relationship but this cannot be taken as absolute, and if possible renal biopsies demonstrating antigen-specific antigens

associated with active immune deposits in the glomerulus are required to support the link (Goldstein et al., 2013). The link between *Borrelia* and nephritis is somewhat tenuous as it is reported that proteinuria may be present in as little as 2% of dogs seropositive for *Borrelia* (Goldstein et al., 2007). Despite lack of causality often being found it is still recommended that treatment for any detected infectious agent is instituted along with standard therapy due to the possibility that the infectious agent could be contributing to glomerular injury (Goldstein et al., 2013). In these cases of glomerular disease in which an infectious trigger is suspected, it is also advised that standard therapy is introduced when the UPC is >0.5 (Goldstein et al., 2013).

1.6 Introduction to urine protein electrophoresis and renal biomarkers

As discussed, renal biopsy is currently required to fully characterise the origin and pathophysiology underlying glomerular proteinuria. However, this is usually perceived as an invasive procedure which is not risk free and therefore, is rarely performed in clinical practice. Recent work has tried to advance fields that may be of assistance in determining the location of renal damage (i.e. tubular versus glomerular) and therefore provide further information in the absence of a histopathological diagnosis. Urine protein electrophoresis (UPE) has previously been utilised to split proteins based on their molecular weight and charge which can then assist in determining their site of leakage and thus can be of value in deciding if the proteinuria is tubular or glomerular. Additionally, there are several urinary biomarkers that have been discovered as markers of tubular damage.

The following will briefly summarise the pre-existing literature on the use of UPE and renal biomarkers (urinary and blood).

1.6.1 Introduction to urine protein electrophoresis

UPE has been utilised in several previous studies to profile the proteins present in canine urine. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) can be utilised to separate urinary proteins based on their molecular weight. When proteins are exposed to an excess of an anionic detergent sodium dodecyl sulphate (SDS) they form complexes. The formation of such complexes leads to the disruption of the normal conformation of the proteins causing them all to assume the same conformation and the same negative charge per mass unit. The process of electrophoresing these proteins then leads to

them being separated out based on their molecular weight. Knowing the molecular weight of the proteins present in the urine can be useful in attempting to localise the source of disease. Earlier work has demonstrated that high molecular weight (HMW) proteins are usually taken to be those >69kDa and are generally associated with glomerular damage, examples of such proteins include albumin and transferrin (Schultze and Jensen, 1998, Suzuki et al., 1985) whilst low molecular weight (LMW) proteins are those <69 kDa and are normally attributable to tubular damage, an example of such a protein would be alpha 1 microprotein (Harrison and Northam, 1966, Boesken, 1975, Bienenstock and Poortmans, 1970, Bazzi et al., 1997). The significance of the presence of specific proteins in veterinary medicine is currently not a topic that has widely been explored. It is unknown, for example, whether the presence of any of these proteins has prognostic implications.

The urine of healthy dogs is expected to contain low levels of protein (as discussed earlier a UPC of <0.2 is considered normal) and it is reported that albumin makes up 40-60% of this low level protein present in normal canine urine (Barsanti and Finco, 1979). Indeed in healthy dogs, the most consistently reported band is one located at approximately the 66kDa mark which is thought to correspond with albumin (Yalcin and Cetin, 2004, Hokamp et al., 2018, Zaragoza et al., 2004, Zaragoza et al., 2003b, Schaefer et al., 2011). Other protein bands have also been intermittently reported in the urine of healthy dogs such as the presence of Tamm-Horsfall protein (approximately 100kDa) (Hokamp et al., 2018) and, in male entire dogs, bands at approximately 26kDa, 15kDa and 8kDa have been noted which are thought to represent proteins present in prostatic fluid (Hokamp et al., 2018, Lavoue et al., 2015, Schellenberg et al., 2008).

In veterinary medicine, specific UPE patterns have not been thoroughly researched, for example, to determine if they correlate with a given disease process. In general, UPE has been utilised to simply to determine the presence of glomerular and/or tubular proteins which can then assist in the general localisation of renal lesions. It is reported that the use of UPE leads to reasonable accuracy for the determination of whether glomerular or tubular lesions are present. For example, results from seven out of eight electrophoretograms agreed with histological reports in a group of dogs with proteinuric disease suggesting adequate accuracy (Schultze and Jensen, 1998). Further work has evaluated the sensitivity and specificity of UPE patterns to identify glomerular and tubulointerstitial damage; the sensitivity is reported to be good with rates of 97-100% for glomerular damage and 90-92.6% for tubulointerstitial damage; however, reported specificity is more variable with rates of 40-100% for glomerular damage and 62.5-100% for tubulointerstitial disease (Zini et al., 2004, Hokamp et al., 2018). As previously discussed, historically dogs with a UPC of >2.0 were presumed to have

glomerular proteinuria and hence it would have been expected that only glomerular proteins be detected on UPE analysis. However, recent studies utilising UPE have assessed the protein profile of dogs with CKD and a UPC of >2.0 and report a mixture of tubular and glomerular proteins to be present (Hokamp et al., 2018, Chacar et al., 2017, Ferlizza et al., 2020). Studies assessing a more general population of dogs (i.e. not only those with proteinuric CKD) with a UPC >2.0 are deficient and therefore, a wider sense of what proteins are present in dogs with high magnitude of proteinuria is lacking.

UPE has also been utilised to research conditions other than primary renal disease. For example, several previous studies have been performed to help to determine whether tubular or glomerular proteins are present in association with specific infectious diseases including leptospirosis and leishmania (Zaragoza et al., 2003b, Zaragoza et al., 2003a). Other non-infectious disease processes have also been investigated to determine the types of proteins present including hyperadrenocorticism and pyometra (Zaragoza et al., 2004, Caragelasco et al., 2017).

In human medicine several studies exist attempting to profile the proteins associated with specific renal disease, however, these studies tend to involve full proteomic analysis. For example, Haubitz *et al.* (2005) assessed for protein/polypeptide patterns utilising a method enabling rapid identification/analysis polypeptides (capillary electrophoresis on-line coupled with mass spectrometer) in patients with IgA nephropathy and found that the polypeptide pattern identified was significantly different from that identified from healthy controls and patients with membranous nephropathy. Similar work has also been performed regarding diabetic nephropathy and again a distinct protein profile has been recognised in patients with diabetic nephropathy compared to healthy controls (Rao et al., 2007). Such detailed analysis of the urinary protein profile in dogs is not yet available.

In summary, UPE represents an important tool in the assessment of proteinuria and an area for further research and understanding. As above, research assessing a larger group of dogs with a UPC of >2.0 and therefore suspected glomerular disease is sparse. It is also unknown whether dogs with certain conditions triggering their proteinuria, for example, endocrine disorders or neoplasia may have a different profile compared to those without a trigger. Furthermore, knowledge regarding the significance of the presence of specific proteins is also lacking. Hence, there are several areas where UPE could be further exploited to gain more information regarding proteinuria in the absence of renal biopsies.

1.6.2 Introduction to renal biomarkers: urinary

This discussion will focus on the biomarkers that have been proposed as possible markers of renal tubular damage. There are several markers of potential renal tubular damage that can be considered. As above, UPE can be used to assess for tubular damage; one of the proteins that can be detected on UPE analysis is retinol binding protein (RBP) which can also be quantified using an enzyme-linked immunosorbent assay (ELISA). Other markers of tubular damage that may not be detected on UPE include enzymes such as N-acetyl-β-D-glycosaminidase (NAG) and gamma-glutamyl transpeptidase (GGT). Finally, several studies have been performed assessing the utility of neutrophil gelatinase associated lipocalin (NGAL) as a marker of tubular damage; NGAL is neither an enzyme nor detected on UPE analysis. The use of RBP, NAG, GGT and NGAL is now briefly discussed. Other possible biomarkers such as cystatin C and clusterin are also introduced.

1.6.2.1 Retinol binding protein (RBP)

RBP is primarily synthesised by the liver; in the kidney RBP passes freely into the glomerular filtrate and is later reabsorbed by the tubule cells in the healthy kidney. RBP has been studied in both cases of acute kidney injury (AKI) and CKD in the dog. In dogs with AKI due to pyometra, infectious disease or envenomation, an increase in RBP has been documented which is thought to be associated with tubular damage, this presumption is supported by results from the study of dogs with pyometra in which dogs with severe tubulointerstitial lesions on histopathology had increased urinary RBP levels compared to those with milder lesions (Hrovat et al., 2013, Maddens et al., 2011, Kules et al., 2018). It is suggested that RBP could be used as an early marker for the detection and diagnosis of CKD with RBP being increased in cases of CKD; correlation with creatinine, GFR and histopathology has also been shown in several studies (Nabity et al., 2012, Smets et al., 2010). However, in another study, RBP was not found to be a sensitive enough marker to detect decreasing GFR in dogs with naturally occurring renal disease that had a normal creatinine (Raila et al., 2010). Furthermore, it has been proposed that the increase in RBP in dogs with CKD could be more associated with the presence of proteinuria rather than decreased renal function (Raila et al., 2010).

1.6.2.2 N-acetyl-β-D-glucosaminidase (NAG)

NAG is a lysosomal enzyme that is detected in abundant quantities in the cells of the proximal renal tubule (Bourbouze et al., 1984). In humans, NAG is used to monitor for the

nephrotoxic effects of aminoglycoside antibiotics and heavy metals as well as assisting in the diagnosis of diabetic nephropathy (Kovarikova, 2015). In dogs, NAG has been shown to become significantly elevated in dogs with X-linked hereditary nephropathy; with the elevation in NAG occurring before creatinine or UPC (Nabity et al., 2012). However, NAG's performance was found to be inferior to RBP for the detection of disease progression and was found to be unhelpful in monitoring later stages of CKD (Nabity et al., 2012). The utility of NAG is also limited as substantial overlap has been demonstrated between levels of NAG in healthy controls and in dogs with CKD (Smets et al., 2010). Additionally, poor correlation between NAG and IRIS staging of dogs with proteinuric CKD has been reported (Hokamp et al., 2016). NAG has also been explored as a possible marker for AKI secondary to pyometra and Leishmania and was found to increase significantly in both scenarios (Palacio et al., 1997, Maddens et al., 2010). Endocrine diseases such as hyperadrenocorticism and uncontrolled diabetes mellitus have also been associated with an increased urinary NAG and this finding is presumed to be due to concurrent renal injury (Smets et al., 2012a, Sato et al., 2002, Lapointe et al., 2008).

1.6.2.3 Gamma-glutamyl transpeptidase (GGT)

GGT is another urinary enzyme located in the brush borders of the proximal tubules. As with NAG, no circadian variations have been observed in the levels of GGT measured in canine urine samples although, unlike with NAG, variation with pH is reported with GGT with values being higher in alkaline urine (Uechi et al., 1998, Brunker et al., 2009). Previous research on urine GGT has predominantly assessed its utility for detection of AKI in dogs. GGT has been shown to increase in dogs with gentamicin induced renal injury; the increase in GGT can occur before an increase in creatinine is seen (Greco et al., 1985, Rivers et al., 1996, Grauer et al., 1995). GGT has also been found to increase in association with renal impairment associated with pyometra, Leishmania and envenomation following Adder bite (Deschepper et al., 1989, Palacio et al., 1997, Palviainen et al., 2013). Recently, GGT measurement via a spectrophotometric method was validated and this study also proposed a reference interval for the urine GGT-to-creatinine ratio in healthy dogs (Ilchyshyn et al., 2019).

1.6.2.4 Neutrophil gelatinase associated lipocalin (NGAL)

NGAL is found in granules within neutrophils, however, it is also expressed in other tissue such as the uterus, stomach, lung, liver and kidneys (Cowland and Borregaard, 1997).

Neoplastic epithelial cells and inflammatory processes have been associated with upregulation of NGAL in humans and NGAL is monitored following cardiac surgery as it is utilised as a predictor for development of AKI (Mishra et al., 2005, Friedl et al., 1999, Nielsen et al., 1996, Wagener et al., 2006).

NGAL has been widely studied as a possible tubular biomarker in dogs and has been documented to increase very early after renal insult in some studies, even possibly before creatinine (Lee et al., 2012, Segev et al., 2013b, Kai et al., 2013). Although a lot of the work regarding NGAL (and other renal biomarkers) has been experimental, NGAL has also been found to be increased in dogs with either naturally occurring AKI or CKD; it has been suggested that NGAL may be able to help differentiate between AKI and CKD and also predict risk of progression in dogs with CKD (Steinbach et al., 2014, Kim et al., 2019, Monari et al., 2020, Scheemaeker et al., 2020). The urine NGAL-creatinine ratio has been shown to have a higher sensitivity and specificity when compared to creatinine for the detection of IRIS Stage I CKD (Ko et al., 2021). However, the presence of pyuria or urinary tract infections has been shown to impact NGAL levels and therefore whether the sediment is active is an important consideration if measuring NGAL (Nabity et al., 2012, Daure et al., 2013).

1.6.2.5 Other renal biomarkers

As mentioned above, RBP, NAG, GGT and NGAL are by no means an exhaustive list of the renal biomarkers being investigated currently. Additional biomarkers include clusterin and cystatin C amongst others

Clusterin is a glycoprotein that is produced in the renal tubules and has been previously approved by the US Food and Drug Administration and European Medicines Agency as a biomarker in rats for the monitoring of proximal tubular injury due to drug administration (Dieterle et al., 2010). In dogs, it has also been shown that urine clusterin increases following administration of nephrotoxic drugs and that urine clusterin may be more sensitive than serum creatinine for the detection of drug-induced renal proximal tubular toxicity (Adedeji et al, 2023, Luo et al, 2014, Zhou et al, 2014). Clusterin has also been shown to increase secondary to endotoxaemia or Leishmania induced renal injury in dogs (Garcia-Martinez, 2012, Steblaj et al, 2023).

Cystatin C is a low molecular weight protein which is present in all nucleated cells and is excreted solely by the kidneys, therefore it is proposed to provide an indirect indicator of GFR. Cystatin C levels have been demonstrated to be increased in dogs with CKD and it has been suggested that cystatin C may have better sensitivity and specificity compared with

serum creatinine for the early detection of canine CKD (Almy et al, 2002, Kim et al, 2020, Miyagawa et al, 2009). Cystatin C has also been shown to detect AKI earlier than serum creatinine in critically ill dogs and those with gentamycin induced AKI (Paes-Leme, et al, 2021, Sasaki et al, 2014).

It is clear from the above literature, that most of the previous studies regarding the use of urinary biomarkers have focused on their use in AKIs or for detection of early CKD. What is unknown is the proportion of dogs with presumed glomerular disease that may also have increased markers of tubular damage. Additionally, it is unknown if they are present, whether any of these urinary biomarkers can assist in determining severity or prediction of prognosis of dogs with glomerular disease.

1.6.3 Renal biomarkers – blood

1.6.3.1 Symmetric dimethylarginine (SDMA)

SDMA is a methylated arginine that is derived following intranuclear methylation of L-arginine and is released into the circulation following proteolysis (Kakimoto and Akazawa, 1970). SDMA has been suggested as a possible endogenous marker of GFR due to the fact that it is freely filtered by the kidneys; it has been estimated that the renal excretion of SDMA is at least 90% and it is reported to be minimally influenced by non-renal factors (Schwedhelm and Boger, 2011, Nabity et al., 2015). For example, it has recently been proven that unlike creatinine, lean body mass does not affect SDMA concentrations (Hall et al., 2015). In humans, a large meta-analysis documented that SDMA strongly correlated with inulin clearance and serum creatinine (Kielstein et al., 2006).

It has been proposed that SDMA can be used for the earlier detection of CKD when compared to creatinine. A reduction in <30% of renal function is reported to lead to an increase in SDMA whereas it is thought that approximately 75% of the nephron mass needs to be lost before a rise in creatinine can be seen (Nabity et al., 2015, Braun et al., 2003). In dogs a study has shown that in 19 dogs with CKD, SDMA levels exceeded the reference range before serum creatinine in 17 dogs; this elevation in SDMA occurred on average 9.8 months earlier highlighting SDMA's potential worth as a biomarker for earlier detection of renal disease (Hall et al., 2016). However, not all studies prove SDMA to be as superior, for example, when the trend of SDMA and creatinine was assessed in individual dogs with CKD due to X-linked hereditary nephropathy, on average, SDMA only increased 2 weeks earlier than creatinine (Nabity et al., 2015). Additionally, in a study assessing the use of SDMA and creatinine as markers of reduced GFR in clinically stable dogs, SDMA's diagnostic performance was similar to creatinine (Pelander et al, 2019). Regardless of some literature

questioning the benefit of SDMA, SDMA levels have recently been included in the IRIS CKD staging guidelines. Consistent elevations in SDMA above 14µg/dL are suggestive of decreased renal function and therefore, patients with SDMA of <18µg/dL are included in Stage I even if their creatinine levels are lower than the cut-off of <125µmol/l for this stage (IRIS, 2019). Puppies have been shown to have a slightly higher reference range compared to adult dogs with values of up to 16µg/dL being normal (IDEXX, 2015). As with creatinine, healthy Greyhounds are also reported to have a higher level of normal SDMA compared to other breeds (Liffman et al., 2018).

SDMA has been found to be stable when stored at 20 and 4ºC and was also able to withstand being exposed to at least 3 freeze-thaw cycles (Nabity et al., 2015). The same study validated the measurement of SDMA via liquid chromatography-mass spectrometry (LC-MS) and documented a mean intra-assay precision coefficient of variability of 2.2% with a mean inter-assay precision of 2.7%; both giving an accuracy of 98% or higher (Nabity et al., 2015). However, although LC-MS is considered the gold standard, it can be time consuming and expensive. IDEXX have developed an alternative homogenous immunoassay which utilises glucose-6-phosphate dehydrogenase conjugate with an anti-SDMA monoclonal anti-body to determine levels of SDMA within serum or plasma. The accuracy of the IDEXX test has been compared with liquid chromatography mass spectrometry and found to be a suitable alternative for clinical use (Ernst et al., 2018). SDMA has not been widely studied in groups of dogs with specific renal disease and hence data regarding the use of SDMA in dogs with glomerular proteinuria is lacking.

1.7 Aims and hypotheses

The aims of this master's project were to broaden our understanding of canine glomerular proteinuria and its treatment. There are several statements within this field that are commonly applied in clinical practice, firstly, that a UPC of >2.0 is associated with glomerular disease and, secondly, therapeutic success regarding treatment for glomerular proteinuria is defined by a ≥50% reduction from baseline UPC or achieving a UPC of <0.5. This master's projects aimed to explore these statements in more depth.

Initially, a retrospective study was designed to investigate the proportion of dogs achieving previously outlined therapeutic targets when treated with ACEi therapy for presumed glomerular proteinuria. This study also aimed to assess whether a significant survival benefit was conveyed in the dogs that achieved target. As additional aims, the impact of baseline azotaemia, hypoalbuminaemia, hypercholesterolaemia and magnitude of UPC on survival were also analysed as these were identified as potential confounding factors. Considering several previous canine studies have suggested an improved outcome following treatment with ACEi (Grauer et al., 2000, Grodecki et al., 1997); it was hypothesed that response to ACEi therapy would be associated with improved 12-month survival. Considering baseline azotaemia, hypoalbuminaemia, hypercholesterolaemia and increased magnitude of UPC are thought to suggest more advanced/severe disease it was hypothesised that their presence would be associated with a worse prognosis.

Secondly, a prospective study was designed to further explore and characterise the UPE patterns of dogs with a UPC of >2.0. This prospective study had several elements. Firstly, we aimed to document the UPE patterns of a varied population of dogs with a UPC of >2.0 and determine how frequently tubular proteins were present in this cohort of dogs. We also set out to determine if the presence of tubular proteins on UPE were associated with more advanced or severe disease. To do this we assessed whether the presence of tubular proteins were associated with markers of disease progression (creatinine and SDMA) or markers of disease severity (albumin, cholesterol and UPC). As discussed earlier, pre-existing literature suggests that the presence of persistent high magnitude proteinuria can lead to tubular damage (Nangaku et al., 2002, Ruggenenti et al., 2003b, Liu et al., 2015). Sustained tubular damage will lead to eventual loss of the tubule and a decrease in GFR leading to an increase in creatinine and SDMA. Therefore, it was hypothesised that although no dog with a UPC >2.0 would have only tubular proteins present, some dogs would have evidence of tubular damage (as documented by the presence of tubular proteins on UPE). It was also hypothesised that dogs with such tubular proteins present would have more advanced and severe disease.

To further analyse the UPE results we also wanted to determine if any proteins had specific relevance, for example, if the presence of specific tubular proteins were associated with markers of disease progression or severity. As discused in the introduction, previous studies report conflicting evidence regarding RBP and its association with GFR whilst alpha-1 microprotein is reported to be associated with decreased GFR in people (Grubb, 1992, Smets et al., 2010, Nabity et al., 2012, Raila et al., 2010). Considering the above hypothesis that the presence of tubular proteins are associated with more progressed/severe disease, we hypothesised that the presence of specific individual tubular proteins would also be associated with markers of disease progression and/or severity.

We also set out to determine if dogs without an identifiable trigger had characteristic UPE patterns (dogs with either endocrine disease, neoplasia, an identifiable inflamamtory condition or hyperlipidaemia were considered to have a trigger for their proteinuria). It was hypothesised that dogs without an identifiable trigger would have characteristic UPE patterns which would enable us to diffferentiate clearly between the two populations of dogs (i.e. those with and without a trigger for their proteinuria).

To further assess for evidence of tubular involvement in dogs with a UPC >2.0 we planned to measure urinary biomarkers of tubular damange (GGT and NGAL). Considering we hypothesed that some dogs with a UPC of >2.0 will have tubular proteins present we also expected some dogs to demonstrate evidence of tubular damage via increased activity of GGT and NGAL.

Finally, we wanted to assess factors impacting on survival. Firstly, we aimed to determine if dogs with tubular proteins present had a worse survival compared to those without, with the hypothesis that dogs with the presence of tubular damange will have shorter survival times than those without (based on the theory that dogs with tubular damange have more progressed disease). As well as looking at the overall protein profile, we also aimed to assess whether the presence of specific proteins could provide prognostic information. In human medicine, increased RBP hs been shown to be an indictor of poor prognosis in patients with glomerulopathies (Kirsztajn et al., 2002), whilst alpha-1 microprotein has also been linked with faster progression and higher mortality in people with CKD (Robles et al., 2021), therefore, we hypothesised that the presence of these tubular proteins would be associated with a worse outcome. We also aimed to look at whether the presence of markers of disease progression (creatinine/SDMA) or severity (albumin, cholesterol and UPC) impacted survival; this final aim was performed to tie in with the secondary aims of the retrospecitve study and to determine if similar findings were noted in a population of dogs assessed

prospectively. It was again hypothesised that the presence of azotaemia, hypoalbuminaemia and increased magnitude of UPC would be associated with a negative prognosis.

1.8 Ethical Considerations

Both the retrospective and prospective studies were approved by the University of Glasgow School of Veterinary Medicine Research ethics committee (application ref EA22/21 and EA23/21). For the prospective study, only residual samples left over from clinical testing were utilised.

CHAPTER TWO

RETROSPECTIVE STUDY: RESPONSE OF DOGS TREATED WITH ANGIOTENSIN CONVERTING ENZYME INHIBITORS FOR PRESUMED GLOMERULAR PROTEINURIA AND EFFECT OF A POSITIVE RESPONSE ON SURVIVAL

2.1 Brief background to the study

Persistent proteinuria can lead to a range of complications including development of hypoalbuminaemia, resulting in decreased oncotic pressure and weight loss/muscle wastage (Center et al., 1987, DiBartola et al., 1989) as well as nephrotic syndrome (Klosterman et al., 2011), development of a hypercoagulable state, thromboembolic complications and a worse prognosis (Rasedee et al., 1986, Cook and Cowgill, 1996, Ritt et al., 1997, Klosterman et al., 2011). Therefore, treatment is recommended to try to lower the magnitude of proteinuria.

As already discussed, the ACVIM Consensus recommendations for standard therapy of glomerular disease in dogs states that treatment of proteinuria should include interference with the RAAS as a standard of care (Brown et al., 2013); this is usually achieved via use of an ACEi.

Data is currently limited on how frequently dogs treated with ACEi medications reach the targets outlined by the ACVIM Consensus Statement and if achieving such targets conveys significant survival benefit.

2.2 Aims

i. Determine the proportion of dogs with presumed glomerular proteinuria that respond to treatment with an ACEi within 3-months and to determine whether a positive response to treatment is associated with improved survival.

   Hypothesis: Response to therapy would be associated with improved 12-month survival.

ii. Evaluate whether the presence of baseline clinicopathological abnormalities impacts survival.

   Hypothesis: Dogs with abnormal baseline clinicopathological values (azotaemia, hypoalbuminaemia, hypercholesterolaemia and severe proteinuria) would have a worse prognosis.

The following work was submitted to the Journal of Veterinary Internal Medicine in November 2022 and has been accepted pending revisions:

'Response of dogs treated with angiotensin converting enzyme inhibitors for presumed glomerular proteinuria and effect of positive response on survival.' Fulton, E.A., McBrearty, A.R., Shaw, D.S., Ridyard. A.E.

2.3 Abstract

Background

Angiotensin-converting enzyme inhibitors (ACEi) are a recommended treatment for glomerular proteinuria. Frequency of response to ACEi and the association of achieving proposed urine protein-to-creatinine ratio (UPC) targets on survival is unknown.

Objectives: *To determine response rates to ACEi therapy and whether a positive response is associated with improved survival.*

Animals: *85 dogs with presumed glomerular proteinuria and UPC>2.0*

Methods: *Retrospective study including dogs with UPC>2.0 prescribed an ACEi for treatment of presumed glomerular proteinuria. Baseline creatinine, albumin, cholesterol, UPC and systolic blood pressure were recorded, and cases reviewed to track UPC. Treatment response was defined as achieving a UPC of <0.5 or reduction of ≥50% from baseline within 3-months. Outcome data was collected to determine overall and 12-month survival.*

Results

Thirty-five (41.2%) dogs responded to ACEi treatment. Treatment response was statistically associated with both median survival time (664 days for responders compared to 117 for non-responders) and 12-month survival (78.6% responders alive compared to 27.5% non-responders). Baseline azotaemia or hypoalbuminaemia were also associated with a worse prognosis, with odds ratios of death at 12-months of 5.34 [Confidence interval (CI):1.85-17.32] and 4.51[CI:1.66-13.14], respectively. In the 25 dogs with normal baseline creatinine and albumin, response to treatment was still statistically associated with 12-month survival (91.7% responders alive compared to 53.9% non-responders).

Conclusions and clinical importance

Achieving recommended UPC targets for the treatment of presumed glomerular proteinuria within 3-months appears to be associated with a significant survival benefit. Response to treatment remained significant even when dogs with azotaemia and hypoalbuminaemia were excluded.

2.4 Introduction

Proteinuria is one of the hallmarks of glomerular disease and when the urine protein-creatinine ratio (UPC) is >2.0 in the absence of pre- and post-renal causes, glomerular pathology is often present (Lees et al., 2005). Glomerular proteinuria may be due to familial or acquired glomerular disease, the latter occurring as a result of damage sustained by immune-mediated processes or systemic factors that impact on the glomerulus; immune-complex glomerulonephritis is thought to account for up to 50% of dogs with acquired glomerular disease (Schneider et al., 2013). While renal biopsy is required for a definitive pathological diagnosis thereby informing treatment choices, it is infrequently performed, and when the UPC is >2.0, a presumptive diagnosis of glomerular proteinuria is usually reached by non-invasive exclusion of pre- or post-renal causes of proteinuria, although rare cases with primary tubulointerstitial disease without glomerular pathology have been shown to have UPCs of this magnitude (Littman et al., 2013, Schneider et al., 2013, Hokamp et al., 2016).

Glomerulopathies are a major cause of chronic kidney disease (CKD) and eventual renal failure in the dog (Macdougall et al., 1986), with proteinuria per se likely contributing to progressive renal pathology (Jerums et al., 1997, Tang et al., 1999, Ruggenenti and Remuzzi, 2000). Persistent proteinuria can also lead to the development of hypoalbuminaemia, resulting in decreased oncotic pressure and weight loss/muscle wastage (DiBartola et al., 1989, Center et al., 1987). Dogs may also develop a hypercoagulable state with the potential for thromboembolic complications and/or may develop nephrotic syndrome both of which are associated with a worse prognosis (Cook and Cowgill, 1996, Klosterman et al., 2011, Rasedee et al., 1986, Ritt et al., 1997). Even in the absence of a histopathological diagnosis, management of cases with presumed glomerular proteinuria is aimed at reduction of proteinuria and management of clinically relevant complications (Pressler et al., 2013, Brown et al., 2013).

The most recent ACVIM Consensus recommendations state modification of the renin-angiotensin-aldosterone system (RAAS) should be standard of care for dogs with glomerular proteinuria and this is often achieved via use of an angiotensin converting enzyme inhibitor (ACEi) (Brown et al., 2013). Benazepril and enalapril are commonly used ACEi; a starting dose of 0.5mg/kg orally once daily is recommended, and providing no adverse effects are seen, incremental dose increases are then recommended every 2-4 weeks based on response to therapy (Brown et al., 2013). The proposed target of ACEi therapy is either a reduction in UPC to <0.5 or a >50% reduction from baseline (Brown et al., 2013). However, despite these recommendations, there is currently limited data on how frequently dogs treated with ACEi

meet such treatment targets and whether achieving these targets impacts on disease progression and overall survival.

The aims of this retrospective study were to determine the proportion of dogs with presumed glomerular proteinuria and UPC>2.0 that achieve the proposed target reduction in UPC within 3-months of starting an ACEi and to determine whether a positive response to treatment was associated with improved survival. To investigate for possible confounding factors, secondary aims were to evaluate whether clinicopathological markers of disease severity in the 30-days prior to treatment commencement had an association with either response to treatment or survival.

It was hypothesised that response to ACEi therapy within 3-months would be associated with improved 12-month survival.

2.5 Materials and Methods

The clinical records of dogs referred to a University Teaching Hospital were searched to identify those with a UPC>2.0 prescribed an ACEi for treatment of presumed glomerular proteinuria between January 2006 and April 2021. Dogs prescribed an ACEi for other conditions, were excluded. Dogs were also excluded if they were receiving concurrent therapy known to affect renal protein loss (tyrosine kinase inhibitors, corticosteroids, or angiotensin receptor blockers [ARBs]) at the time of baseline urinalysis or did not have a baseline urinalysis and biochemistry performed within the 30-days prior to starting ACEi therapy. Urine samples submitted for urinalysis to the laboratory used always have UPC measured. Dogs with incomplete records were not included. Additionally, those without at least one follow-up urinalysis (including UPC measurement) at the university laboratory within 3-months of starting ACEi therapy were also excluded. Screening for infectious diseases was not required for inclusion as the prevalence of infectious diseases that can contribute to proteinuria e.g., leishmania, is low in the UK. Results of any infectious disease screening performed were noted. Cases were not excluded due to the presence of active sediment on urinalysis or the presence of co-morbidities.

Medical records were reviewed and signalment and baseline variables recorded. Baseline variables were defined as the creatinine, albumin, cholesterol, UPC and systolic blood pressure (SBP) values obtained within 30-days prior to ACEi commencement. If several values were available within this time frame, the ones closest to the start of ACEi treatment were used. The choice and starting dose of ACEi (mg/kg/day), concurrent comorbidities and

diagnoses were recorded, as were medications, dietary management and omega-3 fatty acid supplementation already being administered or started at the time of starting ACEi therapy. For clinical threshold analysis, azotaemia was defined as a creatinine >1.4mg/dL (>125µmol/L); hypoalbuminaemia as an albumin <2.5g/dL (<25g/L) and hypercholesterolaemia as a cholesterol >348mg/dL (>9mmol/l)(Klosterman et al., 2011, IRIS, 2019). Severe proteinuria was defined as a UPC ratio >3.5 (Littman et al., 2013). Using the ACVIM Consensus Guidelines for Systemic Hypertension, normotensive or pre-hypertensive dogs were classed as 'non-hypertensive' (SBP<159mmHg) while those with hypertension or severe hypertension were classed as 'hypertensive' (SBP≥160mmHg) (Acierno et al., 2018). The following data was collected from subsequent re-checks: date, UPC, new dose of ACEi if changed and whether ARBs or corticosteroids were started. Dogs were categorized as responders or non-responders. Treatment response was defined as a reduction of UPC to ≥50% from baseline or to <0.5(Brown et al., 2013) at any follow-up visit within 3-months of starting ACEi. If dogs did not achieve these targets by 3-months, they were classed as non-responders. Dogs were also classed as non-responders if they were started on an ARB or corticosteroid within 3-months and prior to the UPC targets being achieved or if ACEi therapy was withdrawn due to progressive azotaemia or hyperkalaemia at any time during the first 3-months prior to the UPC targets being achieved.

To perform survival analyses, the date of death/euthanasia, or the point at which the patient was lost to follow-up (last known to be alive) was recorded. If available, the reason for death or euthanasia was also recorded. Dogs were then additionally classified based on their status 12-months after initiation of ACEi as either alive, dead, or lost to follow-up.

Statistical analysis

Normality testing (using the Anderson-Darling method) indicated that for 3 of the 4 continuous variables (creatinine, cholesterol, UPC) non-parametric statistical analyses was required, therefore, for descriptive statistics the median and range were reported. Pearson Chi-squared analysis was employed to determine the association between baseline clinical threshold variables (baseline azotaemia, hypoalbuminaemia, hypercholesterolaemia, severe proteinuria, and hypertension) and treatment response.

Overall survival data (using all-cause mortality) was assessed using Kaplan Meier analysis with the Log-Rank (Mantel-Cox) test used to assess for a difference in median survival time (MST) between responder and non-responder dogs. The association between the presence of

baseline azotaemia, hypoalbuminaemia, hypercholesterolaemia, severe proteinuria, hypertension and overall survival were assessed similarly.

Pearson Chi-squared analysis was used to assess for a relationship between response to treatment and survival at 12-months. The associations between baseline creatinine, albumin, cholesterol and UPC and 12-month survival were assessed using clinical thresholds and on a continuous basis whilst SBP was assessed on a clinical threshold basis only. For the clinical threshold analysis, Pearson Chi-squared tests were again employed, and Mann-Whitney tests were used to investigate the continuous data.

Univariate logistic regression was carried out to generate odds ratios (OR) and 95% confidence intervals (CI) of survival to 12-months for response to treatment and baseline parameters. Parameters with a P value of <0.2 were eligible for inclusion into the multivariable analysis. Parameters fitting this criterion were entered into a multivariable logistic regression model and terms removed until a minimum model was obtained with only statistically significant ORs remaining.

To further account for possible interplay between baseline parameters and treatment response on 12-month survival, a categorical tree was generated. This analytical technique was chosen due to the unbalanced data set with potentially different combinations of baseline clinical threshold values present in different dogs(Clark, 1997). The categorical tree aimed to assess for the impact of numerous variables (treatment response, baseline azotaemia/hypoalbuminaemia/hypercholesterolaemia or severe proteinuria) on 12-month survival and rank them in order of importance as well as identify combinations of factors leading to the highest risk of death/greatest chance of survival. The categorical tree analysis allowed for binary division of data between groups of categories; the category that led to the biggest difference regarding 12-month survival represented the first division. One subset was then considered, and the model then assessed which category led to the biggest difference in 12-month survival in that sub-population of dogs. The different 'branches' of the tree were independent of each other in terms of what binary partitions were presented. This binary partitioning was continued for smaller and smaller subsets of data until no differentiation in terms of prevalence was possible.

Statistical analysis was performed using commercially available software SPSS (IBM SPSS Statistics 27) and R (v4.2.1© 2021, The R Foundation for Statistical Computing). Statistical significance was taken as P<0.05.

2.6 Results

Case Selection:

The initial database search identified 1,000 dogs with a UPC>2.0. Of these, 245 were prescribed an ACEi. Thirty-one dogs were not prescribed an ACEi for treatment of presumed glomerular proteinuria; these dogs were excluded. Of the remaining 214 dogs, 24 were excluded as they were receiving concurrent medication known to affect UPC, 12 dogs were excluded as their baseline biochemistry or urinalysis was performed >30-days prior to starting the ACEi. A further 93 were excluded due to incomplete clinical records or lack of follow-up within 3-months. Therefore, 85 dogs were included.

Study Population Characteristics:

Of the 85 dogs, 57.6% were female (n=49/85; neutered n=34), and 42.4% were male (n=36/85; neutered n=15). The median age at the time of starting ACEi therapy was 8.70 years (range 0.65-13.85 years). Weight was available for 73 dogs; the median weight was 14.70kg (range 2.30-50.0kg). There were 11 crossbreeds with the remainder of the population being purebred dogs.

At the time of starting ACEi, protein losing nephropathy (PLN) was listed as the diagnosis for 56.5% (n=48/85) dogs. Thirteen dogs (15.3%) had renal diseases other than PLN listed as their diagnosis, 17.6% (n=15/85) had at least one endocrinopathy whilst 11.8% (n=10/85) had a diagnosis of neoplasia (of which 2 had concurrent endocrine disease). One dog was listed as having a type III hypersensitivity reaction as their diagnosis.

Of the 15 dogs with endocrinopathies, 8 had hyperadrenocorticism of which 5 were receiving trilostane treatment prior to ACEi commencement and two were later started on trilostane. All 6 dogs with diabetes mellitus were receiving insulin therapy. One dog had hypothyroidism and was receiving levothyroxine prior to starting ACEi treatment. Regarding infectious diseases, one dog was positive on serology for *Borrelia burgdorferi*, no other cases had infectious diseases documented. None of the dogs included underwent renal biopsy.

The median baseline creatinine was 1.14mg/dL (range 0.51-6.30; 101µmol/l [range 45-557]); 40.0% of dogs (n=34/85) were azotaemic. The median baseline UPC was 6.61 (range 2.15-30.5), 80.0% of dogs had severe proteinuria (n=68/85). The median baseline albumin was 2.5g/dL (range 0.8-3.7g/dL; 25g/l [range 8-37]); 45.9% (n=39/85) of dogs were hypoalbuminaemic. The median cholesterol was 331.5mg/dL (range 83.8-1397.9; 8.5mmol/l [range 2.2-36.2]); 44.7% (37/84) of dogs were hypercholesterolaemic. Baseline SBP

measurement was available for 46 dogs; 52.2% (n=24) of these were classed as being hypertensive (hypertension n=10, severe hypertension n=14) prior to starting ACEi therapy.

Six dogs were prescribed enalapril whilst the remainder received benazepril. The starting dose of ACEi was available for 73 dogs with a median of 0.5mg/kg/day (range 0.16-1.52mg/kg/day). At the time of starting ACEi therapy 8.2% (n=7/85) of dogs were already receiving a renal diet and a further 29.4% (n=25/85) of dogs were started on a renal prescription diet alongside ACEi. None of the dogs were on omega-3 fatty acid supplementation prior to starting an ACEi, however, these were started in 9.4% (n=8/85) of dogs at the same time as the introduction of ACEi, of these, 3 were concurrently started on a renal diet. Three dogs were already on anti-thrombotic medication at the time of starting ACEi; a further 37.6% (n=32/85) were started on such medications at the same time as starting ACEi. At the time of ACEi commencement, 8.2% (n=7/85) were receiving amlodipine therapy, 3 of which had documented hypertension at baseline.

Response to treatment

Table 2 shows the baseline variables of dogs that were classed as responders and non-responders. Thirty-five dogs (41.2%) responded to ACEi therapy within 3-months with only one dog achieving a UPC<0.5. The median number of rechecks performed within 3-months in this group was 2 (range 1-5). The median time to response was 33days (range 3-82days) and the median dose at the time of response was 0.52mg/kg/day (range 0.19-1.32mg/kg/day). Of the dogs that responded, 10 had a further urine sample available within the 3-month period after the one documenting a treatment response. Of these, 30% (n=3/10) had a subsequent UPC that would not have satisfied criteria for successful response to treatment.

Of the fifty dogs (58.8%) that were classified as non-responders; 78.0% (n=39/50) did not reach either UPC target within the first 3-months, 10.0% (n=5/50) had their ACEi therapy withdrawn due to progressive azotaemia, 8.0% (n=4/50) were started on corticosteroid therapy and 4.0% (n=2/50) were started on an ARB before either UPC target was reached. The median number of rechecks performed within 3-months of starting ACEi therapy was 1 (range 1-6). The median maximum dose for dogs that failed to reach UPC targets was 0.55mg/kg/day (range 0.24-2.38mg/kg/day).

One dog that responded to treatment died within 3-months of starting ACEi whilst eight dogs that were classed as non-responders died within this time frame.

Neither the presence of azotaemia (χ^2_1=0.810, P=0.368), hypoalbuminaemia (χ^2_1=1.83, P=0.176), hypercholesterolaemia (χ^2_1=0.498, P=0.480), severe proteinuria (χ^2_1=0.976,

P=0.323) nor hypertension (χ^2_1=0.056, P=0.813) at baseline were found to be significantly associated with response to treatment.

Variables		**Responders (n=35)**	**Non-Responders (n=50)**
Age (years)		9.10 (0.65-13.85)	8.50 (1.49-10.11)
Sex	Female [neutered, %]	23 (65.7%) [14, 60.9%]	26 (52.0%) [20, 76.9%]
	Male [neutered, %]	12 (34.3%) [6, 50.0%]	24 (48%) [9, 37.5%]
Weight (kg), n=73		11.35 (2.3-38.7)	16.15 (2.9-50.0)
Serum biochemistry	Creatinine (mg/dL) [µmol/l]	1.1 (0.6-4.8) [97 (53-424)]	1.2 (0.5-6.3) [106 (44-557)]
	Urea (mg/dL) [mmol/l]	25.2 (6.7-141.7) [4.2 (1.1-23.6)]	30.3 (7.0-128.0) [5.0 (1.2-21.3)]
	Albumin (g/dL) [g/L]	2.7 (0.5-3.7) [27 (5-37)]	2.35 (0.80-3.50) [24 (8-35)]
	Cholesterol (mg/dL) [mmol/l], n=84	338.8 (134.0-660.9) [8.8 (3.5-17.7)]	313.6 (83.1-1397.9) [8.1 (2.2-36.2)]
UPC		6.20 (2.32-20.30)	6.88 (2.04-30.50)
Severe Proteinuria		26 (74.3%)	42 (84.0%)
Hypertension (n=46)		9 (25.7%)	15 (30.0%)
IRIS CKD stage	I	23 (65.7%)	28 (56.0%)
	II	9 (25.7%)	15 (30.0%)
	III	3 (8.6%)	5 (10.0%)
	IV	0	2 (4.0%)
Co-morbidities	Neoplasia	3 (8.6%)	7 (12.7%)
	Hyperadrenocorticism	3 (8.6%)	5 (9.1%)
	Diabetes mellitus	2 (5.7%)	4 (7.3%)

ACEi used	Benazepril	33 (94.3%)	46 (92.0%)
	Enalapril	2 (5.7%)	4 (8.0%)
Starting dose of ACEi (mg/kg/day), n=73		0.44 (0.16-1.27)	0.50 (0.24-1.52)
Starting dose of ACEi <0.5mg/kg/day		17 (48.6%)	19 (38.0%)
Renal support therapies given (started either before or at the time of ACEi)	Renal Diet	14 (40.0%)	18 (36.0%)
	Omega-3 supplementation	1 (2.9%)	7 (14.0%)
	Aspirin	5 (14.3%)	10 (20%)
	Clopidogrel	6 (17.1%)	14 (28.0%)

Table 2: Baseline variables of 85 dogs with presumed glomerular proteinuria and UPC>2.0 that responded and did not respond to angiotensin converting enzyme inhibitors. Treatment response was defined as achieving a UPC of <0.5 or reduction of ≥50% from baseline within 3-months. Data presented as median (range) or n (% of population) as appropriate. Data was available for entire study population (n=85) unless otherwise indicated. ACEi= angiotensin converting enzyme inhibitor; CKD= chronic kidney disease; UPC= urine protein-creatinine ratio

Outcome

Association between treatment response and survival

There was a statistically significant difference in MST between responder and non-responder dogs (664 [95% CI: 459-869] vs 117 [95% CI: 131-223] days, respectively, P=0.009, Figure 4). Of the 68 dogs with a known outcome at 12-months, 41.2% (n=28/68) were responders. Of these, 78.6% (n=22/28) were alive at 12-months whilst 27.5% (n=11/40) of non-responders were alive at 12-months; this difference was statistically significant (χ^2_1=17.2, P<0.001), with the odds of death greatly reduced (0.1) in responders (Table 3).

Association between clinicopathological variables and hypertension on survival

The MST of dogs that were or were not azotaemic at the time of diagnosis also differed significantly (175[95% CI: 62-288] vs 586[95% CI: 398-773] days, respectively), P<0.001, Figure 5). Whilst dogs that were hypoalbuminaemic at baseline had a shorter MST that those that were not (177[95% CI: 115-239] vs 531[95% CI: 373-689] days, respectively), this was not statistically significant (P=0.064). There was no statistically significant difference in the MST for dogs that had hypercholesterolaemia at baseline compared to those that didn't (375 [95% CI: 28-722] vs 412[95% CI: 145-679]days, respectively, P=0.242) nor those with severe proteinuria compared to those that didn't (300[95% CI: 63-537] vs 531[95% CI: 403-659] days, respectively, P=0.916) or hypertension (hypertensive – 251[95% CI: 0=532] vs normotensive – 454[95% CI: 240=668] days, P=0.372).

When assessed as continuous variables, creatinine, albumin and UPC were found to be statistically significantly associated with 12-month outcome whilst cholesterol was not (Figure 6A-D). Furthermore, logistic regression estimated 2.53 and 1.18 increases in the odds of death with each 1.1mg/dL (100µmol/L) increase in creatinine and each unit increase in UPC, respectively, a reduction in odds (0.40) with each 0.1g/dL (1g/L) increase in albumin and no change in odds associated with increasing cholesterol (1.00, Table 3). Qualitatively similar results were obtained when creatinine, albumin, cholesterol, UPC and SBP were assessed in terms of clinical thresholds. The presence of baseline azotaemia (χ^2_1=9.5, P=0.003, OR=5.34 (Table 3)) and hypoalbuminaemia ($\square^2_1$=8.7, P=0.004, OR=4.51) were both negatively associated with 12-month survival, whereas there was no difference in survival at 12-months in dogs with or without hypercholesterolaemia (n=17/36; $\square^2_1$<0.01, P=0.924, OR=0.95) or those with or without severe proteinuria (χ^2_1=2.756, P=0.106, OR 2.91). Twelve-month survival data was known for 38 dogs with baseline SBP readings available; 60% (n=12/20) of the dogs that were not hypertensive were alive at 12-months

compared to 38.9% (n=7/18) of hypertensive dogs. Hypertension at baseline was not associated with 12-month survival (χ^2_1=1.689, P=0.197, OR 2.36).

To remove any impact of baseline azotaemia and hypoalbuminaemia on outcome, 12-month survival analysis was repeated on the sub-population of 25 dogs without these abnormalities present. Response to treatment remained significantly associated with survival at 12-months in this cohort of dogs (χ^2_1=4.425, p=0.035, OR 0.01).

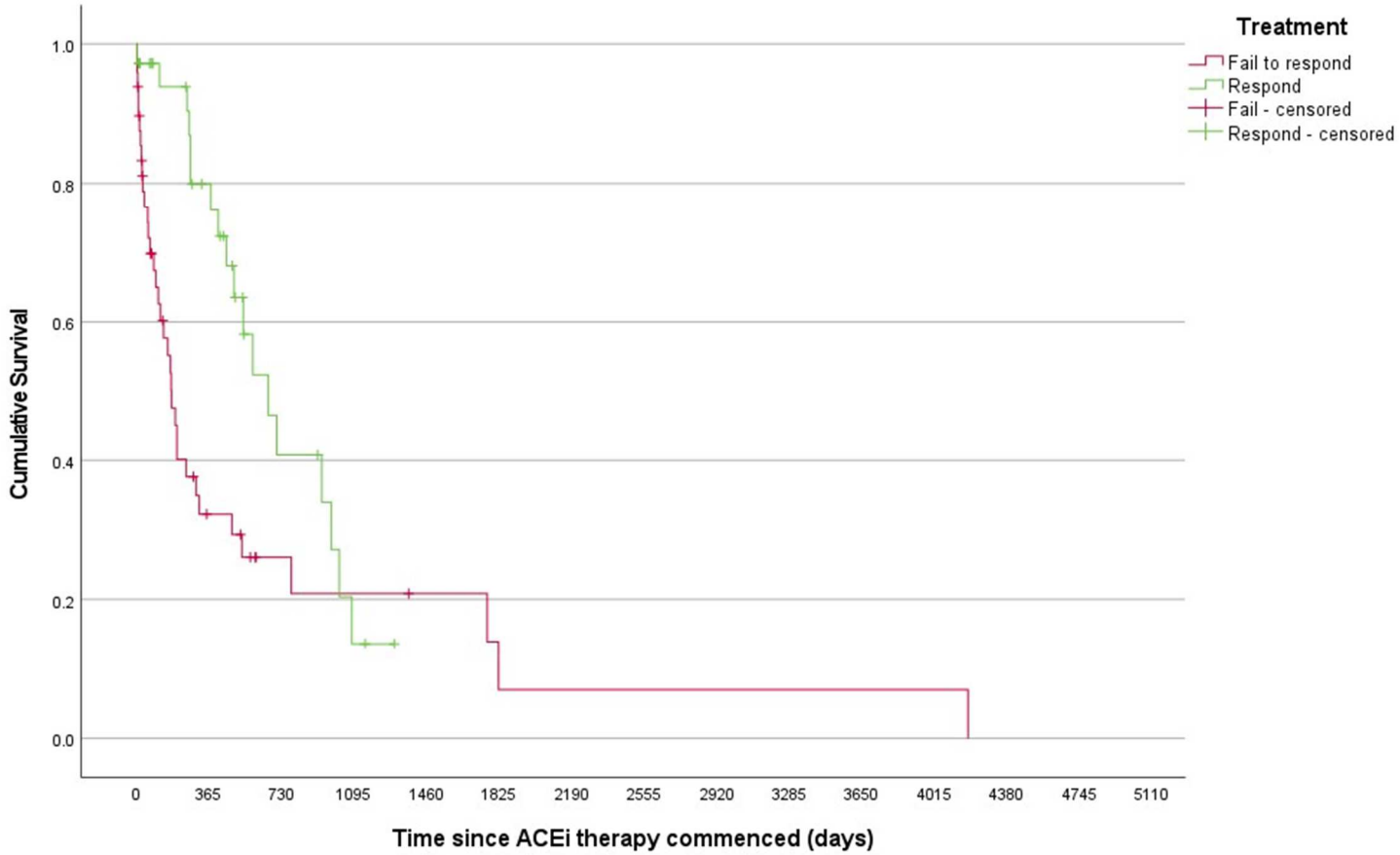


Figure 4: Kaplan-Meier survival curve of all-cause mortality for a cohort of 85 dogs with presumed glomerular proteinuria and a UPC>2.0 that respond to or fail to respond to treatment with ACEi. Treatment response was defined as achieving a UPC of <0.5 or reduction of ≥50% from baseline within 3-months of starting treatment. (Log-rank Mantel Cox p=0.009). ACEi=angiotensin converting enzyme inhibitor; UPC=urine protein creatinine ratio.

Parameter	OR	OR CI	P value
Either UPC target achieved within 3-months	0.10	0.03 – 0.31	**<0.001**
Baseline creatinine			
Values (mg/dL)	2.53	1.38 – 5.61	**0.008**
Azotaemic	5.34	1.85 – 17.32	**0.003**
Baseline albumin			
Values (g/dl)	0.40	0.18 – 0.82	**0.016**
Hypoalbuminaemic	4.51	1.66 – 13.14	**0.004**
Baseline cholesterol (n=84)			
Values (mg/dL)	1.00	1.00 – 1.00	0.742
Hypercholesterolaemia	0.95	0.36 – 2.51	0.924
Baseline urine protein-creatinine ratio (UPC)			
Values	1.18	1.05 – 1.34	**0.010**
Severe proteinuria (UPC >3.5)	2.91	0.84-11.79	0.106
Baseline systolic blood pressure (n=46)			
Hypertensive (systolic blood pressure >159mmHg)	2.36	0.65-9.05	0.197

Table 3: Odds ratios from the univariable analysis of the association between response to treatment with an angiotensin converting enzyme inhibitor and baseline clinicopathological parameters and death by 12-months in a cohort of 85 dogs treated for presumed glomerular proteinuria and UPC >2.0. Parameters were available for all 85 dogs unless otherwise indicated. Treatment response was defined as achieving a UPC of <0.5 or reduction of ≥50% from baseline within 3-months of starting treatment. OR=odds ratio; OR CI=odds ratio 95% confidence interval; P value – associated statistical significance; UPC=urine protein-creatinine ratio]

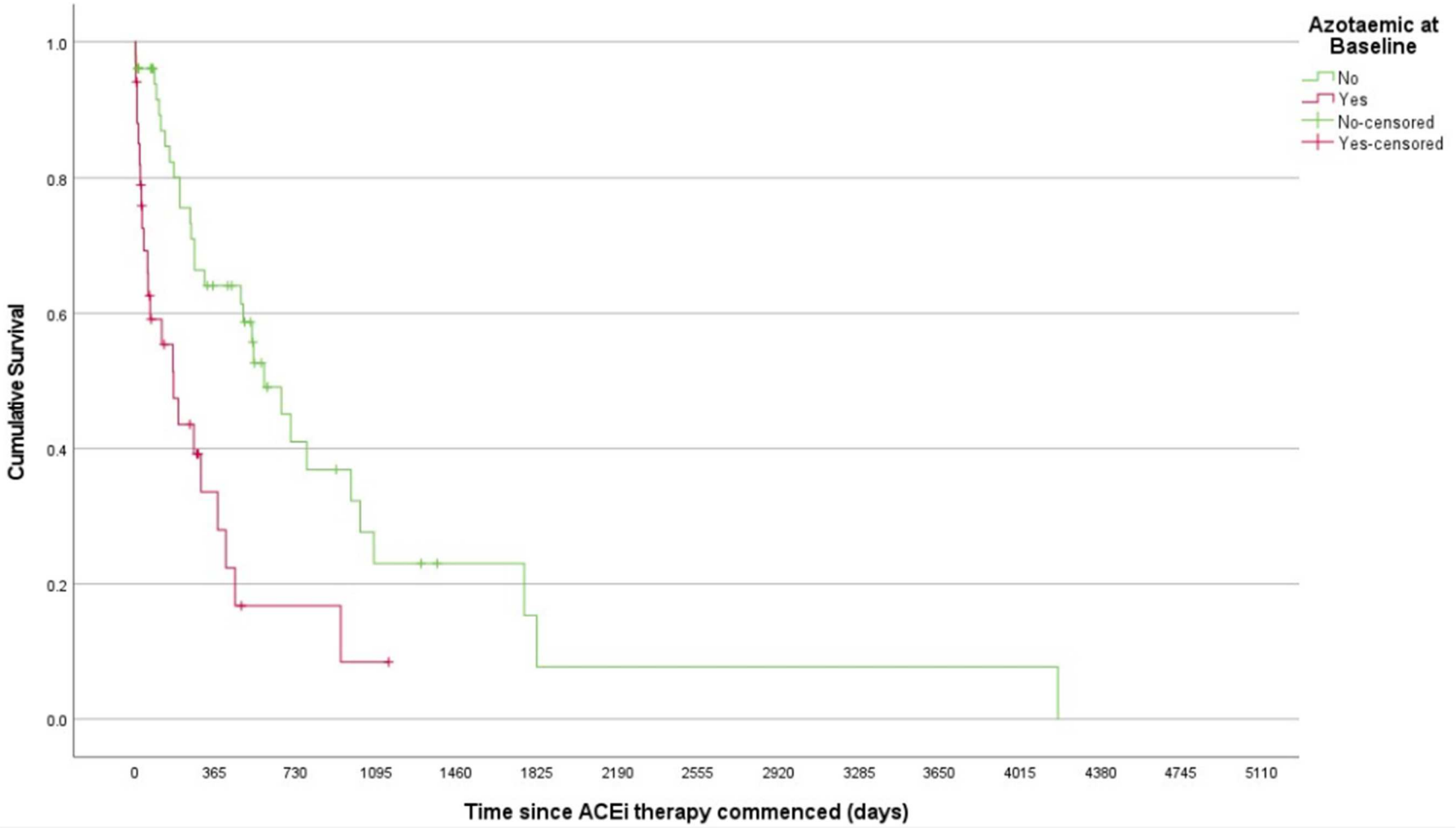


Figure 5: Kaplan-Meier survival curve of all-cause mortality for a cohort of 85 dogs with presumed glomerular proteinuria and a UPC>2.0 with and without baseline azotaemia. (Log-rank Mantel Cox p=<0.001). ACEi=angiotensin converting enzyme inhibitor; UPC=urine protein creatinine ratio

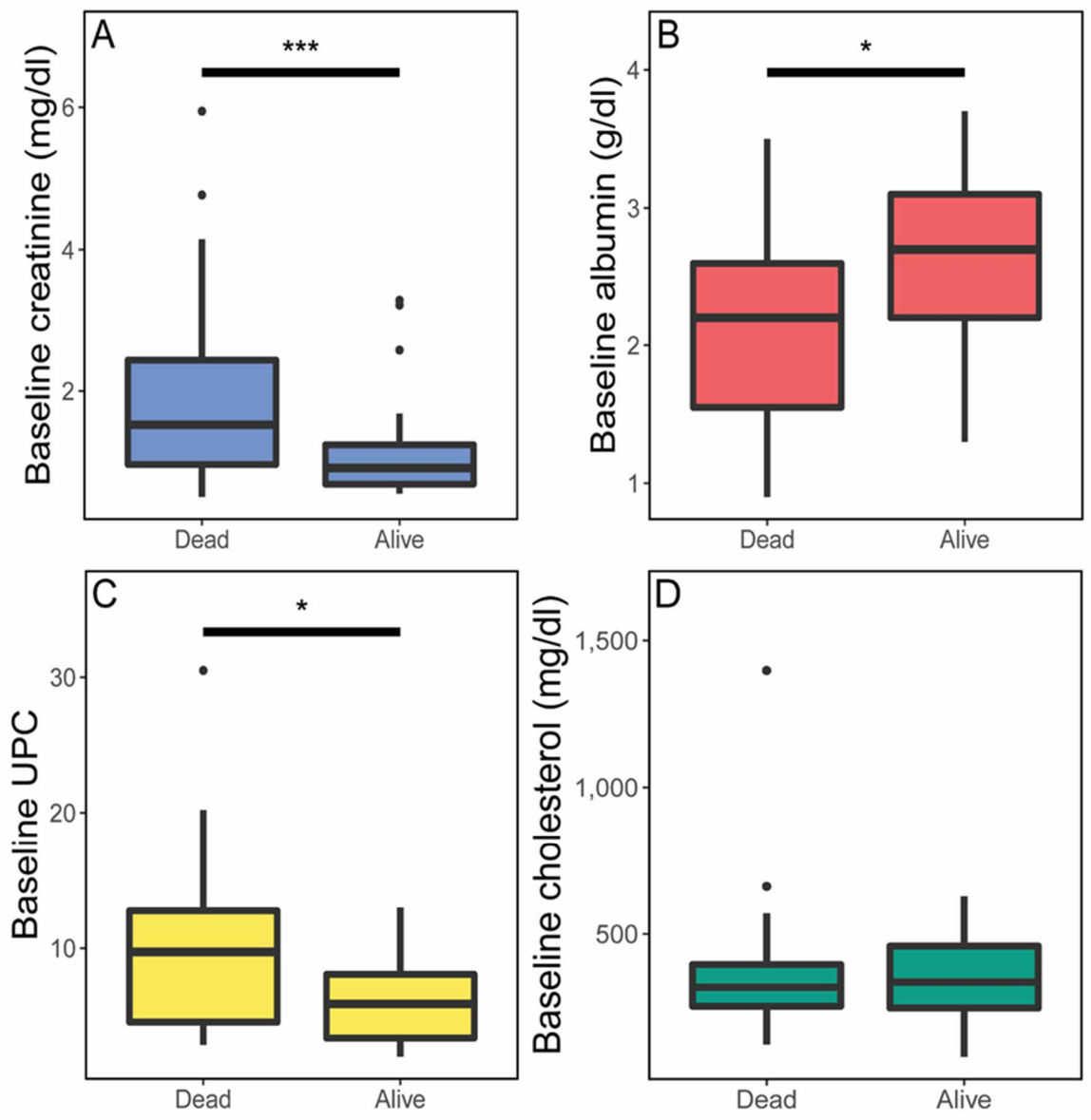


Figure 6 A-D: Box and Whisker Plots for clinicopathological variables at baseline and survival status at 12-months for dogs with presumed glomerular proteinuria and a UPC>2.0 treated with angiotensin converting enzyme inhibition. 12-month survival status was known for 68 dogs. A) Creatinine (mg/dL); B) Albumin (g/dL); C) UPC and D) Cholesterol (mg/dL) l Indicate values > 1.5 x Interquartile Range. Mann-Whitney U test was performed. Statistical significance indicated by *** <0.001, * <0.05. UPC=urine protein creatine ratio.

Reasons for Euthanasia

Thirty-five dogs were known to be dead at the 12-month follow-up date. Of these dogs, the reason for death was known for 24 dogs (68.6%). Progression or presence of renal disease was cited as at least part of the reason for euthanasia in 87.5% of cases (n= 21/24). Reasons for euthanasia in the remaining dogs were progression of neoplasia (n=1), development of cardiorespiratory disease (n=1) and progression of lymphoma along with respiratory compromise (n=1).

Multivariable analysis

Multivariable logistic regression analysis to evaluate the relative importance of azotaemia or hypoalbuminaemia given treatment response found that even considering the reduction in odds of death in responders, the presence of either azotaemia and hypoalbuminaemia at baseline were still associated with increased odds of death at 12-months (7.69 and 4.66, respectively, Table 4).

Categorical Tree

Finally, to evaluate the interplay between the presence of a combination of factors in terms of clustering of clinical thresholds and response to treatment in particular dogs, a categorical tree was generated. Although 12-month survival data was available for 68 dogs, only 67 dogs were included in the categorical tree as cholesterol was unavailable for one dog. Hypertension was not included in this model as SBP was not available for all dogs. The categorical tree determined the principal factor influencing 12-month survival to be treatment response. The worst outcome was seen in dogs that failed to respond to ACEi within 3 months and that were azotaemic; none of these dogs (n=17) were alive at 12-months (Figure 7, [1]). Conversely, the best outcome was seen in dogs that responded to treatment and which had normal albumin and cholesterol at baseline; all dogs (n=8) with this combination of factors were alive at 12-months (Figure 7 [2]).

Predictors	OR	OR CI	P value
Either UPC target achieved within 3-months	0.10	0.02 – 0.34	0.001
Azotaemic at baseline	7.69	1.95 – 39.55	0.007
Hypoalbuminaemic at baseline	4.66	1.32 – 19.04	0.021

Table 4: Factors found on multivariable analysis to be significantly associated with death by 12-months in a cohort of 85 dogs with UPC>2.0 treated with ACEi for presumed glomerular proteinuria. Treatment response was defined as achieving a UPC of <0.5 or reduction of ≥50% from baseline within 3-months of starting treatment. OR=odds ratio, OR CI=odds ratio 95% confidence interval, P value – associated statistical significance, UPC=urine protein-creatinine ratio

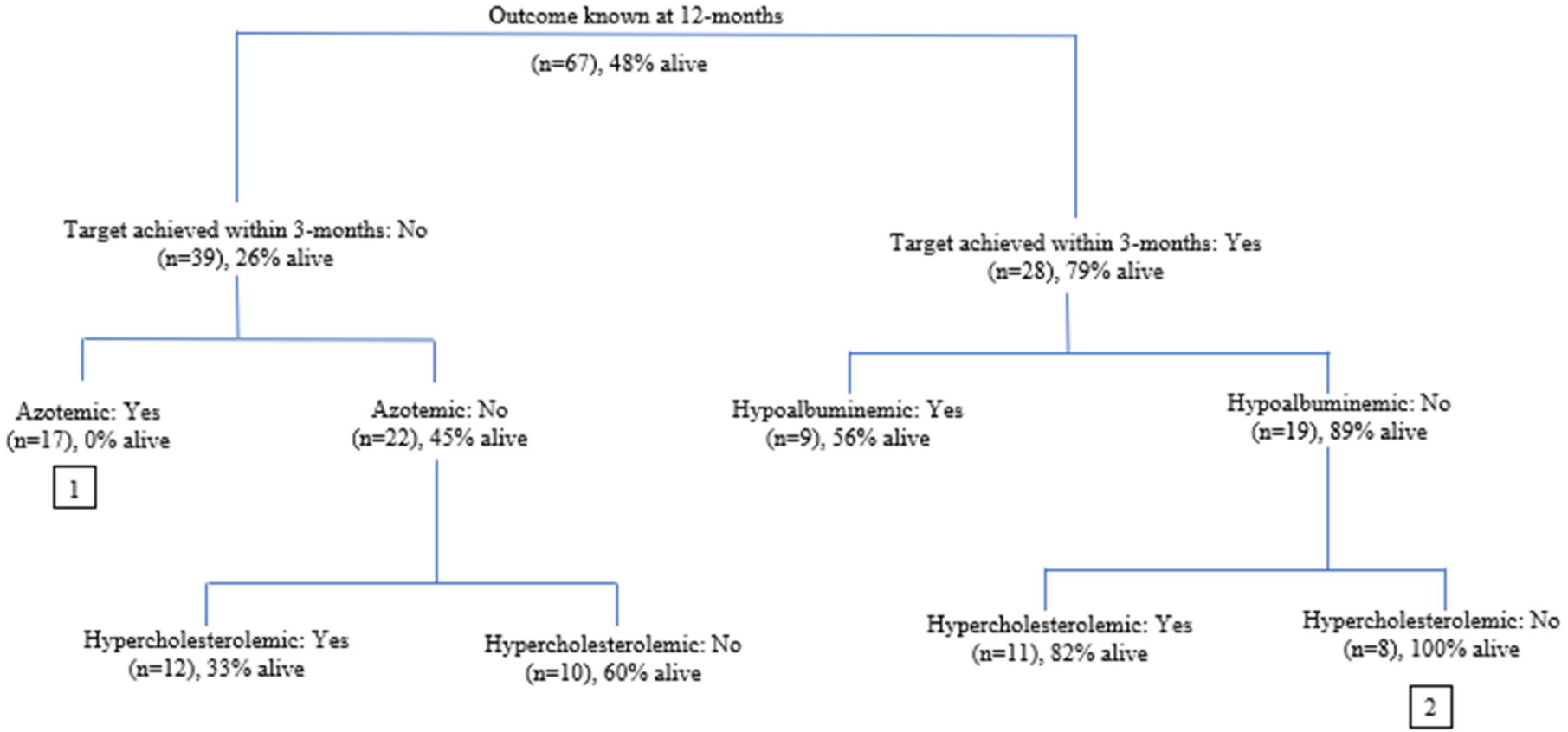


Figure 7: Categorical tree showing assessing impact of achieving target within 3-months and the presence of baseline biochemical abnormalities on survival at 12-months. (n) = number of dogs with that combination of factors; %= % of that combination known to be alive at 12 months. (1 & 2) in boxes see results text.

2.7 Discussion

We set out to evaluate the frequency with which ACEi treatment resulted in a ≥50% reduction in UPC or a UPC<0.5 within 3-months in a cohort of dogs with presumed glomerular proteinuria and UPC>2.0, and to evaluate whether achieving this target was associated with improved survival. Although only 41.2% of our study cohort achieved one of the targets for reduction in UPC within 3-months, those that did were significantly more likely to be alive 12-months after starting treatment with responders also having significantly longer MST. The presence of baseline azotaemia or hypoalbuminaemia, and magnitude of proteinuria, were also associated with a worse outcome although to a lesser extent.

There are numerous studies in human literature supporting the use of ACEi in proteinuric renal diseases, including diabetic nephropathy (Lewis et al., 1993, Amann et al., 2003), non-diabetic nephropathy (Ihle et al., 1996, Maschio et al., 1996, Gansevoort et al., 1993, Ruggenenti et al., 1997a) and IgA nephropathy (Praga et al., 2003, Maschio et al., 1994), where they have been shown to slow the progression to end-stage renal failure. Similarly, there is evidence within veterinary literature to support their use in the management of proteinuric renal disease; Grauer *et al* (2000) showed that enalapril reduced proteinuria and slowed disease progression in some dogs with idiopathic glomerulonephritis (Grauer et al., 2000) and Grodecki *et al* (1997) reported that enalapril delayed the increase in serum creatinine and UPC in dogs with X-linked hereditary nephritis (Grodecki et al., 1997). What is less clear is the whether the benefits of ACEi correlate with the magnitude of reduction in proteinuria. While ACEi reduce proteinuria via their impact on glomerular haemodynamics (Anderson et al., 1986, Brown et al., 1993, Brown et al., 2003), they also lead to a decrease in the production of vasoactive substances implicated in development of glomerulosclerosis, delay the growth and proliferation of mesangial cells, and reduce the degradation of bradykinin, all of which may influence the rate of disease progression independent of the magnitude of reduction of proteinuria (Kagami et al., 1994, Brown et al., 1993, Raij and Keane, 1985, Reddi et al., 1991, Hutchison et al., 1995, Heller et al., 1997). Given that dogs achieving a 50% reduction in UPC were 10 times more likely to be alive at 12-months compared to those that did not and had a significantly longer MST, it appears that outcome is associated with the magnitude of reduction in UPC in response to ACEi. This strong association supports the currently recommended target of >50% reduction in UPC, justifying both the time and financial commitments needed to achieve this target.

Treatment response was not the only variable found to be associated with survival. The presence of baseline azotaemia, hypoalbuminaemia, and magnitude of baseline creatinine,

albumin and UPC were negatively associated with 12-month survival. This is unsurprising as increasing IRIS stage, the presence of nephrotic syndrome and increased UPC are previously reported negative prognostic indicators in a variety of canine renal diseases (Klosterman et al., 2011, Wehner et al., 2008, Rudinsky et al., 2018, Jacob et al., 2005, O'Neill et al., 2013). Similarly, in human IgA nephropathy, baseline creatinine and magnitude of proteinuria are independent predictors of progression to end-stage renal disease (Manno et al., 2009).

Whilst multivariable analysis confirmed that azotaemia and hypoalbuminaemia were independent risk factors for death before 12-months, response to therapy remained positively associated with 12-month survival in the subset of dogs with less severe disease (i.e. with normal creatinine and albumin). Additionally, the categorical tree analysis provided a clear visual representation of the association between the categorical variables assessed and 12-month survival. Although the presence of azotaemia had a negative association and lack of hypoalbuminaemia a positive association, treatment response ranked highest in its association with survival at 12-months. Therefore, although the presence of markers of disease severity (azotaemia and hypoalbuminaemia) appear to be negative prognostic indicators, response to treatment is suggested to have the strongest association with 12-month survival and hence these results again support the currently recommended target of a 50% reduction in UPC regardless of disease severity.

When baseline UPC was assessed as a continuous variable it was found to be associated with 12-month survival, however, when it was assessed as a categorical variable the same significance was not found. Severe proteinuria was defined as a UPC>3.5; as per the ACVIM Consensus Guidelines (Littman et al., 2013). All but 20% of dogs had severe proteinuria and hence it possible that this study was underpowered to detect a difference between the two groups. The cut-off of 3.5 to define severe proteinuria may also be too low in cases of glomerular protein loss; additional studies are required to further interrogate this cut-off.

As reported in people with proteinuric renal disease (Heeg et al., 1987) and in previous canine studies (Grauer et al., 2000, Lourenco et al., 2020), a variable response to ACEi was observed in our study cohort, with only about 40% of dogs reaching the target UPC. This is unsurprising as ACEi therapy does not address the underlying cause of glomerular disease and treatment with ACEi is unlikely to result in complete resolution of glomerular injury (Pressler et al., 2013). Other potential explanations for this inconsistent response include angiotensin converting enzyme (ACE) gene polymorphisms (Hunley et al., 1996, Vleming et al., 1998, Woo et al., 2007, Ruggenenti et al., 2000b), differing aetiology for proteinuria

(Ruggenenti et al., 2000a) and disease severity or magnitude of the proteinuria at time of starting ACEi therapy (Kramer et al., 2003). Lack of response to ACEi could also occur due to incomplete suppression of angiotensin II synthesis either via incomplete inhibition of ACE or production via ACE-independent pathways (Barreras and Gurk-Turner, 2003). Aldosterone breakthrough (increased aldosterone levels despite ACEi treatment) has also been reported to occur in a subset of human patients (Schjoedt et al., 2004, Bomback and Klemmer, 2007, Horita et al., 2006). While this phenomenon has not been widely investigated in dogs, a recent study reported that 34-59% of dogs with proteinuric CKD treated with RAAS inhibition demonstrated aldosterone breakthrough; aldosterone breakthrough has also been reported in 32% of dogs treated with benazepril for cardiac disease (Ames et al., 2017, Ames et al., 2022). Aldosterone breakthrough may also account for the subset of dogs that initially responded to treatment but had subsequent increases in UPC that did not meet either UPC target. The variability in response to ACEi and the clear survival benefit associated with achieving target reductions in UPC, support the recent focus on alternative or adjunctive methods of RAAS suppression when ACEi are insufficient.

In humans, combination therapy of an ACEi with an ARB is reported to have a possible synergistic effect with a recent meta-analysis review suggesting combination therapy to be both safe and effective (Linas, 2008, MacKinnon et al., 2006). Combination therapy has not yet been widely studied in veterinary medicine, however, ARBs, specifically telmisartan, are becoming more frequently prescribed and represent an alternative to ACEi treatment. While literature on the use of telmisartan in dogs is sparse, promising data are now emerging. Lecavalier *et al* (2021) assessed a population of dogs with a UPC of ≥2.0 (if non-azotaemic) or ≥0.5 (if azotaemic) and found that 3-months after telmisartan treatment 68% had reached target UPC (defined as per the current study) whilst a UPC of <0.5 was achieved in 9-14.3% of dogs (Lecavalier et al., 2021). Achieving a UPC of <0.5 was also reported in 21.4% of dogs treated with telmisartan for proteinuric CKD in another study.(Miyagawa et al., 2020) The use of telmisartan was directly compared to enalapril by Lourenco *et al* (2020) who found the change in UPC from baseline to day 30 to be significantly greater in dogs with proteinuric CKD (UPC>0.5 and azotaemic or ≥1.0 if non-azotaemic) treated with telmisartan than those treated with enalapril (Lourenco et al., 2020). Further prospective studies are required to assess the use of RAAS inhibition in a cohort of dogs with known renal pathology to determine response rates and impact on patient outcomes.

Interestingly, response to ACEi in this study was not associated with disease severity as defined by the presence of azotaemia and hypoalbuminaemia. In the human literature, the

use of ACEi has been shown to slow progression of disease even in patients with severe renal failure (Ryan and Tuttle, 2008). Our results suggest that, similarly to human medicine, dogs that are azotaemic at the time of starting ACEi may still show a positive response and benefit from improved survival. This has possible clinical significance as there is often hesitancy to start ACEi therapy in patients with pre-existing azotaemia due to concerns that ACEi-associated alterations in renal haemodynamics will lead to worsening of azotaemia. Further prospective studies are required to assess the benefits and tolerability of ACEi use in dogs with higher IRIS stages. Additionally, response to ACEi was not found to be associated with the presence of baseline hypertension. When the human literature is reviewed this finding is perhaps not surprising with several studies previously showing ACEi to have beneficial effects that are only in part due to the reduction in blood pressure they often concurrently cause (Heeg et al., 1987, Maschio et al., 1996, Ihle et al., 1996).

This study had several important limitations, including the decision to use a UPC of >2.0 as an inclusion criterion for cases with presumed glomerular protein loss. While UPC of >2.0 has been widely accepted as an indicator of glomerular proteinuria (Lees et al., 2005), several more recent studies have reported UPCs >2.0 in dogs with histopathologically-confirmed tubulointerstitial disease (Hokamp et al., 2016, Schneider et al., 2013) and therefore it's possible that some of the dogs in our study did not have primary glomerular disease and that this impacted on our results. Similarly, dogs with confirmed glomerular disease can have a UPC <2.0 (Hokamp et al., 2016) and would have been excluded from our study, again impacting on our conclusions. Given the retrospective nature of this study, with case enrolment starting from 2006, the presence of glomerulonephropathy could not be definitively confirmed or characterized.

Furthermore, due to the retrospective nature of this study, dogs did not undergo a standardized work-up and comprehensive screening for potential triggers was not performed in all cases. Additionally, the starting dose of ACEi and timing of dose escalations was uncontrolled; a substantial proportion of dogs were started on a dose that was below the recommended starting dose and few dogs had their dose of ACEi increased to the upper limits of the dose range. The median dose at the time of response was 0.52mg/kg/day which is the current recommended starting dose (Brown et al., 2013) and median time to response was 33 days. This may account for the low proportion of dogs reaching UPC targets and it is possible that the numbers of responders would have been higher if dogs had been treated more aggressively and further dose escalations pursued. While dose escalation of ACEi has recently been shown to improve response in dogs (Lourenco et al., 2020), an earlier study

questioned the benefit (Grauer et al., 2000). The benefit of ACEi dose escalation in human medicine is also unclear (Schjoedt et al., 2009, Ruggenenti et al., 2003a). It should be noted that the median starting UPC in the canine study that reported an improvement with dose escalation was 2.23, whereas ours was 6.61, and this may have impacted on the likelihood of achieving a treatment response (Lourenco et al., 2020). Further controlled studies are needed to determine whether ACEi dose escalations increase the number of responders and ultimately influences the MST or 12-month survival of responders. The population in this study was heterogenous, particularly with respect to their concurrent diagnoses; numerous dogs had a co-morbidity that could have impacted on the UPC and if such co-morbidities were not well controlled then it is possible that regardless of the efficacy of the ACEi therapy the UPC may have remained increased. Conversely, treatment of underlying diseases could decrease proteinuria independently of ACEi therapy. The study population was chosen to reflect the situation commonly seen in clinical practice, where standard therapies (e.g., ACEi) for glomerular diseases are often started without performing renal biopsies and in the presence of co-morbidities and while, the presence of co-morbidities could also have affected the reliability of survival data, over 85% of the dogs with known cause of death had progression of renal disease listed as at least a contributing factor to the reason behind their euthanasia, supporting this decision.

Concurrent therapy for the treatment of glomerular proteinuria or its complications was also not controlled. Some enrolled dogs were already receiving a prescription renal diet whereas others were started on a renal diet at the time of starting ACEi therapy and others were never fed such a diet. Feeding a renal diet has previously been shown to reduce UPC in dogs with proteinuric CKD (Zatelli et al., 2016). Omega-3 supplementation, antithrombotic and anti-hypertensive therapy were also not controlled. The impact of diet and the aforementioned additional therapies on the UPC and survival in this study is unknown.

In conclusion, the current study suggests response to treatment with ACEi in dogs with presumed glomerular disease and a UPC>2.0 conveys a significant survival benefit, however, target UPCs were only achieved in 40% of dogs treated with ACEi. The presence of baseline azotaemia, hypoalbuminaemia and the magnitude of proteinuria were found to be independent negative prognostic indicators in these dogs. Further prospective studies are required to corroborate the findings of this study and to further our understanding of the variable response to ACEi in dogs with confirmed glomerular proteinuria.

CHAPTER THREE

PROSPECTIVE STUDY: Urine protein electrophoresis in dogs with a UPC >2.0: Assessment of protein patterns and their association with disease stage and prognosis

3.1 Aims

The aims and hypotheses of this prospective study have been previously introduced in detail but are briefly recapped below:

i. Determine how frequently tubular proteins are detected on UPE analysis in dogs with a UPC of >2.0.

   Hypothesis: Tubular proteins will be frequently identified in dogs with a UPC of >2.0

ii. Determine if the presence of tubular proteins on UPE analysis suggests more advanced or severe disease and to determine if the presence of specific proteins has significance.

   Hypothesis: The presence of tubular proteins will correlate with other markers of more advanced disease (for example, increased creatinine) and disease severity (for example, decreased albumin)

iii. Assess whether dogs without an identifiable trigger for their proteinuria have specific UPE patterns.

   Hypothesis: Dogs without an identifiable trigger will have specific UPE patterns enabling them to be distinguished from dogs with a trigger present

iv. Determine how frequently tubular biomarkers (GGT and NGAL) are increased in dogs with a UPC >2.0.

   Hypothesis: Increased tubular biomarkers will be seen in some dogs with a UPC >2.0

v. Evaluate for potential prognostic factors including the presence of tubular proteins on UPE, the presence of specific proteins detected on UPE and the presence of makers of disease progression/severity.

   Hypothesis: Dogs with tubular proteins present on UPE analysis and those with evidence of more advanced/severe disease, such as the presence of azotaemia will have a worse prognosis.

3.2 Materials and Methods

3.2.1 Case Selection

The results of all urine samples submitted to the University of Glasgow's reference laboratory were reviewed daily to identify urine samples with a UPC of >2.0. Full urinalysis results for dogs with a UPC of >2.0 were reviewed and dogs with evidence of an active sediment were excluded. For this study, an active sediment was defined as the presence of either haematuria, pyuria or bacteriuria. Haematuria was defined as >20RBC/high power field (HPF) and pyuria as >5 white blood cells/HPF. The presence of organisms on microscopy is reported semi-quantitatively at the laboratory used for this study; urine samples with occasional organisms per HPF were eligible for inclusion (free catch urine samples were permitted for inclusion and bacteriuria of this magnitude in such samples could be attributable to contamination). Samples with ≥1+ organisms on microscopy reported (equating to approximately >5/HPF) were excluded.

As well as having full urinalysis results available dogs were also required to have serum biochemistry (or renal profile) results within 48-hours of urine collection, any dog without such results was excluded. Pertinent data from the serum biochemistry (albumin, creatinine, cholesterol and globulin) were recorded. Dogs with evidence of hyperglobulinaemia (>47g/L) were excluded due to the possibility of this being associated with a pre-renal proteinuria. Although submission of urine for culture was not a requirement for inclusion, if bacteriology had been requested the results of this were recorded. Clinicians were requested to complete a questionnaire for each potentially eligible dog, at this point the questionnaire results were reviewed. Dogs receiving TKIs or corticosteroids (within 7 days prior to sample collection) were excluded as these drugs are known to affect proteinuria. Similarly, any dogs already receiving treatment for proteinuria (with either ACEi or ARB) were excluded. Dogs that had evidence of lower urinary tract disease (pollakiuria, stranguria or dysuria; abnormal prostate or vaginal/preputial discharge) were excluded as these dogs could have post-renal proteinuria. Dogs that had seizure activity reported in the 24-hours prior to sample collection or those with pyrexia recorded were also excluded as transient, physiological proteinuria could be present in such cases. Finally, dogs with myalgia were excluded as this could suggest the presence of myoglobinuria as a cause of pre-renal proteinuria. For analysis and to stratify the groups, dogs were given a simplified diagnosis based on whether a trigger for proteinuria was present or not. Triggers were defined as being an endocrine, neoplastic, or inflammatory conditions recorded on the clinical history that could contribute to the presence of proteinuria. Additionally, dogs diagnosed with hyperlipidaemia were also included in the

trigger group. Residual blood and urine from dogs fulfilling the inclusion criteria was processed and stored. Urine collected via any method (cystocentesis, free catch or catheterisation) was accepted for inclusion. Urine samples collected at home or during hospitalisation were both permissible.

Urine samples from healthy staff-owned dogs were also collected to provide control samples. Free-catch urine samples were collected, and dogs were required to undergo a physical examination, additionally, owners were asked to fill in a questionnaire. Control dogs were required to be healthy with health status determined by a normal physical examination and consideration of the results of the owner questionnaire. Any dog receiving medications known to affect proteinuria (ACEi, ARBs, corticosteroids or TKIs) was not eligible for inclusion. Urinalysis and urine culture were performed, and the sediment was again required to be inactive for enrolment.

3.2.2 Sample processing

Samples were processed within 48-hours of collection (samples were stored at 4°C degrees until processing). Samples were divided into 1mL aliquots and spun at 2500 revolutions per minute (630g) for 5minutes (PrO-Vet Centrifuge, Centurion); the supernatant was retained and sediment pellet discarded. The supernatant was divided into 0.9mL aliquots and transferred into a plain blood tube with no additive. If available, a second aliquot of 0.9mL was added to a plain blood tube containing 0.13mL of protease inhibitor (PI) (Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets, Roche). The PI had previously been made up to a 7X stock solution and frozen in 0.13mL aliquots, these were defrosted as required at the time processing the urine sample. If the residual sample was plentiful then several 0.9mL aliquots of supernatant were kept. Once the urine had been processed samples were stored at -20°C degrees temporarily before being moved to -80°C degrees for long-term storage. For blood samples, the residual serum and/or plasma was removed and placed into a new blood tube before also being stored initially at -20°C and then at -80°C degrees. Samples were processed within 48-months of being stored at -80°C degrees.

3.2.3 Preliminary testing

Several preliminary tests were performed prior to starting the main experiments for this master's project to allow for optimisation of the methodologies.

3.2.3.1 Preliminary testing for urine protein electrophoresis

For the main study, Hydragel Proteinurie K20 gels (agarose gels) were selected for use. These gels were selected as they have been specifically formulated for use with urine and allow proteins of tubular and glomerular origin to be clearly separated. However, before urine samples were run on these gels preliminary testing was performed. The main aims of this preliminary testing were to compare two methods of protein quantification to see which yielded more appropriate results for electrophoresis; to gain a broad idea of the protein profiles present in each urine sample and to ensure that the urine protein profile did not simply reflect the serum protein profile. The first of these aims was achieved by comparing protein content in the urine via two different methodologies (that performed the University of Glasgow reference laboratory and that generated using a bicinchoninic protein assay). The second aim was achieved by running urine samples on SDS-PAGE gels; whilst these gels worked nicely they were not used for the final study as proteins >62KDa i.e. glomerular proteins were too large for accurate detection. Finally, paired urine and serum samples were run on gels used for serum electrophoresis (Hydragel 7 β1-β2) to address the last aim. Additionally, throughout the preliminary testing, urine samples with and without the addition of a protease inhibitor were compared to see if this affected results.

Samples were run in batches. At the start of the batch run, two aliquots from each dog (one plain and one containing PI) were defrosted on ice and used to aliquot three 100µL samples of plain urine and PI containing urine. The aliquots were placed into conical tubes (Eppendorf®, Sigma-Aldrich) and were then re-frozen at -80°C degrees. These aliquots were made to try to reduce the number of freeze-thaw cycles samples were exposed to. Selected 100µL aliquots (with and without inhibitors) were then used for each analysis.

3.2.3.1.1 Protein Quantification

For reliable electrophoresis, an accurate quantification of the amount of protein in urine samples is required. Urinary protein concentration can be difficult to determine due to the variation in methodology and the potential presence of interfering agents such as reducing compounds. The protein values for the urine samples had been previously determined at the time of initial urinalysis via the Veterinary Diagnostic Service (VDS) (University reference laboratory). To determine if these values were repeatable, samples were re-assessed utilising a different methodology (bicinchoninic acid (BCA) protein assay).

3.2.3.1.1.1 VDS Precipitation Methodology

The VDS use a precipitation method to quantify urine protein. Urine (1.5ml) is centrifuged (3,300g for 240 seconds) before 400µL supernatant is removed for protein quantification. A spectrophotometer is set to a wavelength of 660nm with a maximum absorbance of 0.5. Two cuvettes (Sarstedt® cuvettes, Krackeler Scientific Inc.) are required, one acting as the blank and the other acting as the test. To each cuvette, 200µL of urine supernatant is added; to the blank 600µL of distilled water is also added whilst 600 µL of sulphosalicyclic acid is added to the test, they are then incubated for 5 minutes, mixed and placed onto the spectrophotometer. The galvanometer of the spectrophotometer is zeroed using the blank to account for the effect of the yellow colouration of the urine sediment. The optical density (OD) of the test sample is then determined. This OD is then used to quantify the protein present; the OD is converted into a protein concentration (mg/100mL) utilising a previously produced calibration curve generated by utilising serial dilutions of a sample with a known protein content. To measure urine creatinine (µmol/L) a clinical chemistry analyser was employed (Dimension Xpand Plus, Siemens) and the modified kinetic Jaffe method utilised. To calculate the UPC, the urine protein concentration was divided by the creatinine concentration (following conversion of the latter to mg/100mL).

3.2.3.1.1.2 Bicinchoninic Protein Assay

To perform the BCA protein assay one of the 100µL aliquots was defrosted on ice. A BCA protein assay (ThermoScientific™ Pierce™ BCA Protein Assay Kit) was utilised to generate protein values for each of the urine aliquots. Two 96-well plates were used to run the protein assays; plain urine samples were loaded onto one plate and urine samples containing PI on the other to allow comparisons to be made and determine if the PI appeared to have additional benefit in protein preservation. Bovine serum albumin was used at known variable concentrations (0.05mg/mL, 0.1mg/mL, 0.2mg/mL, 0.4mg/mL, 0.6mg/mL and 0.8mg/mL) to provide standards. Purified water (Milli-Q®, Sigma) was used to provide a blank. Two wells were filled with 25µL of each standard concentration; 25µL of purified water was also pipetted into four wells.

Once defrosted, the tubes containing urine samples were vortexed for 10-20seconds (Rotamixer, Hook & Turner Instruments) to ensure the sample was mixed, 2.5µL of each urine sample was then added to two wells on the plate. Two hundred and fifty microlitres of reagent was then added to each well. The reagent added comprised of 25ml of BCA™ Protein Assay Reagent A and 500µL BCA™ Protein Assay Reagent B to create a ratio of 50:1 as per manufacturers guidelines. The 96-well plate was then mixed at high speed

(300RPM) on a shaker (Shaker S3.01, Progen Scientific LTD) for 20 seconds. The plate was then placed in an incubator (Hybaid Micro-4 incubator) set to 37°C degrees for 30 minutes**.** After incubation, the plate was taken to the microplate reader (LT4500 microplate reader) and read at 570nm using Magellan™ for F50 software. The raw data from the plate reader were then transferred to an Excel spreadsheet for analysis.

For data processing the value obtained from a blank well was subtracted from all the values to correct for background interference. The standard sample absorbance values were then inputted to generate a graph of absorbance against protein concentration from which a gradient was calculated (example shown in Appendix 1). The values for the samples were then inputted into the spreadsheet and the protein quantified by dividing the sample absorbance by the gradient calculated from the standards. An average concentration for each sample was taken from the readings obtain across the two wells. Finally, the stock concentration of the sample was calculated by multiplying the average protein concentration by the dilution to give a protein value of mg/mL.

This value was then compared with the original protein value generated by the diagnostic laboratory. Due to the presence of marked discrepancy between values for some samples further analysis to determine the most appropriate protein value was then performed (Table 5 and Figure 8 and 9).

Study Number	BCA Protein Assay (Plain) mg/ mL	BCA Protein Assay (PI) mg/mL	VDS Quantitative protein (Plain) mg/mL	Difference between BCA and VDS Values (plain urine)
002	18.8	11.95	13.28	+5.52
003	4.7	3.97	1.36	+3.34
004	5.55	5.39	3.16	+2.39
006	14.73	14.79	10.14	+4.59
007	4.4	4.23	2.77	+1.63
008	NA	4.27	2.38	NA
012	4.33	3.33	1.87	+2.46
017	6.44	5.95	1.63	+4.81
027	1.81	1.69	0.30	+1.51
031	1.74	1.54	0.56	+1.18
039	11.21	10.94	9.83	+1.38
040(3)	14.21	13.52	12.97	+1.24
046	7.07	6.74	3.24	+3.83
051 (3)	12.65	12.28	3.64	**+9.01**
054	NA	7.94	3.05	NA
058	7.96	7.45	3.32	+4.64
033 (2)	9.21	8.69	3.87	+5.34
065	NA	NA	0.40	NA
066	8.66	9.66	5.54	+3.12
067	13.30	NA	3.05	**+10.25**
077	8.58	2.32	2.03	+6.55
079	9.54	8.95	3.64	+5.9
085	13.14	5.47	1.22	**+11.92**
088	2.28	5.80	2.52	-0.14
101	NA	14.34	10.46	NA
105	8.86	3.71	1.83	+7.03
108	5.39	5.21	1.36	+4.03
115	5.71	13.44	3.67	+2.04
116	14.17	7.90	1.83	**+12.34**

Table 5: Comparison of the protein content (mg/mL) in each urine sample utilising two different methodologies (precipitation [VDS] versus BCA assay). For the BCA assay urine samples with and without protease inhibitor were also compared. Samples with the largest difference between BCA and VDS values denoted in bold red to correspond to the red dots on the below scatter plot (Figure 9). BCA= Bicinchoninic protein assay; VDS=Veterinary Diagnostic Service

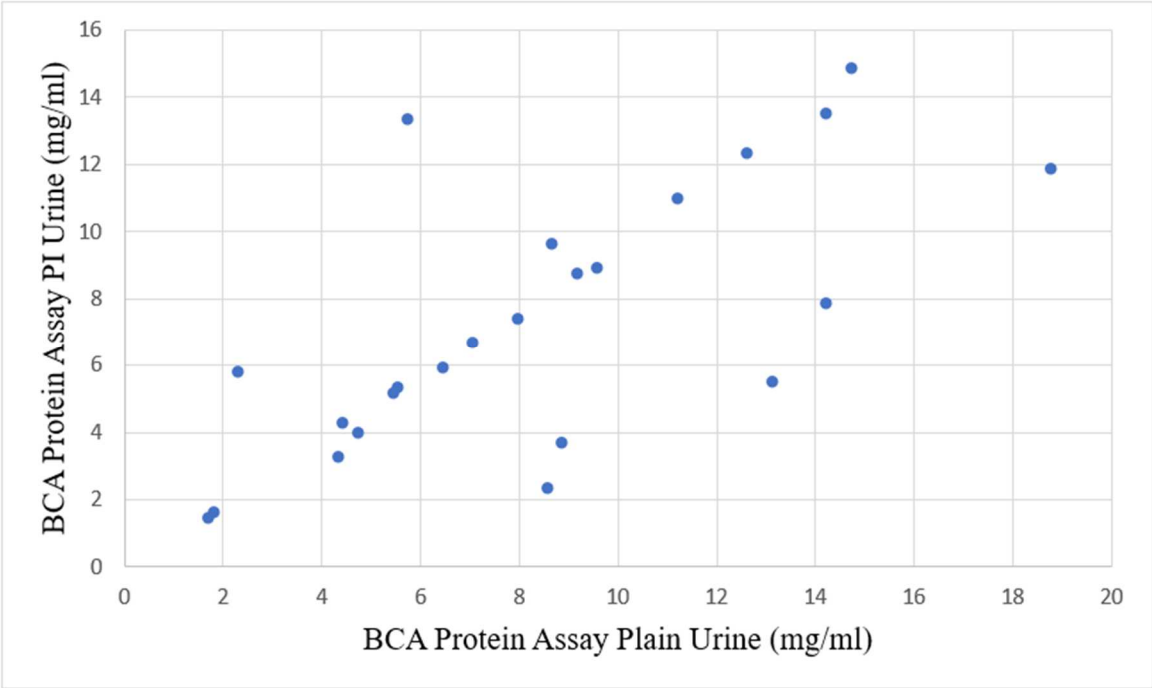


Figure 8: Scatterplot to show the protein assay results (mg/mL) of individual samples when calculated using plain urine and PI urine. The protein content was calculated using the BCA protein assay. Twenty-four paired samples were available for analysis. BCA=Bicinchoninic protein assay; PI=protease inhibitor

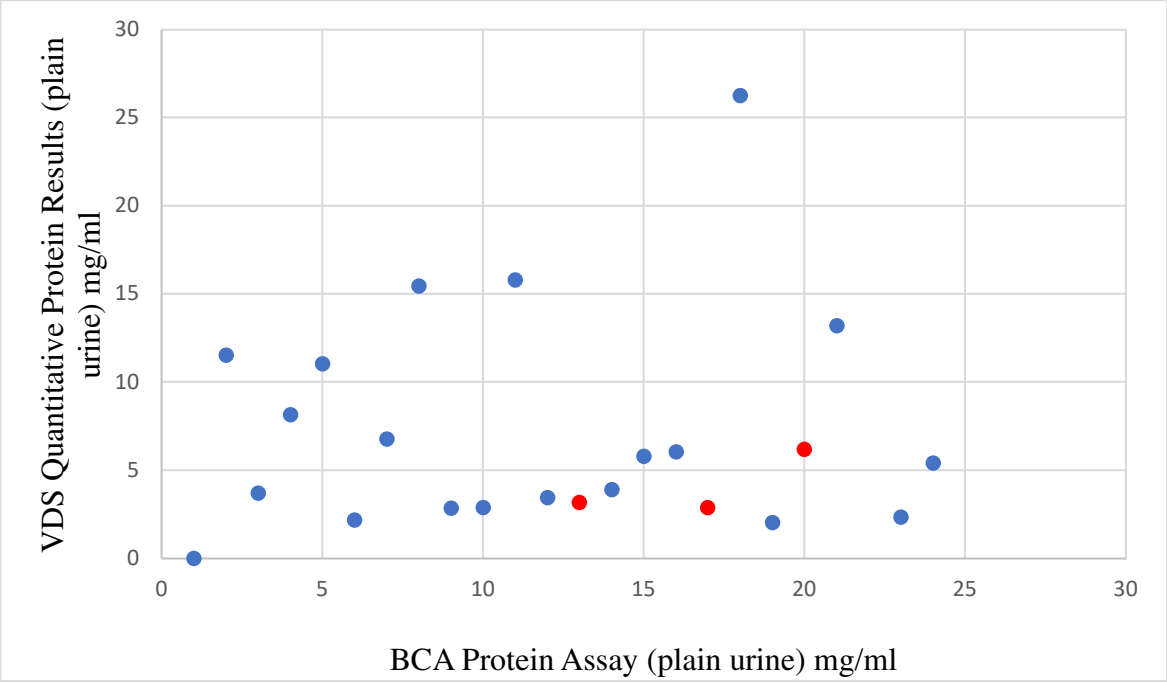


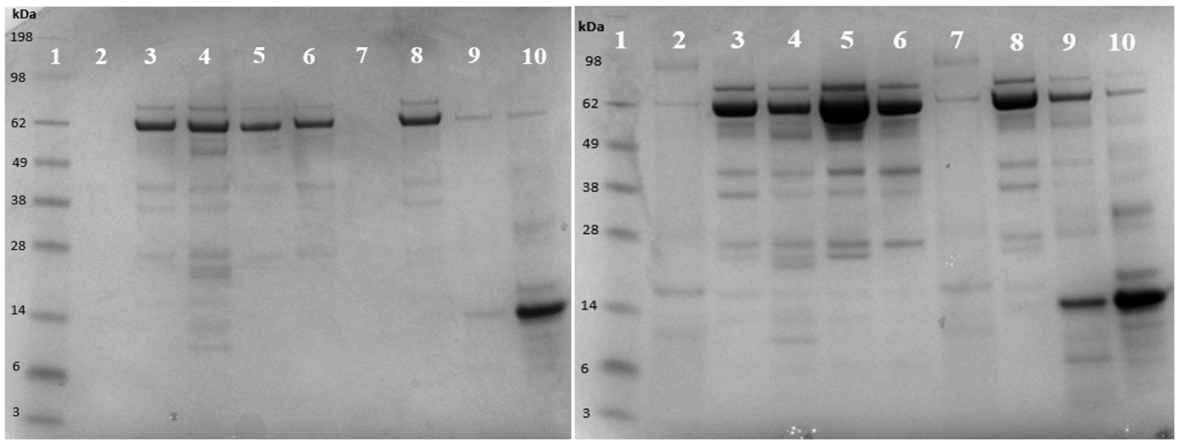
Figure 9: Scatterplot to show the protein assay results (mg/mL) of individual samples (plain urine) when calculated using the BCA assay versus the protein content reported by the VDS quantitative assay. Twenty-five paired samples were available for analysis. Dots in red represent samples with the largest difference between BCA and VDS quantification. BCA=Bicinchoninic protein assay; VDS=Veterinary Diagnostic Service.

3.2.3.1.2 SDS-PAGE Analysis

A Wilcoxon Signed Rank test demonstrated that there was a significant difference between paired sample results generated using the BCA assay and the results generated by the VDS precipitation methodology (p=0.00014). Therefore, to determine the most appropriate protein concentration, the best and worst corresponding samples were selected for both plain urine and urine containing PI. These samples were then used to run comparative gel electrophoresis.

To perform gel electrophoresis, 10-well precast polyacrylamide gels (NuPAGE Novex 4-12% Bis/Tris 1.5mm Protein Gel, ThermoFisher Scientific) were utilised. The maximum capacity for the wells of this gel was 24µL. Samples were diluted with purified water as appropriate to achieve a maximal protein concentration per well of 10µg/mL (samples with an initial concentration of >10 µg/mL were diluted whilst those with an initial concentration of <10µg/mL were loaded neat); the sample was made up to a final volume of 24µL by the addition of the appropriate volume of commercial Laemmil buffer (BioRad) containing the 40mM reducing agent dithiothreitol (DTT). The samples were then denatured by heating at 96°C degrees for 4 minutes before repeat pulse centrifugation was performed.

Gels were removed from the packaging and rinsed, the comb removed and NuPage MES buffer x1 used to fill the wells and remove and debris/residue. Samples were loaded into the wells and 4µL of a protein marker (SeeBlue™ Plus 2 marker) were added to the first well of each gel. One gel was used to run samples with volumes calculated using the BCA assay and one gel was used to run samples with volumes calculated using the diagnostic laboratory values. The gels were run at 145v constant for 60 minutes or until the dye front reached the bottom of the gels. The gels were then removed from the cassette and placed in Coomassie stain overnight. The following day the gels were removed and placed in de-staining solution (50% H20, 10% acetic acid and 40% methanol) for four hours. Gels were scanned on a flatbed scanner and the digitised images saved as greyscale. The results of the gels are shown in Figure 10.



BCA **VDS**

Lane	Sample ID	BCA protein value (mg/mL)	VDS protein value (mg/mL)
1	Marker	NA	NA
2	C02 PI	18.16	0.32
3	031 PI	1.54	0.56
4	077 PI	2.32	2.03
5	079 PI	8.95	3.64
6	116 PI	7.9	1.83
7	C02	19.24	0.32
8	031	1.74	0.56
9	105	8.86	1.83
10	088	2.28	2.52

Figure 10: Comparison of SDS-Page analysis using protein quantifications generated from BCA assay (left) and VDS precipitation method (right) – greyscale. Lane 1: Protein marker (SeeBlue™ Plus 2), lanes 2-10 matching clinical samples. kDa scale given on the far left. Samples are listed in the table with the protein value generated by each method. Samples with the prefix 'C' represent control samples. BCA=Bicinchoninic protein assay; kDa=Kilodalton; PI=protease inhibitor; VDS=Veterinary Diagnostic Service.

Based on the comparisons of the SDS-page gels, the VDS precipitation method values appeared most appropriate as the protein bands on these gels were more prominent and numerous. Hence, it was determined that the VDS precipitation protein quantification would be used for any further calculations required. There did not appear to be a large difference between the protein levels in urine with and without PI when protein quantification with the

BCA assay was performed. Therefore, it was decided that plain urine samples would be utilised. This decision was made in part due to the minimal difference seen with protein quantification with the BCA assay but additionally, it was thought that the PI may interfere with some of the enzyme-based testing that was planned for the second part of the study.

Preliminary testing of all plain urine samples available at that time was then carried out utilising a larger Criterion XT Precast gel, 4-12%Bi/Tris gel. Final sample volumes of 15µL containing a maximum of 10µg of protein were used and BioRad XT MES buffer used for electrophoresis. The results of the preliminary run are shown in Figure 11 and 12. To provide a comparison, some of the final lanes of the second gel were loaded with urine containing PI: again, there was no clear difference between the urine samples with and without PI included. Samples from control dogs were also run on the second gel, as expected, the control dogs showed fewer and much less intense staining bands. These gels were run to perform a preliminary survey screening of the urine samples, the gels mainly detected smaller proteins (<62kDa) as they are not specifically designed for urine protein electrophoresis.

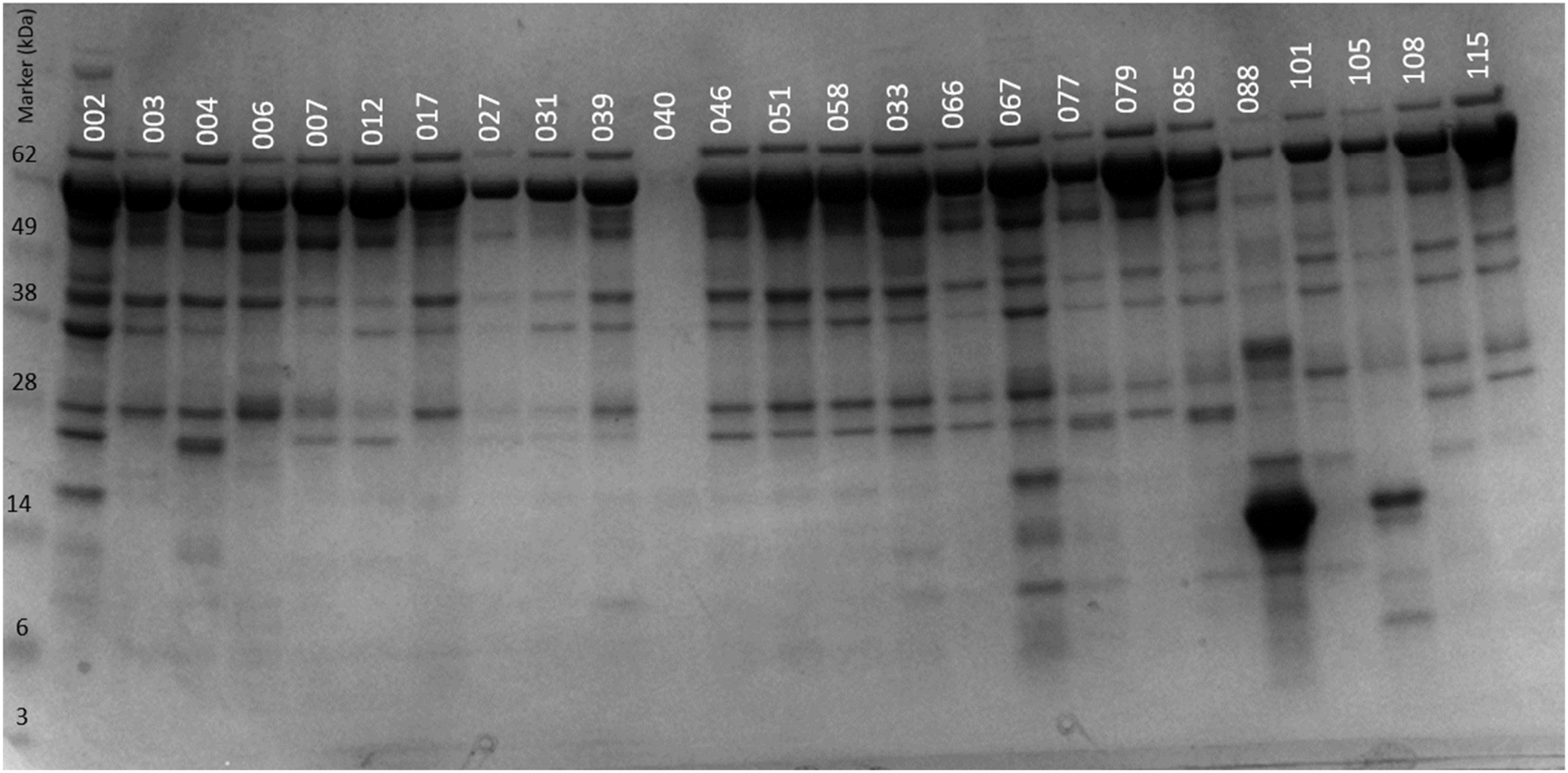


Figure 11: Preliminary gel 1 in grayscale (plain urine samples only). The first lane contained a marker and the kilodaltons (kDa) are included on the left. The lanes are labelled with the corresponding clinical sample number to enable comparison where appropriate with the second gel (Figure 12)

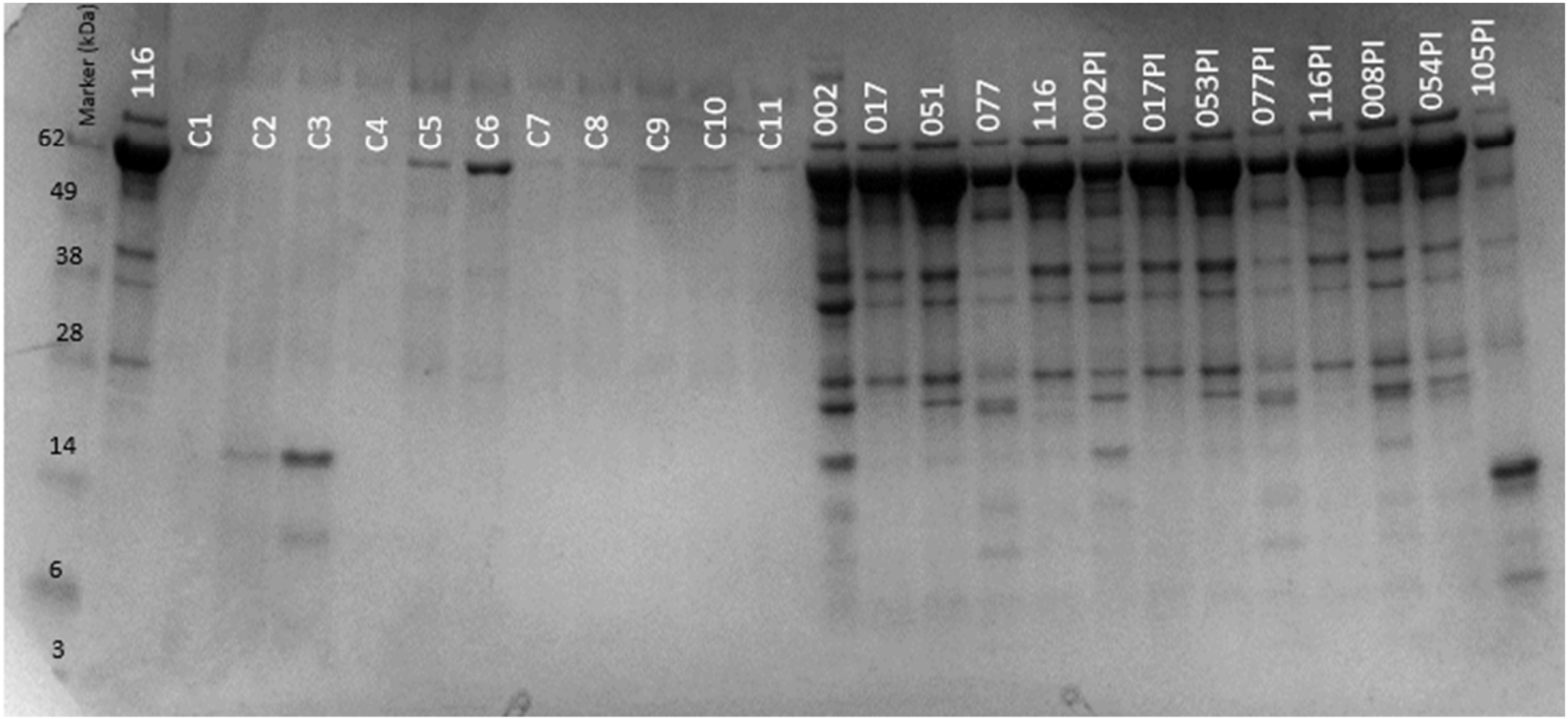


Figure 12: Preliminary gel 2 in grayscale. The first lane again contained a marker and the kilodaltons (kDa) are included on the left. The lanes are labelled with the corresponding clinical sample number so that lanes can be compared with the first gel where appropriate. Samples with the prefix 'C' denote control samples. PI=protease inhibitor.

3.2.3.1.3 Testing on serum protein gels (Hydragel 7 β1-β2)

To establish the viability of running urine samples using agarose/acrylamide commercial gels, a preliminary test was performed using the gel system optimised for serum (Hydragel 7 β1-β2). The gels were prepared as per the manufacturer's guidelines. Briefly, the gel was removed from the outer packaging and loaded onto the set frame which is part of the gel applicator. A control marker was loaded into the first lane followed by a clinical sample in the second lane. In the remaining lanes three urine samples were loaded, two of these urine samples were run alongside a paired serum sample from the same dog. Paired serum and urine samples were loaded to ensure that that the protein content in the urine was not an exact mirror image of what was detected in the serum. Samples were loaded onto the gel and it was transferred to an electrophoresis densitometer rack filled with buffer (Tris Barbital). The rack was placed into the densitometer for 20minutes (settings: 90V, 12+/- 3mA) before the gel was then immersed in a fixative solution (ethanol/acetic acid). After fixing, the gel was placed into an incubator to dry completely before being stained/de-stained. When the gel was dry and cool it was immersed into Amidoblack stock solution before being moved into a beaker of de-staining solution (the gel was continually placed in a fresh beaker of de-staining solution until the solution remained clear after immersion of the gel). The gel was then left to dry before being interpreted. From the results of the trial run (Figure 13), it was seen that 2 out of the 3 urine samples ran successfully, however, one urine sample did not develop any bands at all. Importantly, what was also clear from the results of this gel was that the protein content of the urine was not an exact mirror image of what was present in the serum. Urine is a more readily available biomarker compared to blood and the confirmation of the lack of similarity between blood and urine electrophoresis results confirmed that pursuing further research centred on urine was valid.

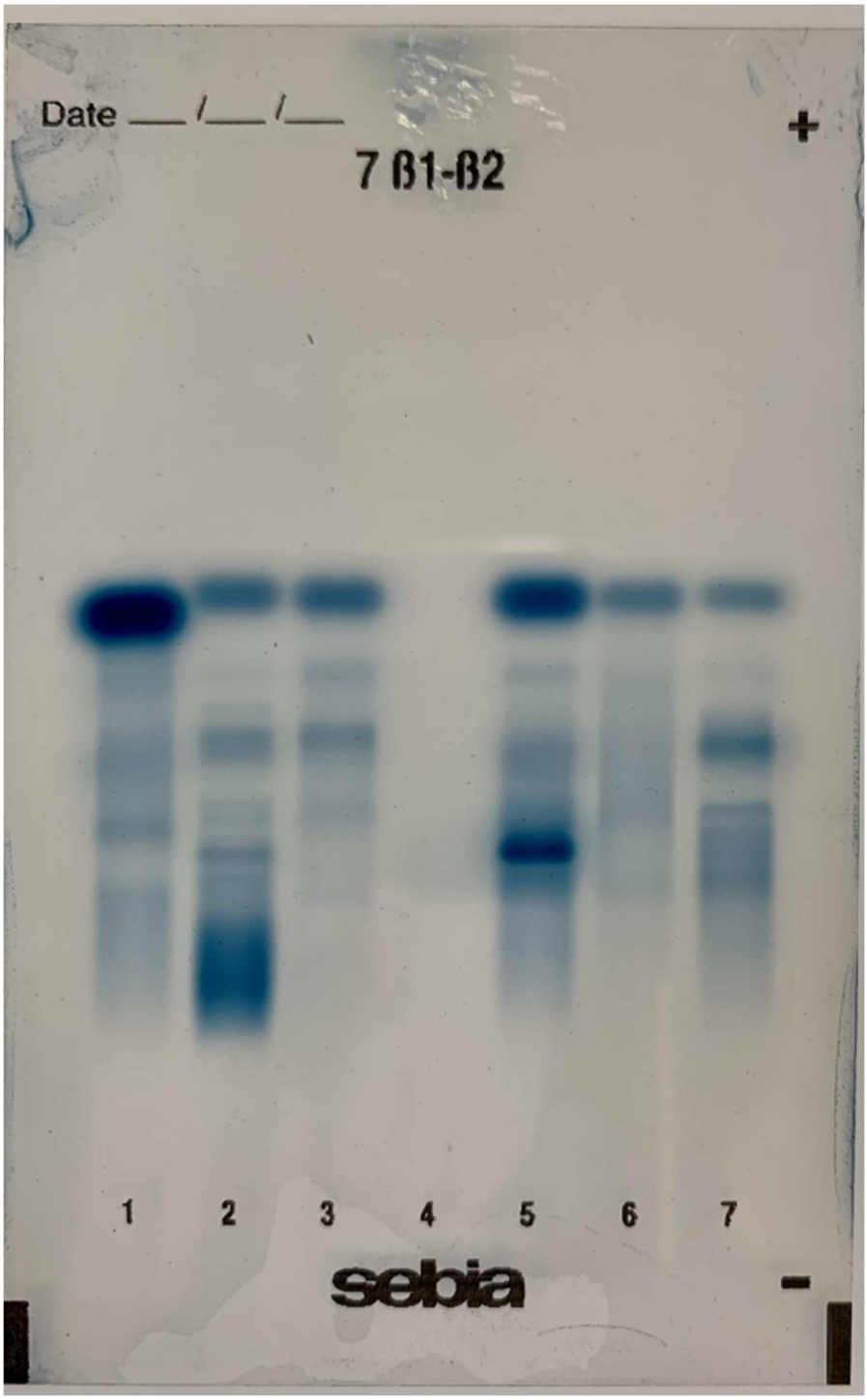


Figure 13: Hydragel 7 β1-β2 trial gel. Lane 1= quality control; lane 2= clinical sample, lane 3=urine (study sample 002), lane 4=urine (study sample 088), lane 5=serum (study sample 088), lane 6=urine (study sample 040) and lane 7=serum (study sample 040)

3.2.3.1.4 Optimisation of Hydragel Proteinurie K20 Gels

After the preliminary testing described above, the samples were run on the main test gels (Hydragel Proteinurie K20, Sebia). These gels are agarose gels that have been formulated specifically for use with urine as they enable proteins of tubular and glomerular origin to be separated. Testing was performed as per the manufacturer's guidelines. Briefly, samples were first diluted if required to achieve a maximal concentration of 2mg/mL; dilution was performed by the addition of the appropriate amount of sodium chloride. Samples not requiring dilution (i.e. with an initial concentration of <2mg/mL) were loaded neat. Samples were then added to diluent (a pre-made diluent solution was provided with the kit containing buffer solution pH 7.0±0.5; bromophenol blue) and vortexed before a set volume of the combined urine and buffer solution was added to the gels. The samples were left to diffuse on the gel for 10 minutes before the gels were placed in the chamber. The guidelines recommended use of the SEBIA K20 chamber, however, this was not available and thus a Beckman Electrophoresis Densitometer chamber was instead used. The chamber was filled with buffer solution (SDS stock solution buffer) and the gel inserted before the chamber was placed in the Beckman Densitometer. The recommended migration conditions were 60 minutes at 60V, however, the unit available did not have 60V as a possible setting, therefore gels were exposed to 50V for 60 minutes. After removal from the chamber, the gels were placed on a pre-heated glass plate and left to dry in an incubator. Again, the guidelines had to be modified slightly; the recommendation was to place the gels in an incubator-dryer at 80°C degrees for 20 minutes. However, the incubator available was unable to be increased above 60°C degrees, hence, gels were stored under these conditions until dry. After drying, the gels were placed in staining solution (Acid violet stain) for 30 minutes and were then rinsed and placed in de-staining solution for 45 minutes. A preliminary run of the gels showed that following the above methodology even with the described modifications, bands were visible in the appropriate gel lanes. However, with 45 minutes of de-staining the gels remained very purple. To try to improve the quality of the final gel they were left in de-staining solution overnight which led to considerable improvement of the clarity of the gel and no deterioration to quality (Figure 14). After de-staining the final steps involved placing the gel into a storage solution (15% glycerine solution) for 15 minutes before placing the gel in 80°C degree hot air stream (this was not available and hence the gels were dried by placing them back in the 60°C incubator). Ten gels were available each with 5 lanes, a protein marker (SeeBlue™ Plus2 Pre-stained Protein Standard, Invitrogen) was placed in the first lane of each gel; to act as a quality control the protein marker was placed into lane 1 of each gel.

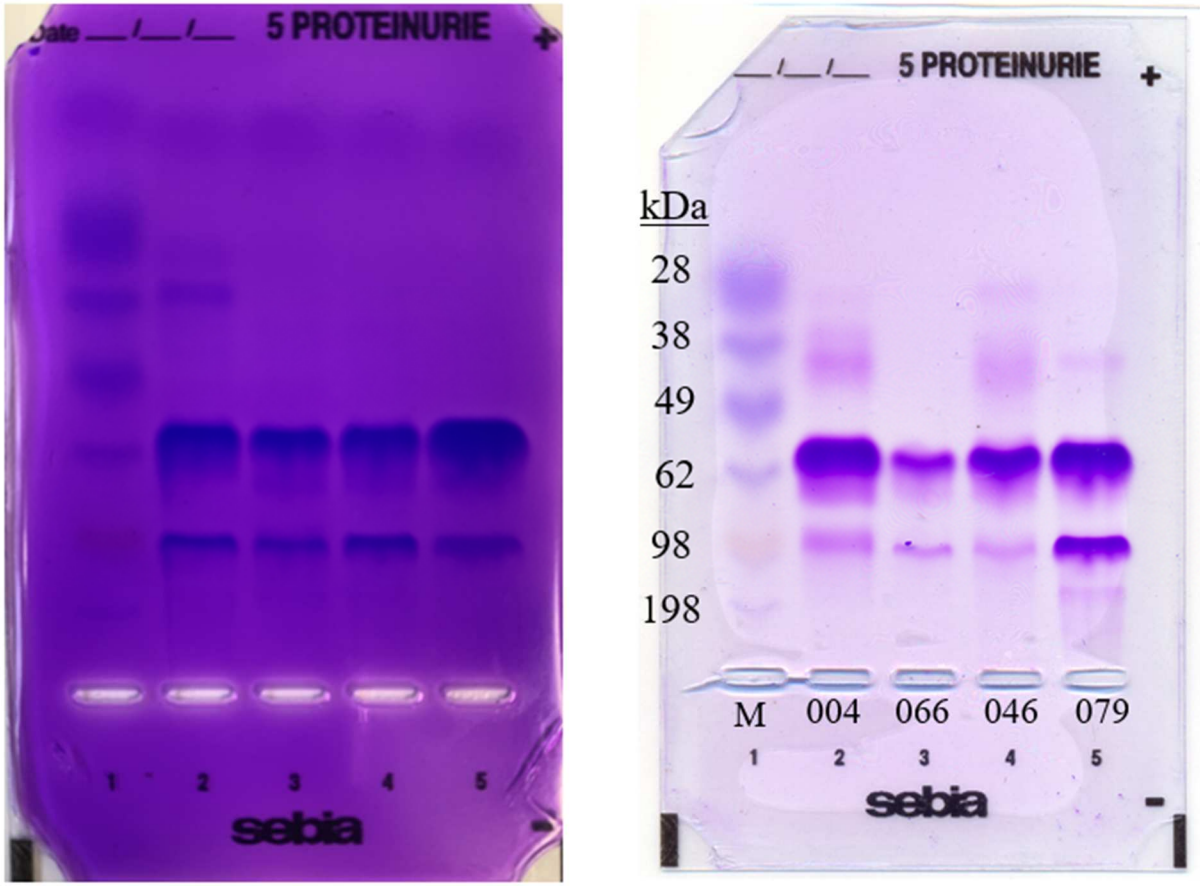


Figure 14: Examples of gels after 45 minutes of de-staining (left) and overnight de-staining (right). The gel on the right is labelled with the kilodalton (kDa) scale. Lane 1 contained a marker (M), lanes 2-5 contained study samples (study number provided)

Acetone precipitation of control samples

To ensure that the protein patterns detected on the gels from study samples were not the same as those in healthy dogs, samples from two control dogs were run. Initially, the control samples showed essentially no banding on the gels. To concentrate the protein present, acetone precipitation was then performed. Following precipitation, a band was visible at the position of albumin and transferrin (Figure 15). To perform acetone precipitation, an aliquot of urine (500µL) was mixed with an equal volume of 100% ice-cold acetone before being incubated at -20°C degrees for at least 60 minutes. After this time, the mixture was centrifuged at 13,000g for 10 minutes at 4°C degrees. Following centrifugation, the pellet was then washed thoroughly with the equivalent volume of 80% ice-cold acetone before the mixture was then again centrifuged (under the same settings). After the second centrifugation, the supernatant was decanted, and the tubes were left to air-dry for 5 minutes at room temperature before the pellet was suspended with 50µL of 50mM HEPES buffer. Following precipitation, the control urine samples were then re-run on a subsequent gel.

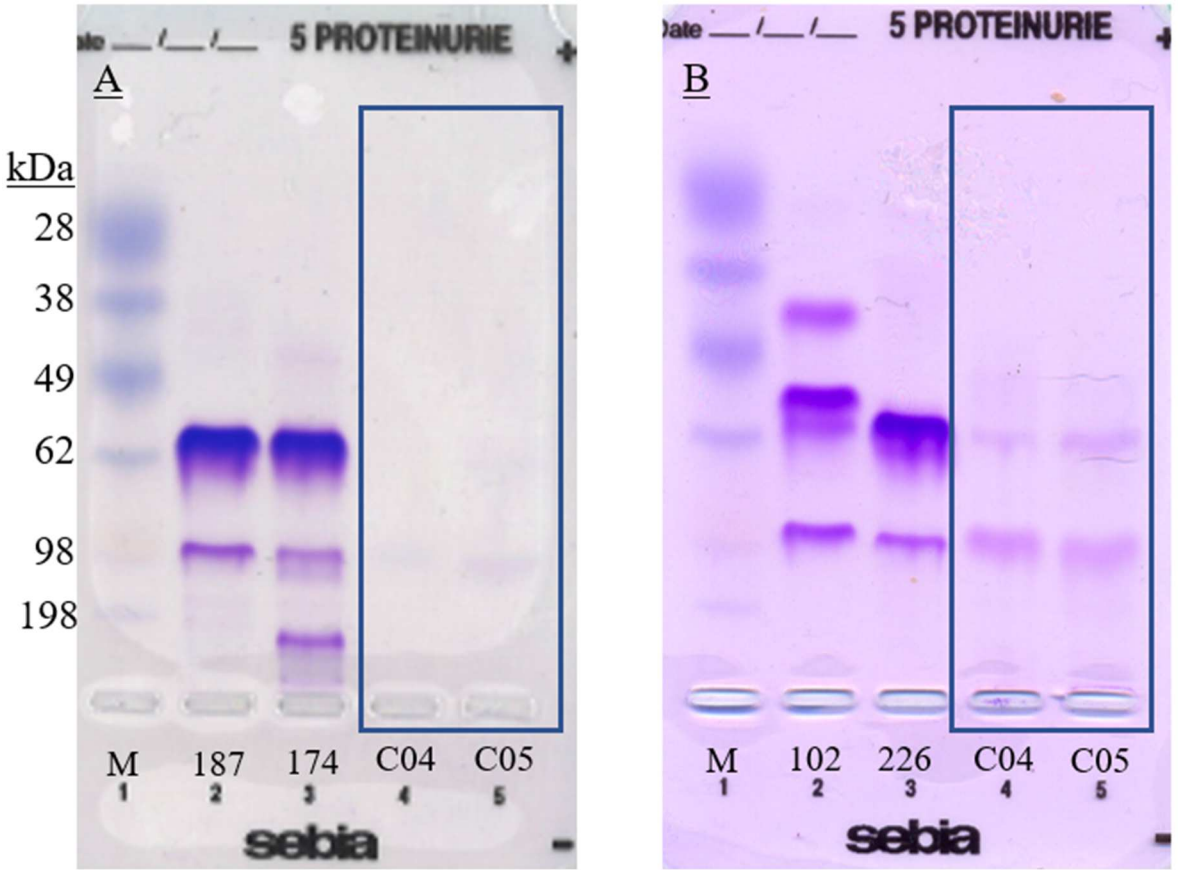


Figure 15: Gels representing control samples before and after acetone precipitation. Lane 4 and 5 on gel A represent before precipitation and land 4 and 5 on gel B represent after. The kilodalton (kDa) scale is given on the left, M=marker, samples with prefix 'C' denote control samples. Lanes 2 and 3 for both gels represent study samples (study number provided).

3.2.3.1.5 Interpretation of the Hydragel Proteinurie K20 Gels

3.2.3.1.5.1 Quantification of the protein bands

To provide a method for numerical semi-quantification of each protein present, UPE gels were assessed utilising ImageJ software. This software allowed for the pixels present in each protein band to be quantified giving a numerical number for each band. The total pixel number per sample was determined by adding the values generated for all bands and the percentage contributed by each protein was then determined (an example is given in Figure 16). As the proteins albumin and transferrin were always present on UPE analysis the percentage these proteins contributed was used for statistical analysis.

3.2.3.1.5.2 Interpretation of the protein bands

Guidelines for the interpretation of the protein bands on the Hydragel Proteinurie K30 were provided by the manufacturers. A migration pattern was provided within the product insert depicting which proteins could be expected at each band level. The migration pattern supplied by the manufacturer is shown in Figure 17a whilst Figure 17b shows an example of a study gel interpretation.

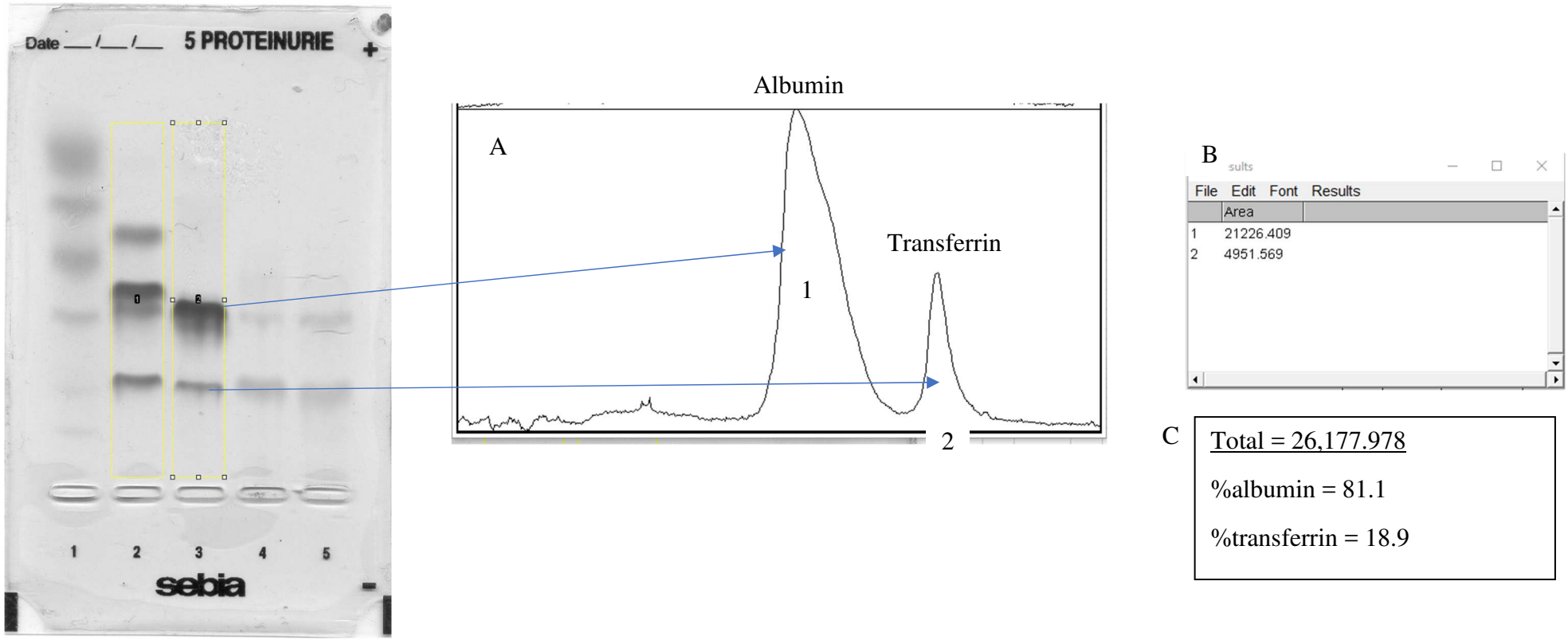


Figure 16: Example of the use of ImageJ software. Lane 3 is analysed, and the two bands represented as two peaks (A), the area under the peaks is calculated and a numerical number generated (B). These two numbers would be added together to give the total 'protein count' for the lane and then the percentage that each protein contributed calculated (C).

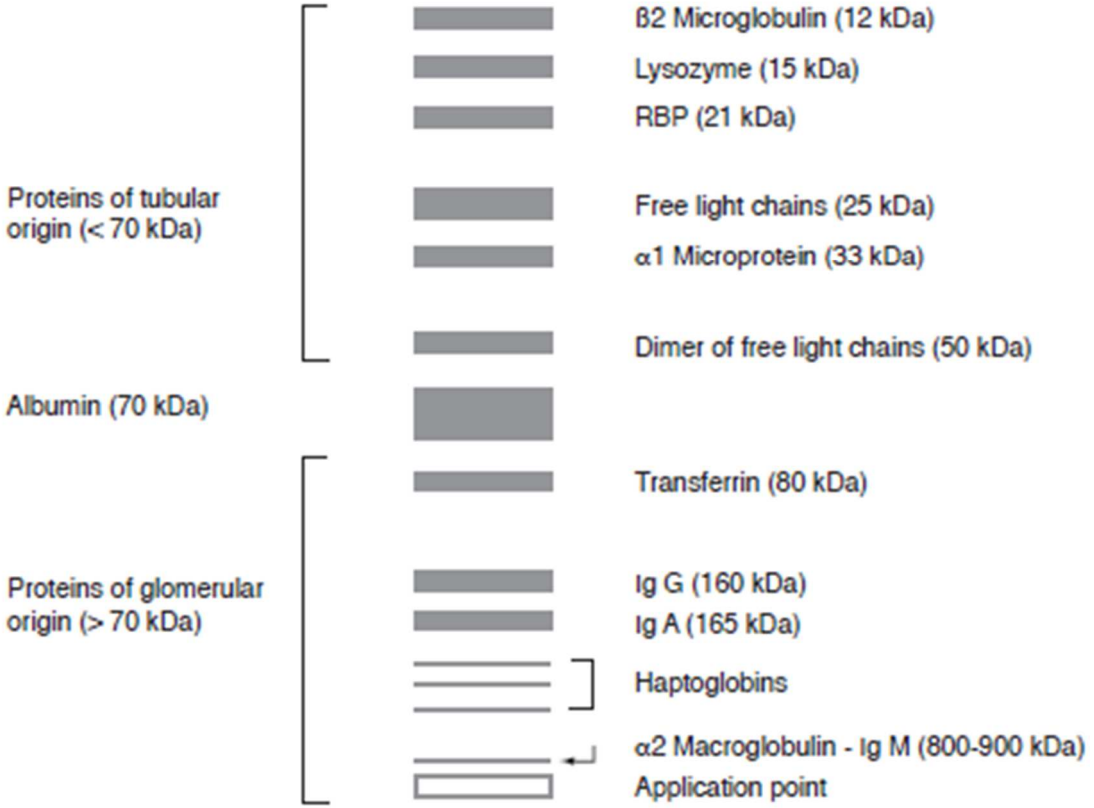


Figure 17a: Migration pattern for protein bands on Hydragel Proteinuria K20 gels (taken from product insert)

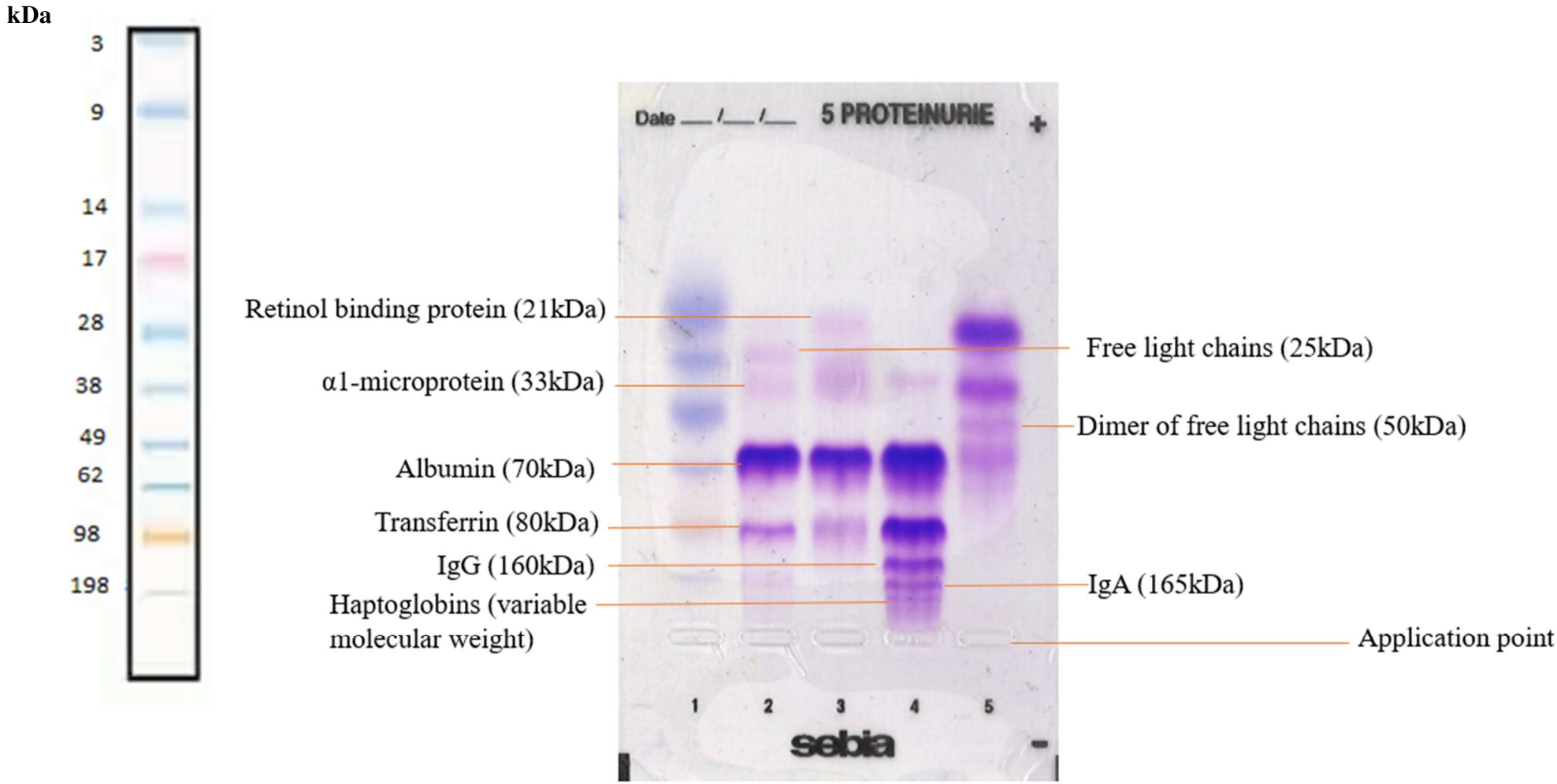


Figure 17b: An example of gel interpretation. Lane 1 of the gel contains a marker, the key for which is provided on the left. kDa=Kilodaltons

3.2.3.2 Preliminary testing biomarkers

In addition to characterising the UPE of dogs with a UPC >2.0, the presence/absence of certain urinary biomarkers was also assessed. Preliminary work to optimise measurement of GGT and NGAL was therefore performed; this work demonstrated that GGT was unstable after freezing at -20°C degrees and hence GGT analysis was not taken further. The NGAL ELISA required multiple runs to determine the best dilutions for the study. The preliminary work/methodology for both GGT and NGAL measurement is described below.

3.2.3.2.1 GGT

Urine GGT was measured using an integrated chemistry system (Dimension Xpand Plus, Siemens). To obtain a GGT measurement, a 100µL aliquot of urine was defrosted on ice and then loaded into the chemistry system.

It had been planned to batch run urine samples for GGT measurement, therefore, twenty-six urine samples from dogs with UPC >2.0 were submitted for GGT analysis along with 11 samples from healthy controls. The median value for the 26 study samples was 8 (range 2-168) whilst the median value for control samples was also 8 (range 7-33). Following preliminary evaluation of the results concern was raised regarding the stability of urine GGT in the samples. Concern arose due to the identical median values between dogs with a UPC >2.0 and control. However, more importantly, GGT had been run on some of the control samples prior to freezing and this showed that the values were considerably different to those obtained following freezing. There were 6 control samples that had GGT values available on both fresh and frozen samples (Figure 18); the median for the fresh samples was 45.5 (range 33-52) and the median for the frozen was 12.50 (range 7-19).

Therefore, an additional study was performed to determine the stability of urine GGT under various storage conditions. The findings of this study are briefly reported below.

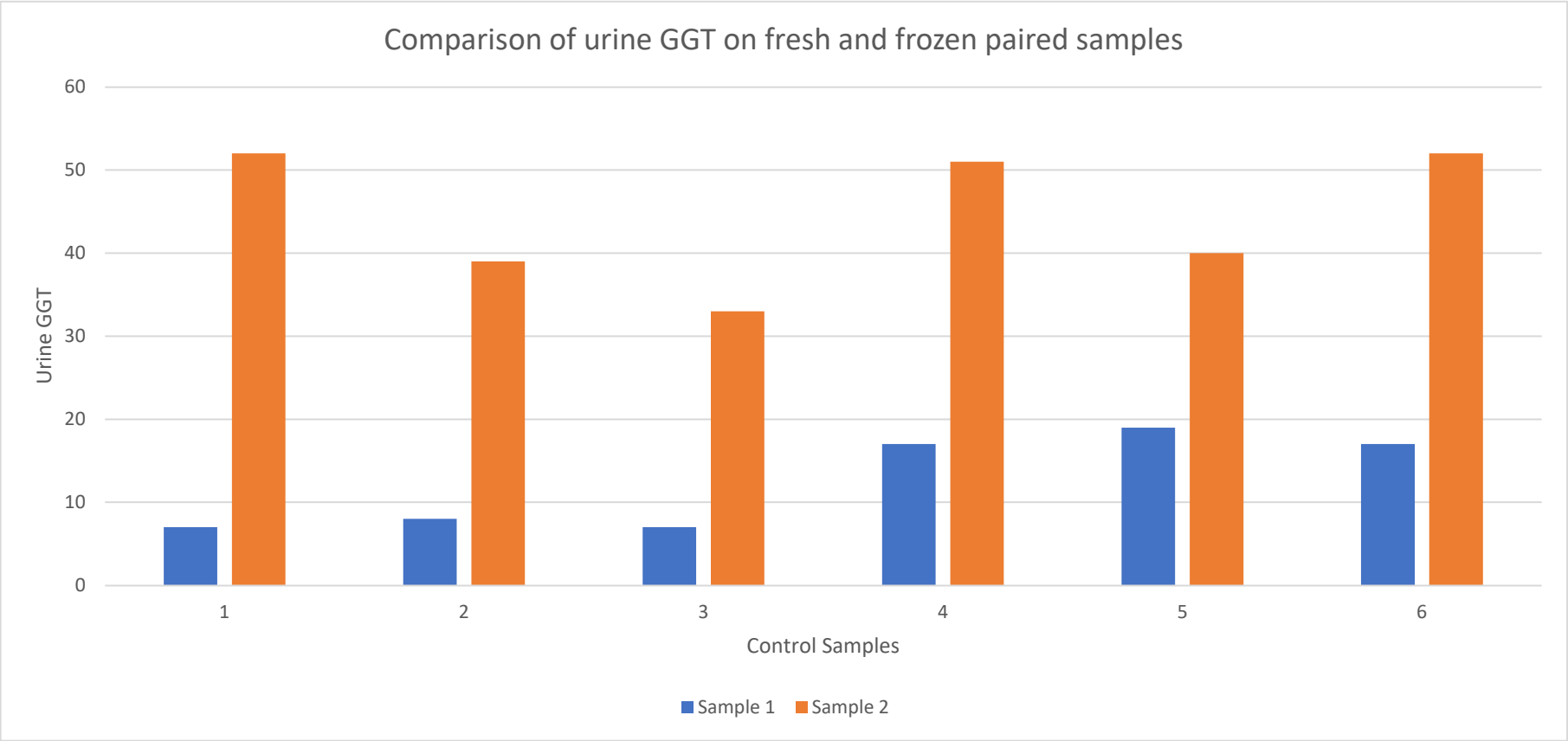


Figure 18: Bar chart comparing GGT measurements on frozen urine (sample 1) compared to fresh urine (sample 2) in six canine urine samples. GGT= gamma glutamyl transferase

3.2.3.2.1.1 Stability of urine GGT under different conditions

Due to the above, a sub analysis was performed assessing the stability of urine GGT under different conditions. Urine was collected from 12 dogs with a variety of co-morbidities, as this study was looking only at GGT stability the concurrent conditions of the dogs was not thought relevant. Full urinalysis (including GGT) was performed within 24-hours of collection. Urine was then aliquoted and subjected to different storage conditions (fridge [4°C degrees], -20°C degrees and -80°C degrees). Repeat GGT measurement was made after samples had been stored for 48 hours (at 4°C degrees), 7 days (at room temperature, fridge, -20°C and -80°C degrees), 14 days (-80°C degrees) and one month (-80°C degrees). For the samples frozen at -20°C degrees, the addition of a PI was also assessed to see if this could delay the degradation of GGT. All 12 samples were run at each time point apart from at -20°C degrees after 7 days with PI and both at -80°C degrees after 14 and 30 days; only 6 samples were run at these time points.

The results of the GGT levels at the various time points for each sample are provided in Appendix 2. To investigate statistical difference between the baseline samples and the samples taken at subsequent time points a Wilcoxon signed-rank test was performed (Table 6). Urine GGT appeared stable when stored in the fridge or at room temperature for up to 7 days, it was also stable at -80°C degrees for up to one month. Storing urine at -20°C degrees at 7 days or longer appeared to lead to considerable degradation of GGT ($p=0.002$ showing significant statistical difference between paired samples). The addition of a PI did not preserve urine GGT levels when frozen at -20°C degrees. There are sparse previous reports in the literature regarding the stability of urine GGT. GGT was found to be unaffected by the presence of haematuria, haemoglobinuria or bacteriuria in a previous study which also found that GGT levels were found to significantly decrease following storage at -20°C degrees (as with our study it was found to be stable at warmer temperatures; as it was reported to be stable at both 4°C and 20°C degrees for up to 3 days) (Ilchyshyn et al., 2019). Gossett *et al.* (1987) have also documented that GGT is stable refrigerated for at least 4 days and that a single sample correlated well with excretion over 24 hours.

The results of the above analysis suggest that urine GGT is not stable when frozen at -20°C degrees and that the addition of a PI does not significantly deter degradation. All the urine processed for analysis for this thesis was initially stored at -20°C degrees and therefore, further analysis was not performed.

	Median GGT U/L (Range)	Wilcoxon Signed-Rank P value
Baseline (n=12)	41.5 (17.0-121.0)	-
Fridge within 48h (n=12)	38.5 (16.0-112.0)	0.645
Room temperature within 7 days (n=8)	34.5 (9.0-112.0)	0.078
Fridge within 7 days (n=12)	37 (15.0-113.0)	0.181
Freezer -20 within 7 days (n=12)	7.5 (5.0-35.0)	**0.002**
Freezer -20 within 7 days + PI (n=6)	8 (5.0-36.0)	**0.043**
Freezer -80 within 7 days (n=12)	37.5 (16.0-113.0)	0.098
Freezer -80 within 14 days (n=6)	64.5 (17.0-112.0)	0.528
Freezer -80 within 30 days (n=6)	33.4 (19.0-54.0)	0.753

Table 6: Median and range of GGT (U/L) in samples stored under different conditions and results of Wilcoxon Signed-Rank (p value) when results are compared with baseline GGT values. Significant results highlighted in bold red. GGT= gamma-glutamyl transferase, P value – associated statistical significance.

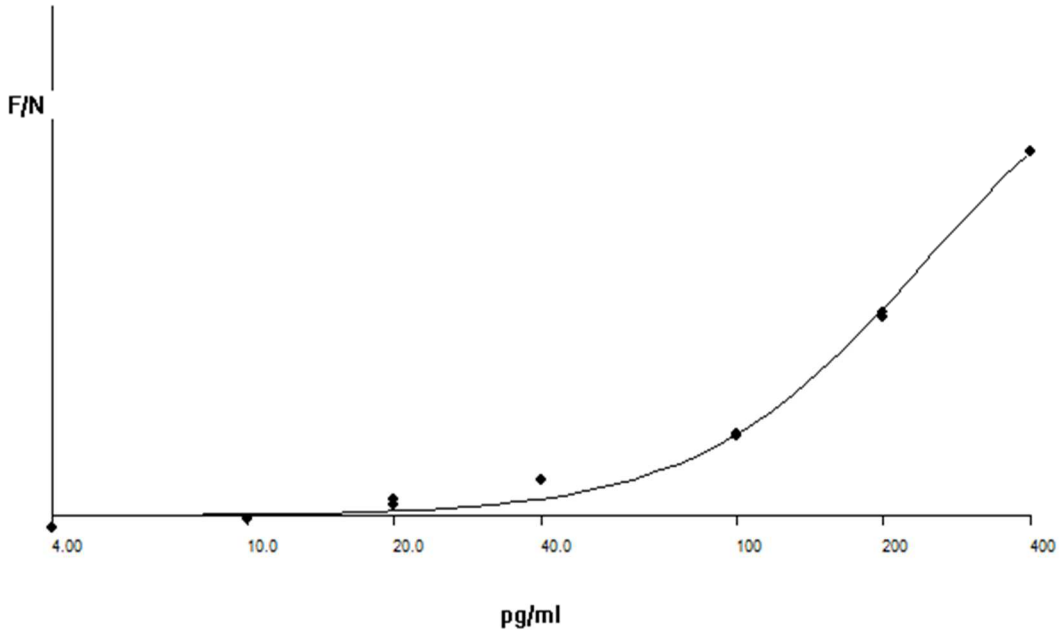
3.2.3.2.2 NGAL ELISA

To determine NGAL concentrations in the urine samples a commercially available ELISA was used (BioPorto Diagnostics, 96-well plate). The ELISA was performed as per the manufacturer's guidelines. Briefly, the urine samples were defrosted slowly and all kit reagents brought to room temperature. An initial screening dilution of 1/100 was recommended and therefore 10mL of each urine sample was combined with 990mL of prediluted sample diluent (supplied in the kit). One hundred microlitres of each calibrator dilution and diluted samples was then pipetted into appropriate wells. The wells were then covered and incubated at room temperature for 1 hour, during this time the plate was placed on a shaking platform (Grant Bio PMS 1000 microplate shaker) set at a speed of 200/minute. After this initial incubation, the contents of the wells were then washed with 300mL of diluted wash solution. Following the wash, 100mL of Biotinylated Dog-NGAL antibody was pipetted into each well and a repeat incubation performed under the same settings as above. A repeat wash was then performed before 100mL of HRP-Streptavidin was added to each well and a third incubation performed followed by a third wash. Next 100mL of 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well and the plate was incubated in the dark for exactly 10minutes. After this exact time, 100mL of stop solution was added to the wells and the plate then gentle shook for 20 seconds. The absorbance values were then read within 30minutes at 450nm.

3.2.3.2.2.1 Preliminary NGAL Testing

The first ELISA was run exactly as per manufacturer guidelines as a preliminary run. The initial results of the absorbance values were assessed first using the software AssayZap. A standard sigmoid curve was potted to generate results (Figure 19a), utilising this approach only five samples had a value within the readable range. Two of the control dogs had a value too low for the limit of detection whilst all the other samples were off scale. To try to gain further information a different method of assessment was then utilised. An alternative software (PRIZM) was employed and a second order polynomial plot was used instead of a sigmoidal plot (Figure 19b). Utilising this method provided a few more results that fell within an acceptable range of the standard, however, a significant number of samples remained unreadable. The available results from the initial ELISA are reported provided in Appendix 3.

a.



b.

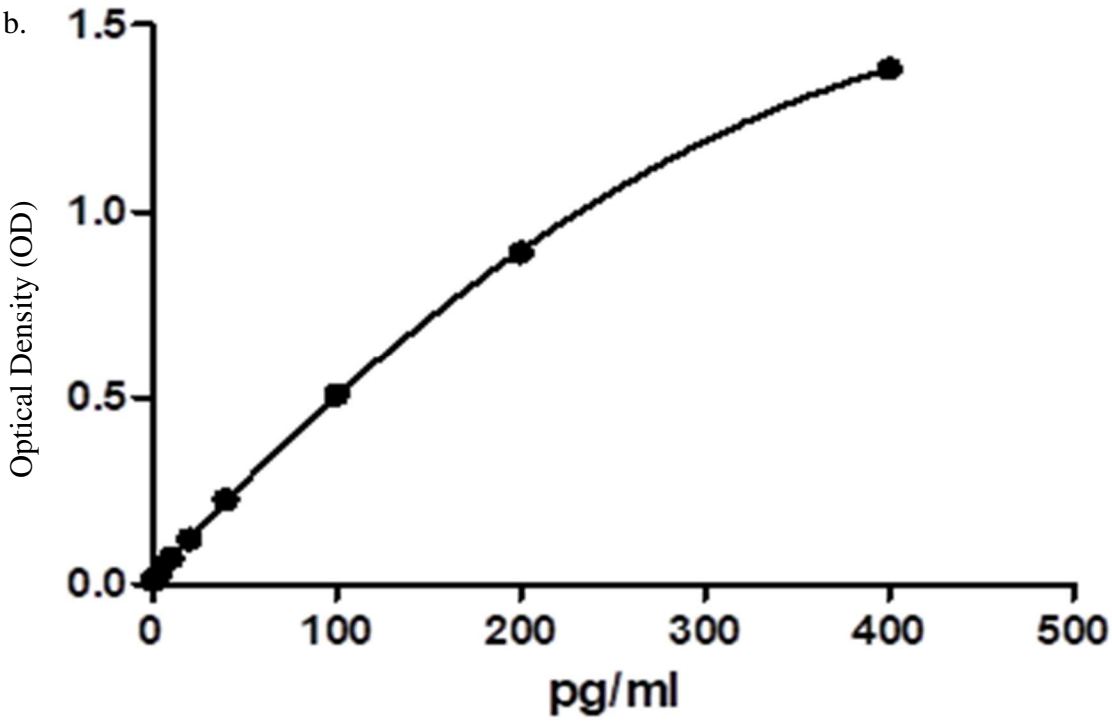


Figure 19: a=AssayZap Sigmoid Curve and b=PRIZM curve, both generated from 96-well neutrophil gelatinase associated lipocalin (NGAL) ELISA assay.

Since many samples were not within the detectable range of the ELISA, a second 96-well plate was obtained to repeat the analysis after samples had been additionally diluted. Three samples were selected that had previously given a result on the first ELISA so that these could act as control samples to compare for variability between the two assays. Using the original OD generated for each sample from the first ELISA run, dilution factors were calculated for each sample aiming to achieve an OD of 0.5. For the control samples, the manufacturers recommended 1/100 dilution was still used. For samples that had not been run on the initial ELISA, a 1/400 dilution was used. The five urine samples that had the highest OD on the original ELISA also underwent a further second dilution (double that calculated to achieve an OD of 0.5).

The second ELISA was run with the new dilution factors to bring readings on scale. However an error was made in the methodology where the pre-equilibration step of the antibody complex formation was omitted (i.e. the ELISA plate was not incubated with the samples alone for one hour prior to the addition of the biotinylated dog-NGAL antibody). Despite this, the samples repeated from ELISA 1 gave similar values (Table 7) and most samples were on scale.

Sample	1st Assay Reading	2nd Assay Reading	Difference between readings	Difference between readings (%)
1	55.3	49.3	-6	10.8
2	208.9	205.1	-3.8	1.8
3	329.1	294.5	-34.6	10.5

Table 7: Percent difference between the first and second readings for three samples acting as controls between the two neutrophil gelatinase associated lipocalin (NGAL) ELISA assays.

To eliminate any possible erroneous values due to the previous methodology problems described above, a third ELISA was run. The final dilutions utilised for this final ELISA were determined by evaluation of the results of the previous two ELISAs. In short, the previously generated OD values were analysed, and samples were then grouped so that they were either subjected to no further dilution past the one recommended by the manufacturer or a further 1/2, 1/10 or 1/20 dilution.

Previous studies have reported a range of values for NGAL in healthy dogs from 0.04-12.9ng/mL (Monari et al., 2020, Davis et al., 2021, Cobrin et al., 2016, Steinbach et al.,

2014). Therefore, an NGAL result of greater than 13ng/mL was used to define an increased NGAL. We also included seven control dogs with an inactive urine sediment and a sterile urine culture to assess the NGAL results for healthy dogs utilising the above method and test the reference range previously reported. The median NGAL for these 7 dogs was 11.1ng/mL (range: 0.74-16.8). Considering the cut-off for normal we used for this study, two control dogs had a minimally elevated NGAL result (13.4ng/mL and 16.8ng/mL).

3.2.4 SDMA

SDMA was the only non-urine parameter run. No preliminary testing was performed for the analysis of SDMA. SDMA was run in batches. Blood samples that had been stored at -80°C degrees were defrosted on ice and submitted for analysis in groups. Serum samples were submitted to Nationwide Laboratories for analysis of SDMA. This reference laboratory use liquid chromatography – mass spectrometry for their SDMA assay which is the reference method for analysis of this parameter. As SDMA can be affected by GFR, for the SDMA study, additional data were collected at the time of enrolment. Dogs that were receiving drugs affecting GFR (glucosamine, levothyroxine or diuretic medications) were not eligible for SDMA measurement. The age of the dogs was recorded as it has been suggested that puppies may have a slightly higher reference range than adult dogs, as these data is yet to be officially published dogs were not excluded based on age. Information regarding whether the clinician deemed the dog to be dehydrated at the time of presentation; whether intravenous fluid therapy (IVFT) had been administered or sedation performed within 48 hours of sample collection was also noted.

3.2.5 Definitions

Reference is made to markers of disease progression (/more advanced disease) and disease severity through the following results section. Markers of disease progression include creatinine and SDMA as these are indirect markers of GFR whilst markers of severity include albumin, cholesterol and UPC. Creatinine, SDMA, albumin and cholesterol were all measured on serum samples. Azotaemia was defined as a creatinine of >125µmol/L, hypoalbuminaemia as an albumin of <25g/L whilst hypercholesterolaemia was taken as a cholesterol of >9mmol/L (Klosterman et al., 2011, IRIS, 2019). These cut-offs were also selected to align with the previously reported retrospective study. Severe proteinuria was defined as a UPC ratio >3.5 (Littman et al., 2013). An SDMA of >18µg/dL was considered elevated as per the IRIS guidelines for CKD (IRIS, 2019). As above, a urinary NGAL result of greater than 13ng/mL was used to define abnormal for this study. For UPE analysis, dogs were referred to as having either a glomerular protein UPE pattern (only glomerular proteins present, referred to as G-UPE) or a mixed UPE pattern (both glomerular and tubular proteins present, referred to as M-UPE).

3.2.6 Statistical analysis

The statistical analysis for the prospective study largely mirrored that performed in the retrospective study. Normality testing (using the Anderson-Darling methodology) showed that for most of the continuous variables (weight, creatinine, cholesterol and UPC) non-parametric statistical analysis would be appropriate and therefore for descriptive statistics, the median and range were reported.

To determine if the presence of a M-UPE pattern was associated with more advanced disease an association between G-UPE versus M-UPE patterns and creatinine and SDMA was assessed; creatinine and SDMA were evaluated on a continuous basis using a Mann-Whitney U analysis and on a categorical basis using a Chi-squared analysis. A similar evaluation was also performed assessing albumin, cholesterol and UPC. Definitions for categorical variables have been described above.

To determine if specific proteins were correlated with more advanced disease a similar approach was utilised; an association between the presence/absence of specific proteins (RBP, a1 microprotein or IgG) and the same markers of disease progression/severity were assessed again classing the variables as either continual (Mann-Whitney U) or categorical (Chi-squared). A slightly different methodology was required to assess associations with albumin and transferrin as these proteins were present in all samples. These two proteins were considered as continuous variables themselves and an association between the presence/absence of azotaemia, increased SDMA, hypoalbuminaemia, hypercholesterolaemia or severe proteinuria and the percentage of the total of protein that albumin or transferrin accounted for assessed using the Mann-Whitney U analysis. The same markers of disease progression/severity were also assessed as continuous parameters for an association with the magnitude of the percentage of albumin and transferrin utilising the Pearson Correlation test. The methodology to obtain the percentage of albumin/transferrin present is described above.

To determine if the magnitude of NGAL was associated with markers of disease progression/severity, NGAL was considered as a continuous variable and evaluated against the presence/absence of azotaemia, increased SDMA, hypoalbuminaemia, hypercholesterolaemia and severe proteinuria again utilising a Mann-Whitney U analysis. To allow for assessment of the magnitude of NGAL and the markers of disease progression/severity when they were considered as continual variables a Pearson Correlation test was again performed. The magnitude of NGAL and the presence of a G-UPE versus M-UPE pattern was evaluated utilising a Mann-Whitney U test. As the majority of the NGAL

results were above the reference range NGAL itself was only assessed as a continuous parameter. Regarding GGT, due to changes in the planned analysis (described above) the only statistical analysis performed was a Wilcoxon Signed-Rank test analysis to compare GGT values following various storage conditions.

Several survival analyses were performed considering different parameters. Overall survival was always assessed using Kaplan-Meier analysis and Log Rank (Mantel Cox) testing to determine for a statistical difference in median survival times. For the retrospective study, 12-month survival was also assessed in addition to overall survival, however, for the prospective study 6-month survival was instead assessed to increase the sample size available for analysis. Six-month survival was assessed using Pearson Chi-squared analysis. Variables assessed for possible statistical significance with survival were the presence of G-UPE versus M-UPE protein pattern and the presence of specific proteins (RBP, a1 microprotein and IgG) and the presence of markers of disease progression/severity.

Statistical analysis was performed using the commercially available software SPSS (IBM SPSS Statistics 27). Statistical significance was taken to be $P<0.05$.

3.3 Results

3.3.1 Sample population

Urine samples were collected over the study period from 15th of November 2021 to 31st May 2022. During this period 299 canine urine samples were identified with a UPC of >2.0. Twenty-two of these samples were excluded as a full urinalysis was not performed whilst 92 samples were excluded due to the presence of an active sediment. A further 16 samples were excluded as there was no concurrent serum biochemistry or renal profile performed; an additional 21 samples were excluded as the 'concurrent' blood testing was performed >48hours before/after urine sample collection. One urine sample was excluded as the results were reported 5 days after collection due to a Bank Holiday. Five samples were excluded as they had a globulin above the cut-off outlined for this study. After these exclusions were made, the study population was checked for duplicates; 35 dogs had multiple urine samples within the timeframe of study period, for these dogs, only the earliest urine sample was considered for inclusion. Forty-seven samples were excluded due to medications that the dog was receiving (corticosteroids n=17, ACEi n=14, TKI n=9 and ARB n=7). Five samples were excluded due to signs suggestive of lower urinary tract disease whilst 2 samples were excluded as the dogs were reported to have myalgia. This left 53 samples that were eligible for inclusion. Residual urine was available for 36 of these dogs. On review of the data, one dog had immune-mediated haemolytic anaemia (IMHA) and therefore should not have been included as the proteinuria was considered pre-renal. This dog was not included in description of the study population but did have a UPE run. The UPE profile was run for this dog for interest as dogs with IMHA are suspected to have pre-renal proteinuria rather than renal. Similarly, two samples from dogs with multiple myeloma had a UPE profile run although they were again not included in the analysis. The inclusion of these two dogs was also for interest and to screen for the protein profile present in another type of disease process that would be expected to produce pre-renal (and hence mainly low molecular weight) proteinuria. This left 35 dogs with presumed glomerular proteinuria, results from UPE were available for 32 of these dogs and these dogs therefore made up the final study population. Due to the cost of the Hydragel Proteinurie K20 gels only 10 gels were available; each gel could be used to run a total of four samples (with one lane being used for the protein marker). Thirty-two clinical samples were run, four lanes were utilised for control samples (two lanes pre and two lanes post precipitation), finally three sample suspected to have pre-renal proteinuria were run for interest and one sample with organisms on sediment examination was run in error.

The median age of the final study population was 2964 days (8.2 years) (range: 548-4760 days; 1.5-13.2 years). The median weight at the time of sampling was 15.5kg (range: 4.3-65.0kg). There were 8 crossbred dogs in the study population; purebred dogs represented by more than one were the Border Collie (n=4), Yorkshire Terrier (n=3), Cocker Spaniel (n=3), Miniature Schnauzer (n=2) and Lhasa Apso (n=2). Of the study population, 50.0% were male (n=16/32, neutered n=9) and 50.0% female dogs (n=16/32, neutered n=14). Regarding the urine samples, 71.9% (n=23/32) were collected in the hospital whilst 28.1% (n=9/32) were collected at home. Fifty-six percent (n=18/32) of samples were collected via cystocentesis; 40.6% (n=13/32) were free catch samples and 3.1% (n=1/32) via urinary catheter.

Fifteen dogs had a concurrent urine culture performed; the culture was sterile in 93.3% (n=14/15) of dogs. The dog with the positive urine culture was included as its sediment fulfilled our criteria to be classed as 'inactive'. On evaluation of the serum biochemistry, 37.5% (n=12/32) of dogs were azotaemic (median creatinine 110µmol/L, range: 52-415) and 17.1% (n=5/35) were hypoalbuminaemic (median albumin 30g/L, range: 15-40). Cholesterol was available for 96.9% (n=31/32) dogs and 44.1% (n=13/31) were hypercholesterolaemic (median cholesterol 7.96mmol/L, range 4.89-17.7). The median UPC of the study population was 4.87 (range 2.03-26.26); severe proteinuria was present in 62.5% (n=20/32). Appendices 4 and 5 detail the study population demographics and biochemical parameters.

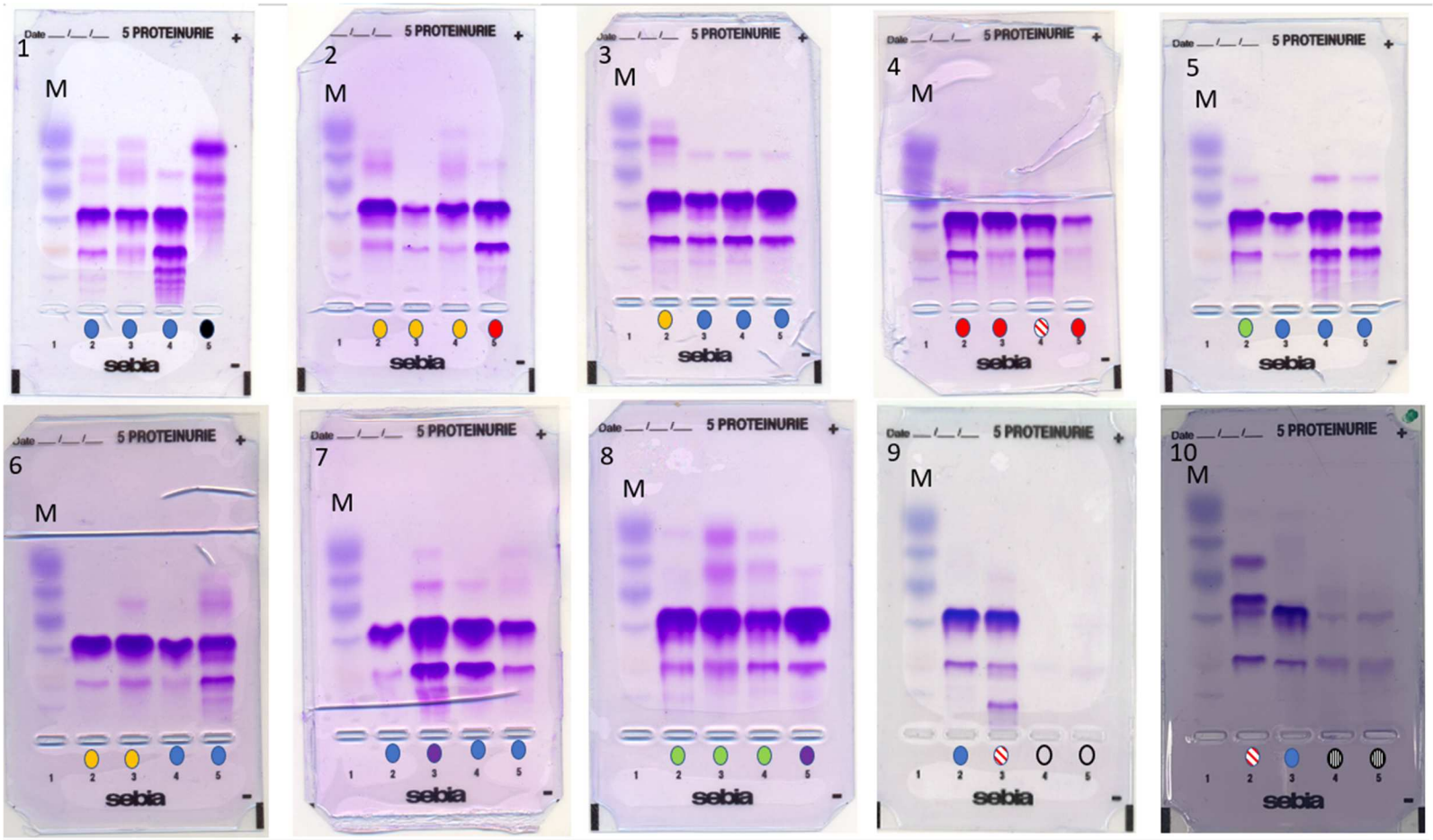
SDMA measurement was available for 71.9% (n=23/32) of dogs. SDMA was increased in 52.2% (n=12/23) (median 15.3µg/dL, range: 6.2-57.4). When the clinician questionnaire results were taken into consideration; 3 dogs (13.0%) of dogs with an SDMA measurement were reported to be dehydrated; 3 dogs (13.0%) had received IVFT and 2 (8.7%) had been sedated in the preceding 48-hours. No dogs were excluded on the basis that they were receiving medication that could impact on GFR. The median age for the SDMA population was 2952 days (range: 451-4725); as previously mentioned, different reference ranges have been suggested for puppies, however, as none of the population with SDMA were less than 1-year of age, in addition to the fact altered reference ranges based on age need to be more widely established, all dogs were included for SDMA analysis regardless of age.

The medical records of the included dogs were reviewed, and dogs split into whether or not there was a trigger evident for their proteinuria (endocrinopathy, neoplasia, significant inflammatory disease or hyperlipidaemia). There were 50.0% (n=16/32) of dogs with an identifiable trigger (endocrine n=6, neoplasia n=4, inflammatory n=4 and hyperlipidaemia n=2). No trigger was identified in 50.0% (n=16/32) of the study population. Diagnostic

investigations suggested to screen for a trigger for glomerular proteinuria have been previously outlined (Littman et al., 2013). Recommended screening includes thoracic and abdominal imaging, blood pressure analysis and screening for appropriate infectious disease. Records were assessed to review the investigations dogs with no trigger identified underwent to determine to what extent a trigger had been screened for; 43.8% (n=7/16) of dogs with 'no trigger' had thoracic imaging within 6 weeks of their urine sample being submitted. All dogs had abdominal imaging performed; 93.8% (n=15/16) had abdominal imaging performed within 6 weeks, one dog had abdominal imaging 54 days prior to the urine sample being collected. Fourteen dogs had a blood pressure measurement obtained, all but one of these measurements was performed within 6 weeks of the included urine sample being collected; one dog had a blood pressure assessment 68-days after the urine sample was collected. Four dogs were reported to be hypertensive (systolic blood pressure >160mmHg). Finally, screening for an infectious trigger (via a SNAP 4DX test), was performed in 75% (n=12/16) of dogs; all dogs bar one had this performed within 6 weeks, one dog had a SNAP 4DX performed 54 days after the included urine sample. All SNAP 4DX results were negative apart from one dog that tested positive for antibodies against *Borrelia burgdorferi.*

3.3.2 SDS Hydragel Proteinuria K20 – initial interpretation

In total, 40 samples were run on the Hydragel Proteinuria K20 gels. Gels were run between April and June 2022. Of these 40, 32 lanes represented dogs with a UPC >2.0. Four gel lanes were utilised for control dogs, three gel lanes were used to run urine samples from dogs with expected pre-renal proteinuria for interest and one lane was used in error for a sample that should have been previously excluded due to the presence of ≥1+ organisms on microscopy. An overview of the 10 gels is provided in Figure 20.



Key

M – denotes lane with maker

- No trigger
- Neoplasia
- Endocrine
- Inflammatory
- Hyperlipidaemia
- IMHA (excluded)
- Neoplasia but excluded*
- Control dogs
- Control dogs after precipitation

Samples excluded from analysis

- Gel 1, lane 5 – IMHA

- *Gel 4, lane 4 – neoplasia with organisms on microscopy (sample wrongly run)

- *Gel 9, lane 3 – Multiple myeloma (Bence Jones protein negative)

- *Gel 10, lane 2 – Multiple myeloma (Bence Jones protein positive)

Figure 20: An overview of the 10 gels generated. Lane 1 of each gel represents the protein marker. Lanes 2-5 represent samples included in the study (apart from where indicated in the key).

3.3.3 Final Study Results

3.3.3.1 Assessment of UPE in dogs with UPC >2.0: evaluation of the frequency of the presence of tubular proteins

Of the 32 samples run in dogs with a UPC >2.0, the majority (71.9%, n=23/32) had both glomerular and tubular proteins present (M-UPE) whilst 28.1% (n=9/32) had only glomerular proteins present (G-UPE) (for example, Figure 20: Gel 6, Lanes 2 and 4). No dog had only tubular proteins present. There were 16 dogs with an identifiable potential trigger for their proteinuria and 16 dogs without such a trigger. Markers of disease progression/severity were available for all dogs aside from one dog that did not have a cholesterol result available. Additionally, SDMA results were only available for 23 dogs.

3.3.3.1.1 Glomerular protein UPE pattern

As above, there were 9 dogs with a G-UPE pattern; albumin and transferrin were the only protein bands visible in all 9 of these dogs. These proteins were also the only ones detected in the control samples albeit at much lower concentrations requiring precipitation for detection. The median and range values for the markers of disease progression/severity for the dogs with G-UPE are provided in Table 8. Of the dogs with G-UPE, 22.2% (n=2/9) were azotaemic; no dog was hypoalbuminaemic and 55.6% (n=5/9) had hypercholesterolaemia. Severe proteinuria was present in 55.6% of dogs with a G-UPE. SDMA was available for 8/9 dogs and 25% (n=2/8) had an increased SDMA.

3.3.3.1.2 Mixed protein UPE pattern

Most dogs (71.9%) had a M-UPE pattern with both glomerular and tubular proteins present. While the mixture of proteins present within this group was variable, there were three distinct protein combinations observed in several samples. Again, the median and range values for the markers of disease progression/severity for the dogs with M-UPE are provided in Table 8. Of the dogs with M-UPE 47.8% (n=11/23) of dogs were azotaemic; 26.1% (n=6/23) hypoalbuminaemic and 36.4% (n=8/22) of this group of dogs had hypercholesterolaemia. Severe proteinuria was present in 65.2% (n=15/23) of dogs. SDMA results were available for 15/23 dogs and the result was increased in 53.3% (n=8/15) of cases.

3.3.3.2 Assessment of whether the presence of tubular proteins on UPE analysis in dogs with a UPC>2.0 is associated with more advanced/severe disease.

To evaluate whether the presence of tubular proteins on UPE analysis correlated with more advanced disease, the relationship between UPE protein pattern and creatinine and SDMA was analysed. Despite there being a substantial difference in the proportion of dogs that were azotaemic in each group (22.2% of dogs with glomerular protein versus 47.8% of dogs with mixed protein UPE pattern), there was no association between creatinine and UPE protein pattern when assessed on either a continual or categorical basis (Table 8 and 9). Similarly, although there was no statistical association between SDMA and the UPE protein pattern when assessed on either a continual or categorical basis, as with creatinine, increased SDMA was seen more frequently in dogs with a mixed protein UPE pattern (53.3% of dogs with M-UPE pattern had increased SDMA compared to 25.0% with a G-UPE pattern). We also assessed whether the type of UPE pattern was associated with serum albumin, cholesterol or UPC (assessed categorically and continually) and again, despite differences on descriptive analysis, no statistical significance was detected between dogs with a G-UPE or M-UPE pattern (Table 8 and 9).

Clinicopathological Parameter	**Median (Range)**		**Mann-Whitney**	**P value**
	Glomerular protein pattern	Mixed protein pattern		
Creatinine (µmol/L)	87 (52-202)	113 (58-415)	69.0	0.148
SDMA (µg/dL) (n=23)	12.0 (6.2-32.1)	18.8 (7.4-57.4)	38.5	0.165
Serum Albumin (g/L)	31 (26-39)	30 (15-39)	70.0	0.158
Cholesterol (mmol/L) (n=31)	10.50 (6.10-12.06)	7.80 (4.89-17.70)	77.0	0.338
UPC	3.91 (2.80-6.23)	5.85 (2.03-26.26)	74.0	0.216

Table 8: Comparison between markers of disease progression/severity (assessed continually) in dogs with glomerular or mixed (glomerular and tubular) protein patterns on urine protein electrophoresis analysis. Parameters were available for all dogs (n=32) unless otherwise indicated. P value=associated statistical significance; SDMA=symmetric dimethylarginine; UPC=urine protein creatinine ratio.

Clinicopathological Parameter	Pearson Chi Squared	OR (95% CI) †	P value
Azotaemia (Y/N)	1.758	-	0.185
Increased SDMA (Y/N) [n=23]	1.704	-	0.192
Hypoalbuminaemia (Y/N)	2.890	-	0.089
Hypercholesterolaemia (Y/N) [n=31]	0.966	-	0.326
Severe proteinuria (Y/N)	0.258	-	0.612

Table 9: Comparison between disease progression/severity (assessed categorically) between dogs with glomerular or mixed (glomerular and tubular) protein pattern on urine protein electrophoresis analysis. †Estimated only if $p<0.05$. Parameters were available for all dogs (n=32) unless otherwise indicated. 95% CI= 95% confidence interval; OR=Odds ratio, P value=associated statistical significance; SDMA=symmetric dimethylarginine.

3.3.3.3 Evaluation of the association between the presence of individual proteins detected on UPE analysis and markers of disease progression/severity.

To perform more in-depth analysis of our UPE results, we assessed whether the presence of any specific protein was suggestive of more advanced or severe disease.

3.3.3.3.1 Tubular proteins

The UPE gels utilised in this study screened for the following tubular proteins: RBP, free light chains, a1 microprotein and dimer of free light chain. From the total study population 28.1% (n=9/32) had an RBP band present. Free light chains were present in 6.3% (n=2/32) whilst a1 microprotein was the most frequently detected tubular protein present in 68.8% (n=22/32) of samples. Dimer of free light chain was not present in any sample. Table 10 shows the presence of markers of disease progression/severity in dogs with and without these individual tubular proteins. SDMA is not included in this table as SDMA was not available for all dogs. Twenty-three dogs had SDMA results available. Of the dogs with RBP detected, SDMA results were available for 6 dogs and 50.0% (n=3/6) had an increased SDMA. Both dogs with free light chain present had SDMA results available; 50% (n=1/2) had an increased SDMA. Fifteen of the dogs with SDMA results had a1 microprotein present and 53.3% (n=8/15) had an increased SDMA.

Table 11 and 12 show the results of statistical testing to look for an association between the presence of individual tubular proteins and markers of disease progression/severity (assessed both as continual and categorical variables). The only result reaching statistical significance was that the presence of a1 microprotein was associated with an increasing creatinine when assessed on a continuous basis (p=0.049) (Figure 21). However, it is interesting to note that although a statistical significance was not reached, no dog without a1 microprotein was hypoalbuminaemic compared to the 27.3% of dogs with a1 microprotein present that were hypoalbuminaemic.

			Azotaemic (%)	Hypoalbuminaemic (%)	Hypercholesterolaemic (%)	Severe proteinuria (%)
Tubular proteins	**RBP**	Yes (n=9)	5 (55.6)	2 (22.2)	2 (22.2)	5 (55.6)
		No (n=23)	8 (34.8)	4 (17.4)	11 (47.8)	15 (65.2)
	Free light chain	Yes (n=2)	2 (100.0)	0 (0.0)	0 (0.0)	1 (50.0)
		No (n=30)	11 (36.7)	6 (18.8)	12 (37.5)	19 (59.4)
	A1-microprotein	Yes (n=22)	11 (50.0)	6 (27.3)	8 (36.4)	14 (63.6)
		No (n=10)	2 (20.0)	0 (0.0)	5 (50.0)	6 (60.0)
	Dimer of free light chain	Yes (n=0)				
		No (n=32)	13 (40.6)	6 (18.8)	13 (40.6)	20 (62.5)
Glomerular proteins	**IgG**	Yes (n=11)	6 (54.5)	4 (36.4)	4 (40.0)	9 (81.8)
		No (n=21)	7 (33.3)	2 (9.5)	9 (42.9)	11 (52.4)
	IgA	Yes (n=3)	3 (100.0)	1 (33.3)	2 (66.6)	1 (33.3)
		No (n=29)	10 (34.5)	5 (17.2)	11 (38.0)	19 (59.4)
	Haptoglobin	Yes (n=2)	2 (100.0)	0 (0.0)	1 (50.0)	0 (0.0)
		No (n=30)	11 (36.7)	6 (20.0)	12 (37.5)	20 (62.5)
	A1 macroglobulin	Yes (n=0)				
		No (n=32)	13 (40.6)	6 (18.8)	13 (40.6)	20 (62.5)

Table 10: The presence of markers of disease progression/severity in samples with and without specific proteins present. IgA= immunoglobulin A; IgG= immunoglobulin G; RBP= Retinol binding protein,

Protein	Categorical Parameter	Chi-squared	P-value	OR (95% CI) †
RBP	Azotaemia (yes/no)	1.157	0.282	-
	Increased SDMA (yes/no) [n=23]	0.140	0.708	-
	Hypoalbuminaemia (yes/no)	0.099	0.753	-
	Hypercholesterolaemia (yes/no) [n=31]	2.024	0.155	-
	Severe proteinuria (yes/no)	0.258	0.612	-
a1-microprotein	Azotaemia (yes/no)	2.565	0.109	-
	Increased SDMA (yes/no) [n=23]	1.704	0.192	-
	Hypoalbuminaemia (yes/no)	3.357	0.067	-
	Hypercholesterolaemia (yes/no) [n=31]	0.394	0.530	-
	Severe proteinuria (yes/no)	0.039	0.844	-
IgG	Azotaemia (yes/no)	1.347	0.246	**-**
	Increased SDMA (yes/no) [n=23]	1.806	0.179	**-**
	Hypoalbuminaemia (yes/no)	3.413	0.065	**-**
	Hypercholesterolaemia (yes/no) [n=31]	0.023	0.880	**-**
	Severe proteinuria (yes/no)	2.669	0.102	**-**

Table 11: Chi-squared analysis to assess for association between specific proteins and the presence/absence of markers of disease progression/severity and 6-month survival. Parameters were available for all dogs (n=32) unless otherwise indicated. †Estimated only if $p<0.05$; 95% CI = 95% confidence interval; IgG=immunoglobulin G; P value=associated statistical significance; RBP=retinol binding protein; SDMA=symmetric dimethylarginine.

Protein	Continuous Parameter	Median (Range)		Mann-Whitney U	P-value
		Protein present	Protein absent		
RBP	Creatinine (µmol/L)	138 (58-400)	94 (52-415)	76.5	0.258
	SDMA (µg/dL) (n=23)	18.8 (11.5-57.4)	14.9 (6.2-32.9)	39.0	0.401
	Serum Albumin (g/L)	28 (19-36)	30 (15-39)	94.0	0.689
	Cholesterol (mmol/l) (n=31)	6.55 (4.90-12.25)	9.78 (5.57-17.7)	60.0	0.090
	UPC	5.42 (2.03-15.80)	4.31 (2.19-26.26)	103.0	0.983
a1-microprotein	Creatinine (µmol/L)	**126 (58-415)**	**79 (52-202)**	**61.50**	**0.049**
	SDMA (µg/dL) (n=23)	18.8 (7.4-57.4)	12 (7.4-57.4)	38.5	0.165
	Serum Albumin	30 (15-39)	31 (26-39)	84.5	0.298
	Cholesterol (mmol/l) (n=31)	7.96 (5.50-17.70)	9.78 (4.89-12.06)	98.0	0.767
	UPC	5.63 (2.0-26.26)	4.11 (2.24-13.21)	93.0	0.489
IgG	Creatinine (µmol/L)	142 (60-415)	87 (52-400)	75.0	0.108
	SDMA (µg/dL) (n=23)	20.30 (10.6-29.0)	14.9 (6.2-57.4)	46.0	0.366
	Serum Albumin	**28 (15-32)**	**31 (19-39)**	**63.0**	**0.036**
	Cholesterol (mmol/l) (n=31)	8.26 (5.57-17.70)	7.74 (4.89-12.25)	92.5	0.597
	UPC	5.85 (2.19-26.26)	3.91 (2.03-15.8)	87.0	0.258

Table 12: Mann-Whitney U analysis to assess for association between specific proteins and makers of disease progression/severity assessed on a continuous basis. Parameters in bold red represent statistical significance. Parameters were available for all dogs (n=32) unless otherwise indicated. IgG=immunoglobulin G; P value=associated statistical significance; RBP=retinol binding protein; SDMA=symmetric dimethylarginine; UPC=urine protein creatinine ratio

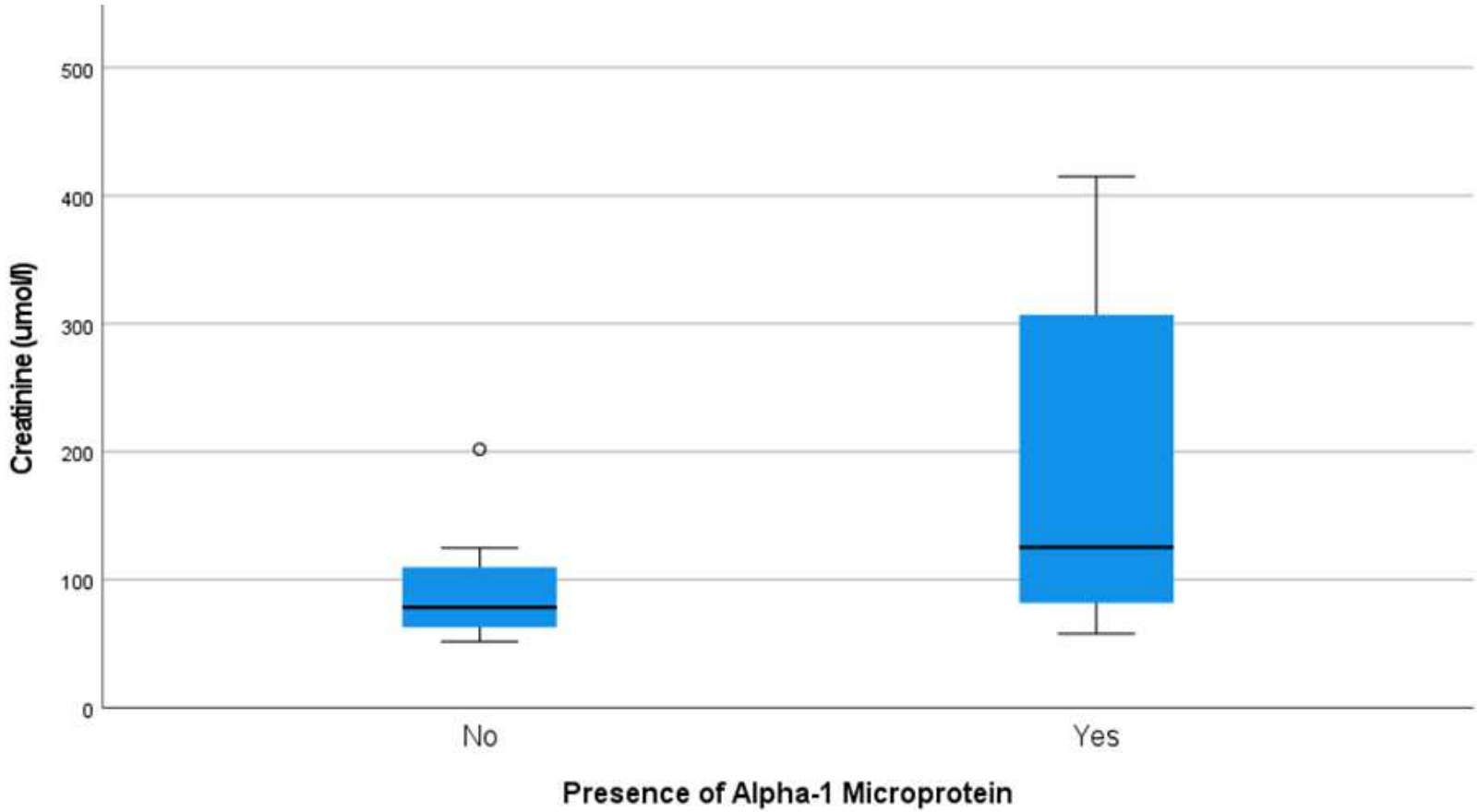


Figure 21: Box and Whisker blot demonstrating creatinine levels in dogs with and without the presence of alpha-1 microprotein. Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The circle represents an outlier.

3.3.3.3.2 Glomerular proteins

The UPE gels utilised in this study screened for the following glomerular proteins: albumin, transferrin, IgG, IgA, haptoglobin and a2 macroglobulin. As discussed previously, albumin and transferrin were present in all samples and therefore, these proteins are discussed below. IgG was present in 34.4% (n=11/32) of samples whilst IgA was present in 9.4% (n=3/32) of dogs. Haptoglobin was present in 6.3% (n=2/32) whilst a2 macroglobulin was not detected in any sample. The presence/absence of markers of disease progression/severity in association with these glomerular proteins is presented in Table 10. Again, SDMA is not presented here as results were only available for 23 dogs. Of the dogs with IgG detected, SDMA was available for 8 dogs, 62.5% (n=5/8) had an increased SDMA. Two of the dogs with IgA had SDMA results available, one of which had an abnormal result. One dog with haptoglobin had an SDMA result which was also increased above the reference range. Only IgG was included in statistical analysis due to low numbers of samples with IgA and haptoglobin. The results for statistical testing for an association between the presence of IgG and markers of disease progression/severity are presented in Table 11 and 12. The main finding was that the presence of IgG showed statistical significance with serum albumin (when assessed as a continuous variable) (Figure 22).

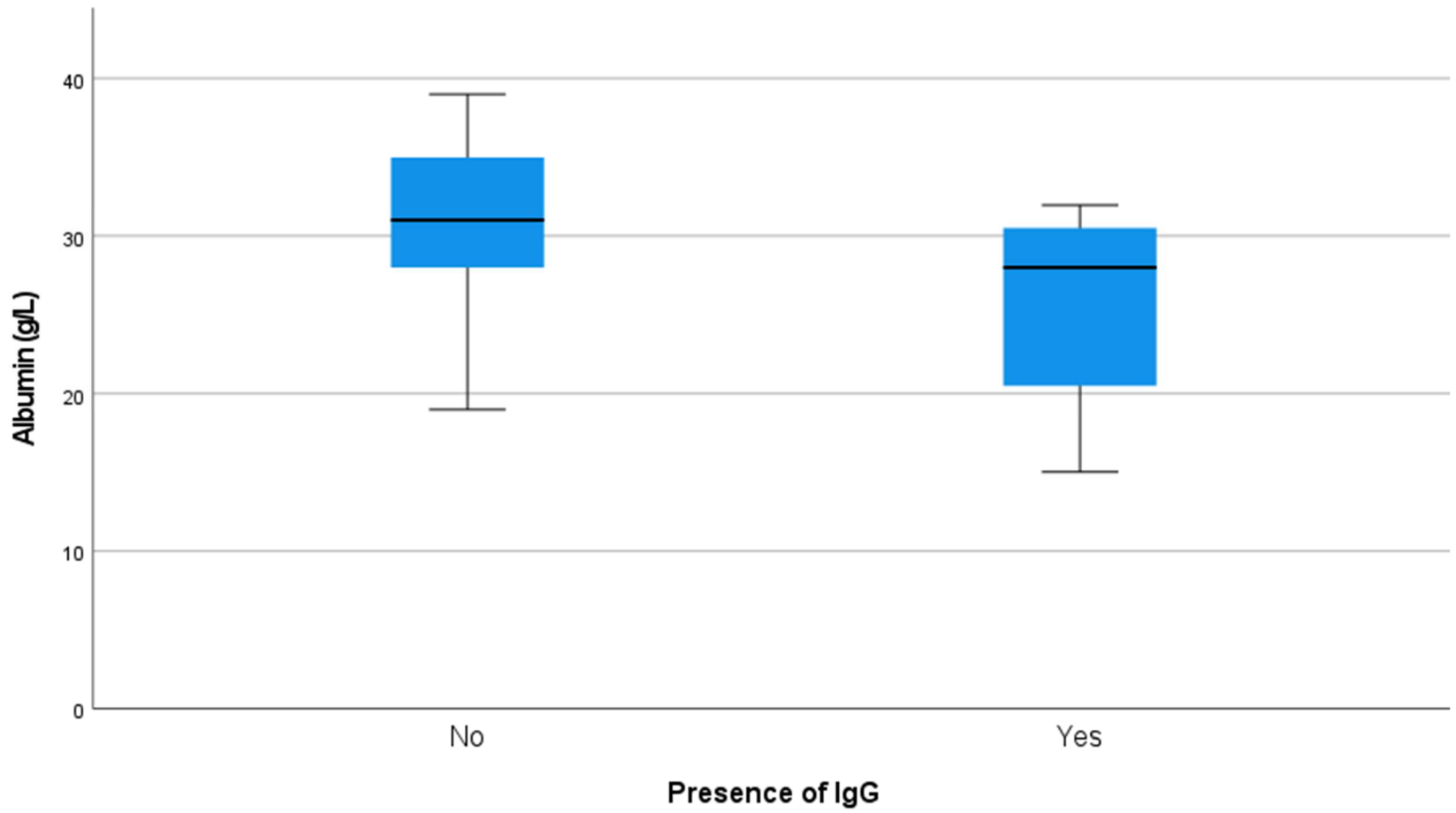


Figure 22: Box and Whisker blot demonstrating serum albumin levels in dogs with and without the presence of immunoglobulin G (IgG). Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box.

As albumin and transferrin were glomerular proteins that were present in all samples they were assessed as continuous variables with the percentage of albumin and transferrin as a proportion of the total protein present being considered. (The percentage of albumin/transferrin was calculated using ImageJ software as previously described). Albumin was always the most predominant protein present whilst transferrin was the second most predominant protein in 90.6% (n=29/32); a1 microprotein replaced transferrin as the second most prominent protein in 3 cases. The percent contributed by each protein in individual samples is provided in Appendix 6.

Albumin

The percentage of urine protein present made up by albumin varied from 42.5-90.3%. The percentage of urine albumin and association with the presence/absence of azotaemia, increased SDMA, hypoalbuminaemia, hypercholesterolaemia and severe proteinuria was analysed. The percentage of urinary albumin present was associated with the presence of azotaemia (MW-U 45.0, p=0.003) and hypoalbuminaemia (MW-U=25.0, p=0.011) but not increased SDMA (MW-U=37.0, p=0.082), hypercholesterolaemia (MW-U88.0, p=0.246) or severe proteinuria (MW-U=111.0, p=0.726). Dogs with azotaemia or hypoalbuminaemia had a lower percentage of urinary albumin making up their total protein content compared to dogs that were not azotaemic or hypoalbuminaemic (Figure 23 and 24). When markers of disease progression/severity were assessed continually, the percentage of urine albumin was also shown to correlate with creatinine (Pearson Correlation Score -0.497, p=0.004); as the percent of urine albumin decreased the creatinine increased. There was also a statistically significant correlation between urine albumin and serum albumin (Pearson Correlation Score 0.458, p=0.008) in that as the percentage of urine albumin decreased the serum albumin also decreased. There was no correlation between the percentage of albumin and SDMA, cholesterol or UPC when these parameters were assessed continually.

Transferrin

The percentage of protein that transferrin contributed ranged from 9.7-51.9%. The percentage of transferrin present was not associated with the presence of azotaemia (MW-U 98.0, p=0.328), increased SDMA (MW-U 54.0, p=0.495), hypoalbuminaemia (MW-U=71.0, p=0.735), hypercholesterolaemia (111.0, p=0.810) or severe proteinuria (MW-U=94.0, p=0.312). When the same parameters were assessed continually, there was no correlation between the percentage of transferrin and the magnitude of creatinine (Pearson Correlation Score 0.122, p=0.507), SDMA (Pearson Correlation Score -0.046, p=0.833), serum albumin (Pearson Correlation Score -0.290, p=0.107) or cholesterol (Pearson Correlation Score 0.036, p=0.848).

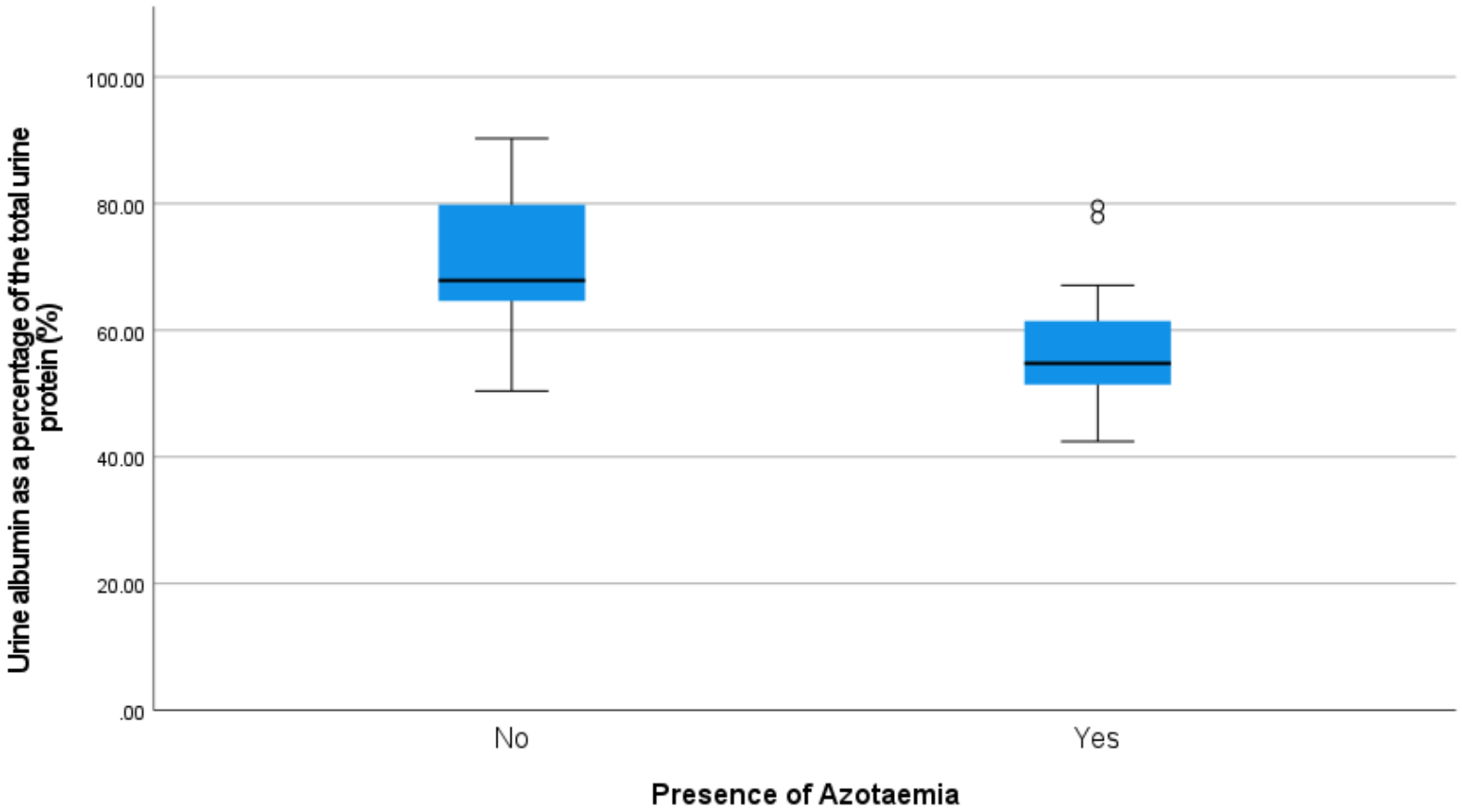


Figure 23: Box and Whisker Plot showing the percentage of total protein present made up by albumin in dogs with and without azotaemia (defined as a creatinine >125µmol/L). Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The circles represents mild outliers.

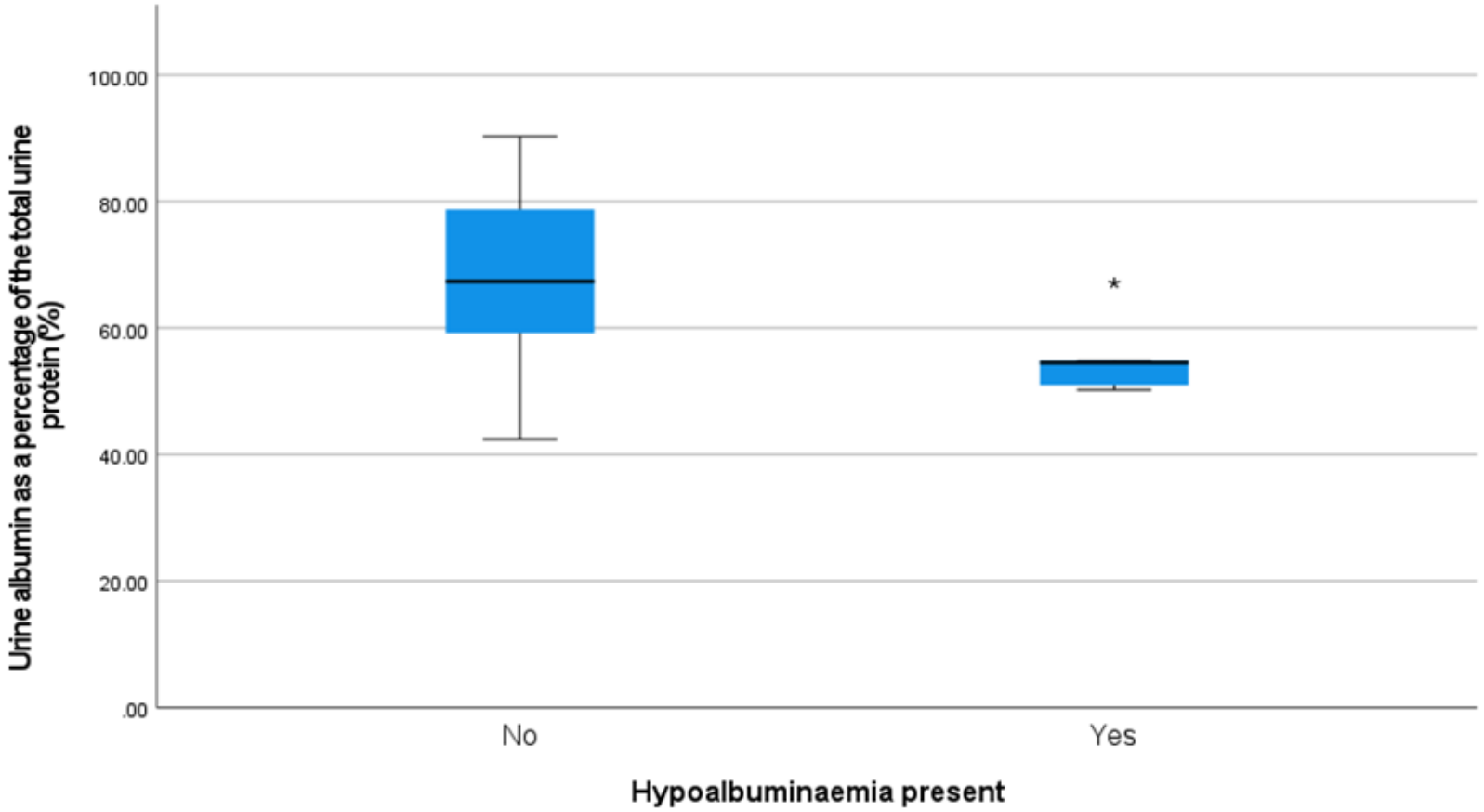


Figure 24: Box and Whisker Plot showing the percentage of total urine protein present made up by albumin in dogs with and without hypoalbuminaemia (defined as a serum albumin <25g/L). Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The asterix represents an extreme outlier.

3.3.3.4 Evaluation of the use of UPE to distinguish between dogs with and without a trigger present for their proteinuria.

Fifty percent (n=16/32) of the study population had a potential trigger for their proteinuria identified whilst the remaining 50% were deemed to have no underlying trigger. Of those dogs with a trigger these were grouped into the following categories: endocrine (n=6), neoplasia (n=4), inflammatory (n=4) and hyperlipidaemia (n=2). The markers of disease progression/severity for dogs with and without a trigger are compared in Table 13. Dogs with no trigger identified were found to have significantly higher creatinine and SDMA and a lower albumin compared to those in which a trigger was identified. The UPE patterns (G-UPE versus M-UPE) and the presence of specific proteins were then compared between dogs with and without a trigger.

There was no clear distinction between dogs with and without a trigger when the UPE pattern was considered; of the dogs with only a G-UPE pattern (n=9); 44.4% (n=4/9) dogs had a trigger identified (endocrine n=2, neoplasia n=2). Whilst, of the dogs with a M-UPE pattern (n=23); 52.2% (n=12/23) dogs had a trigger identified (endocrine n=4, inflammatory n=4, hyperlipidaemia n=2 and neoplasia n=2).

Of the tubular proteins, RBP was present in 9 dogs; 66.7% of these had a trigger (inflammatory n=3, endocrine n=2, hyperlipidaemia n=1). The two endocrine conditions with RBP present were not the same (diabetes mellitus n=1, hyperadrenocorticism n=1). Free light chains were present in just 2 dogs; one of these had a trigger (endocrine). A1 microprotein was present in 22 dogs; 50% of these had a trigger identified (endocrine n=4, inflammatory n=3, hyperlipidaemia n=2, neoplasia n=2). The glomerular protein IgG was present in 11 dogs; 36.4% had a trigger present (neoplasia n=2, inflammatory n=1, hyperlipidaemia n=1). IgA was present in 3 dogs; none of which had a trigger identified. Finally, haptoglobin was also present in 2 dogs neither of which had a trigger found. While overall there was no clear distinction in the UPE pattern between dogs with and without a trigger the fact that no dog with either IgA or haptoglobin present had a trigger detected is interesting and perhaps an area of future research with a larger study cohort.

Several recurrent combinations of proteins were detected on UPE analysis; however, these dogs could fall into either the ‘trigger’ or ‘no trigger’ group suggesting that UPE protein combinations could not differentiate between the presence or absence of an underlying trigger. There were three UPE protein combinations that were detected in more than one dog. Combination one included a1 microprotein, albumin, transferrin and IgG; this was present in 8 dogs of which 37.5% (n=3/8) had a trigger identified (neoplasia n=2, inflammatory n=1). Combination two included RBP, a1 microprotein, albumin and

transferrin; this pattern was identified in 5 dogs of which 60.0% (n=3/5) had a trigger (inflammatory n=2, endocrine n=1). Finally, combination three included a1 microprotein, albumin and transferrin; this was present in four dogs of which 75.0% (n=3/4) had a trigger (endocrine n=2, hyperlipidaemia n=1).

Even when the dogs with the same trigger were compared a variety of UPE patterns were seen within each subcategory.

Parameter	Trigger Cohort	No Trigger Cohort	Mann-Whiteny U	P-value
Creatinine (µmol/L)	79 (52.0-415.0)	134 (62.0-400.0)	62.0	**0.013**
SDMA (µg/dL) (n=23)	13 .0 (6.2-29.7)	24.2 (9.4-57.4)	29.0	**0.023**
Albumin (g/L)	31 (19.0-39.0)	28 (15.0-39.0)	74.0	**0.041**
Cholesterol (mmol/L) (n=31)	11.10 (4.9-17.7)	7.69 (5.5-13.45)	93.5	0.295
UPC	4.87 (2.0-26.3)	4.86 (2.2-15.8)	120.0	0.763
Glomerular protein UPE Pattern (%)	4 (25.0%)	5 (31.3%)		
Mixed protein UPE Pattern (%)	12 (75.0%)	11(68.7%)		

Table 13: Comparison between markers of disease progression/severity in dogs with and without a trigger for their proteinuria. Parameters were available for the entire study population (n=32) unless otherwise indicated. Median values (range) or N (% of population) are given as appropriate. Significant results highlighted in bold red. SDMA=symmetric dimethylarginine; UPC=urine protein creatinine ratio; UPE= urine protein electrophoresis.

3.3.3.5 Assessment of NGAL in dogs with a UPC >2.0

NGAL was detectable in all samples, however, due to errors in sample dilution, 2 dogs were excluded from statistical analysis as their NGAL content was either unknown (n=1) or above the limit of detection (n=1). The median NGAL for the remaining study population (n=30) was 74.28ng/mL (range: 5.88-919.25). All the dogs apart from two had a high NGAL suggesting the majority (93.8%) of the study population had at least some degree of tubular damage. The two dogs for which NGAL was normal both had a G-UPE pattern.

To assess if NGAL was associated with more advanced disease we looked for an association between NGAL and creatinine and SDMA. When NGAL was assessed as a continuous variable and compared to the presence or absence of azotaemia or increased SDMA an association was not seen (Table 14). Similarly, when creatinine and SDMA were assessed as continuous variables, no significant correlation was reported with the magnitude of the NGAL result (Figure 25). When the reciprocal of creatinine and SDMA were assessed, there was also no correlation seen with NGAL (creatinine reciprocal – Pearson Correlation Score: -0.246, p=0.191; SDMA reciprocal – Pearson Correlation Score: -0.291, p=0.188).

We also investigated whether the magnitude of NGAL correlated with serum albumin, cholesterol or UPC (Table 14). There was no association between cholesterol and NGAL. However, there was a correlation noted between serum albumin and NGAL when serum albumin was assessed both continually and categorically (Figure 26). The Pearson Correlation score demonstrates increasing NGAL was associated with decreasing serum albumin, however the score of -0.533 suggests only a moderate correlation is present. There was also an association noted between NGAL and UPC when UPC was assessed on a continual basis only (as UPC increased so did NGAL). Finally, we also investigated whether there was evidence of a relationship between the NGAL level and the UPE protein pattern present. The NGAL level was found to be significantly different between the groups of dogs with a glomerular UPE pattern and those with a mixed protein UPE pattern (Mann-Whitney U 9.0, p=0.0002) (Figure 27).

Parameter		Mann-Whitney U	Pearson Correlation Score	P-value
Creatinine (µmol/L) (n=30)	Categorical	97.0		0.572
	Continuous		0.231	0.219
SDMA (µg/dL) (n=22)	Categorical	38.00		0.147
	Continuous		0.159	0.480
Serum Albumin (g/L) (n=30)	Categorical	29.00		**0.026**
	Continuous		-0.533	**0.002**
Cholesterol (mmol/L) (n=29)	Categorical	64.0		0.092
	Continuous		-0.197	0.306
UPC (n=30)	Categorical	86.00		0.352
	Continuous		0.526	**0.003**

Table 14: Assessment of the relationship between NGAL values and markers of disease progression/severity assessed on both a categorical and continual basis. NGAL values were available for statistical analysis for thirty dogs overall; cholesterol values were missing for one of these dogs and SDMA results available for 22 of these dogs. Significant results highlighted in bold red. NGAL=Neutrophil gelatinase associated lipocalin; P value=associated statistical significance; SDMA=symmetric dimethylarginine.

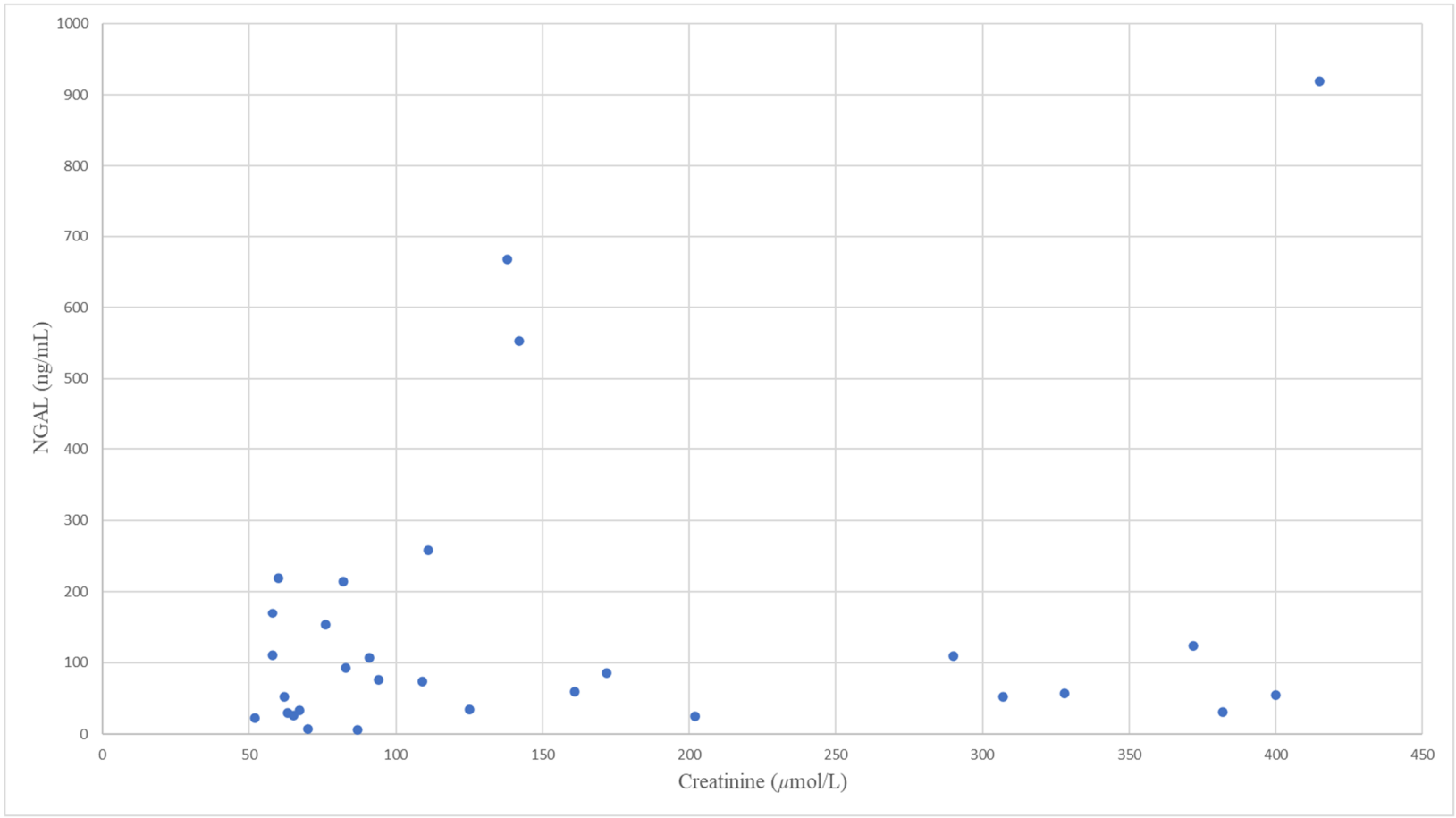


Figure 25: Scatterplot demonstrating lack of clear correlation between creatinine (µmol/L) and neutrophil gelatinase associated lipocalin NGAL (ng/mL).The results of Pearson Correlation testing showed a small, positive correlation between creatinine and NGAL (r=0.231,n=30), however this correlation was not significant (p=0.219) .

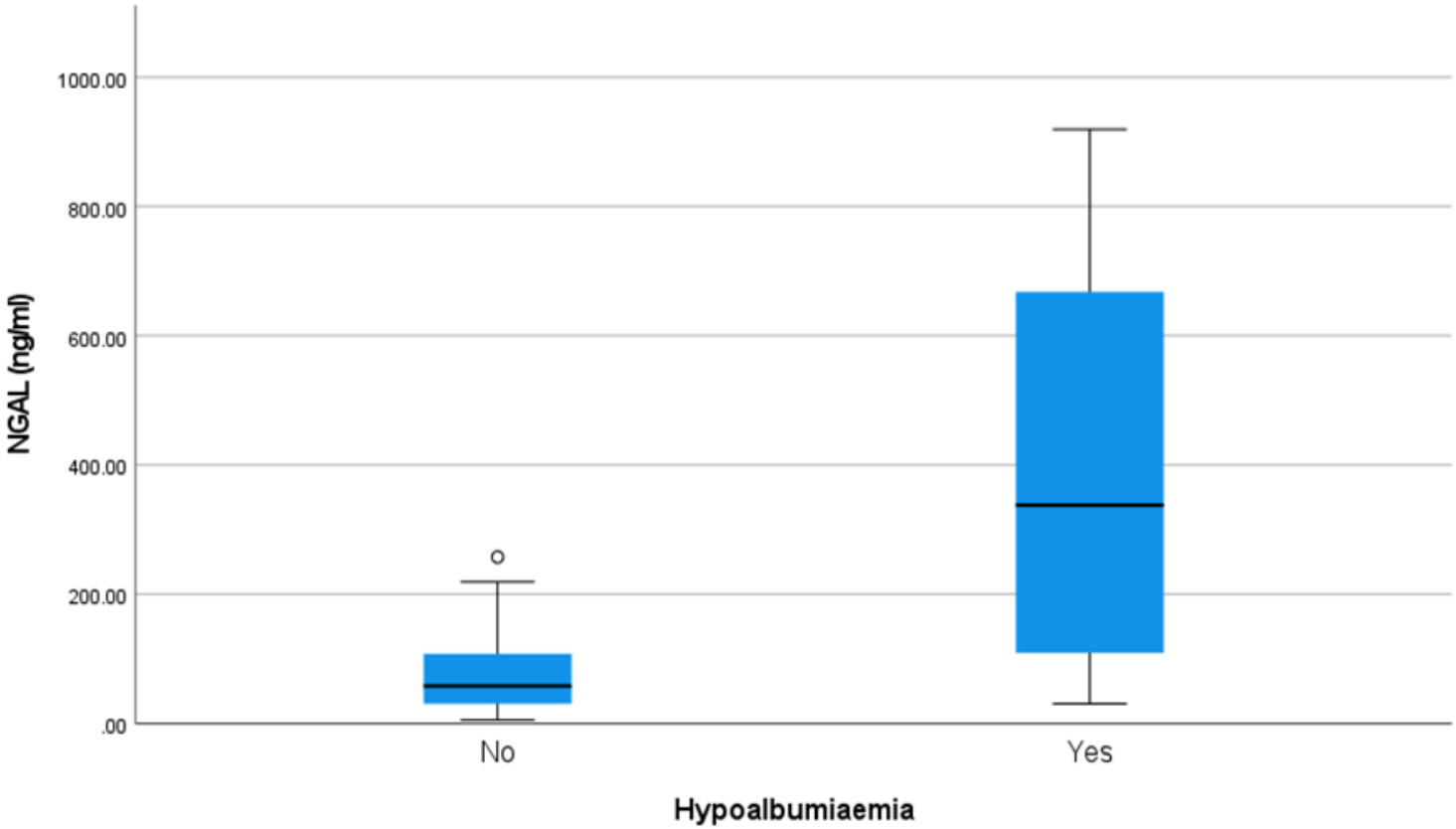


Figure 26: Box and Whisker plot comparing the neutrophil gelatinase associated lipocalin (NGAL) with the presence or absence of hypoalbuminaemia (defined as a serum albumin <25g/L). Dogs with hypoalbuminaemia had significantly higher NGAL values (Mann-Whitney 29.0, p=0.026). Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The circles represents a mild outlier.

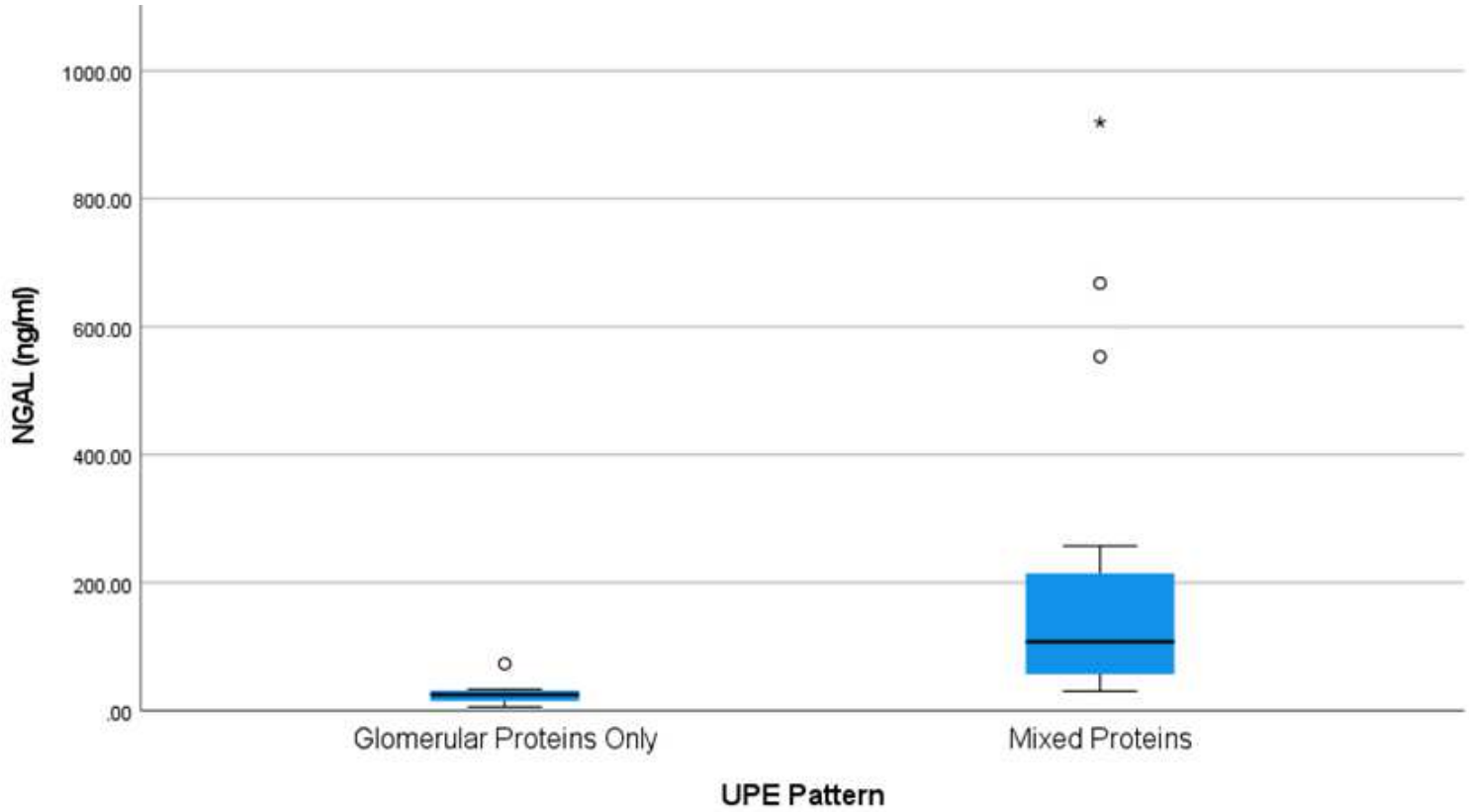
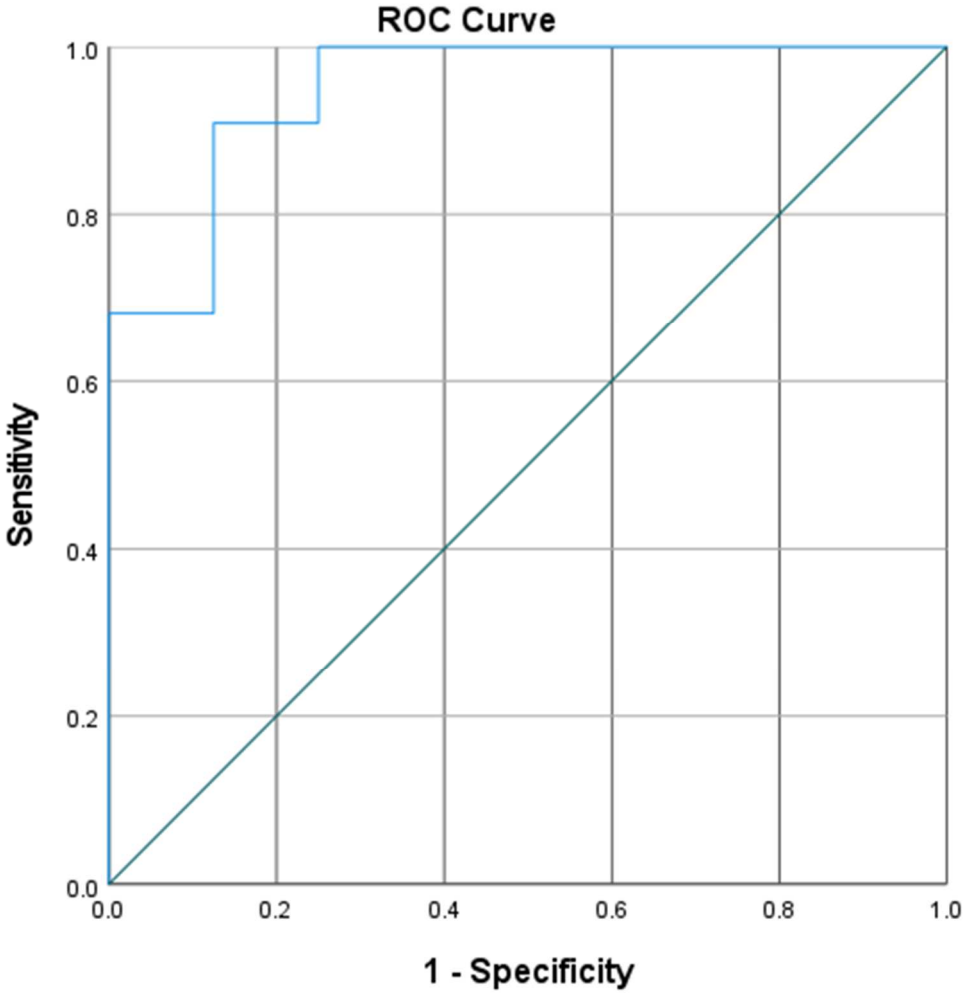


Figure 27: Box and Whisker plot displaying NGAL values for dogs based on the UPE pattern present. Dogs with glomerular proteins only on UPE had significantly lower NGAL compared to those with a mixed (glomerular and tubular proteins) pattern (Mann-Whitney U 9.0, p=0.0002). NGAL= neutrophil gelatinase associated lipocalin; UPE=urine protein electrophoresis. Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The circles represents mild outliers and the asterix major outliers.

It was noted that no dog with an NGAL of >75ng/mL had only glomerular proteins present whilst 68.2% of dogs with a mixed UPE had an NGAL above this level. Therefore, to evaluate whether we could generate an NGAL cut-off that could be used to predict the presence of a mixed protein pattern a receiver operating characteristic (ROC) curve analysis was performed (Figure 28). The table of the co-ordinates of the curve of the ROC curve output is provided in Appendix 7. Using an NGAL reading of 74.2ng/mL to indicate the presence of a mixed protein pattern was associated with a sensitivity of 68.2% and a specificity of 100.0%. To achieve a sensitivity of 100.0% a cut-off of 29.97ng/mL was required, however, this gave a specificity of just 25.0%.



Area	Std. Error	Asymptotic Sig.	Asymptomatic 95% Confidence Interval	
			Lower bound	Upper bound
0.949	0.043	0.0002	0.864	1.000

Figure 28: ROC curve analysis for the use of NGAL to predict the presence of a mixed pattern on the UPE analysis. NGAL=neutrophil gelatinase associated lipocalin; ROC= receiver operating characteristic; UPE=urine protein electrophoresis

3.3.3.6 Evaluation of markers of prognosis

To evaluate markers of prognosis, we assessed whether the protein pattern present on UPE (G-UPE versus M-UPE) was associated with long-term or 6-month survival. We also evaluated (where numbers allowed) if the presence of a specific protein could provide prognostic information. Finally, the presence of markers of disease progression/severity were also evaluated for prognostic significance.

3.3.3.6.1 Evaluation of the impact of the presence of tubular proteins on survival

Firstly, we assessed if there was a survival difference between dogs with a G-UPE versus a M-UPE pattern. Overall survival was not statistically impacted by the UPE pattern present (Log rank Mantel-Cox p=0.133); the median survival time for dogs with a G-UPE pattern was 623 days (95% CI was not available) versus 317 days (95% CI 0-662 days) for dogs with a M-UPE pattern. Six-month survival was known for 84.4% (n=27/32) dogs. Of the dogs with survival data available, 37.0% (n=10/27) were dead at 6-months. Of the dogs that had a G-UPE pattern (n=5), none were dead at 6-months; of the dogs that had a M-UPE pattern 45.5% (n=10/22) were dead at 6-months. There was no statistically significant difference (χ^2_1= 3.610, p=0.057) in 6-month survival between the dogs with either glomerular or mixed protein pattern present.

3.3.3.6.2 Evaluation of the impact of the presence of a specific protein on survival

RBP was present in 9 dogs with 6-month survival data present; 44.4% (n=4/9) dogs were dead at the 6-month point. Alpha-1 microprotein was present in 21 dogs with survival data available; 47.6% (n=10/21) were dead at 6-months. Free light chains were present in 2 dogs with 6-month survival data present; 50% (n=1/2) were dead at follow-up. Ten dogs had IgG present and 6-month survival data available; 50% (n=5/10) were dead at 6-months. Haptoglobin was present in 2 dogs with 6-month survival data; both were alive at the 6-month mark.

Due to the low numbers of dogs with some specific proteins present, only the presence of RBP, a1 microprotein and IgG were considered for statistical analysis. None of these proteins were found to be associated with long-term survival whilst the presence of a1 microprotein was found to be associated with 6-month survival; of the dogs without a1 microprotein present, all were alive at 6-months whilst 47.6% of dogs with a1 microprotein present were dead (Table 15).

		Specific Proteins		
		RBP	**a1-microprotein**	**IgG**
Long-term survival	**Log Rank (Mantel-Cox)**	0.381	0.055	0.356
	MST (95% CI) with protein present	317 (0-875)	188 (0-462)	188 (71-305)
	MST (95% CI) without protein present	459 (241-677)	623 (NA)	623 (NA)
Short-term (6-month survival)	**Chi-squared**	0.318	**4.538**	1.144
	P-value	0.573	**0.033**	0.285
	OR (95% CI) †	-	**1.545 (1.088-2.195)**	-

Table 15: Long-term and short-term survival considering the presence or absence of specific proteins (RBP, a1 microprotein and IgG). Significant results in bold red. For some, the MST and/or 95% CI was not available due to the fact the 45th percentile was not reached for this subset of data and therefore, the MST was not reached. 95% CI= 95% confidence interval, IgG=immunoglobulin G, MST=median survival time, RBP=retinol binding protein.

3.3.3.6.3 Evaluation of the significance of the presence of a trigger for proteinuria on survival

Briefly, we also assessed whether the presence of a trigger for the proteinuria appeared to impact on survival. The presence or absence of a trigger was not associated with long-term survival (Log Rank Mantel-Cox p=0.518); the median survival time for dogs without a trigger was 188 days (95% CI 0-390) whilst the median survival time for dogs with a trigger was 459 days (95% CI not available). There were 16 dogs with a trigger present that had 6-month survival data available; of these 31.3% (n=5/16) were dead at 6-months. There were 11 dogs without a trigger with 6-month survival data available; of these 45.5% (n=5/11) were dead at 6-months. The presence or absence of a trigger was not associated with 6-month survival (χ^2_1=0.564, p=0.453).

3.3.3.6.4 Evaluation of markers of disease progression/severity and impact on survival

Overall survival

Finally, the presence or absence of markers of disease progression/severity were assessed to determine their impact on survival. Both long-term and 6-month survival were assessed. The presence of azotaemia and hypoalbuminaemia were both associated with overall survival (Figures 29 and 30); increased SDMA, hypercholesterolaemia or severe proteinuria were not associated with overall survival (Table 16). When the population was split into whether there was a trigger present the same results were found. Azotaemia and hypoalbuminaemia remained associated with survival in both cohorts of dogs whilst neither the presence of hypercholesterolaemia or severe proteinuria were associated with overall survival (Table 16). SDMA was not statistically assessed when the population was split into trigger versus no trigger due to low numbers.

Six-month survival

When six-month rather than overall survival was assessed, similar results were found. Creatinine and serum albumin were associated with death by six-months when assessed both on a continuous and categorical basis; increasing creatinine and decreasing serum albumin were associated with a worse prognosis. SDMA, cholesterol and proteinuria were not associated with six-month survival (Table 17 and 18, Figures 31 and 32). The population was not split into trigger versus no trigger for assessment of survival as there were only 11 dogs with no trigger and 6-month survival data available.

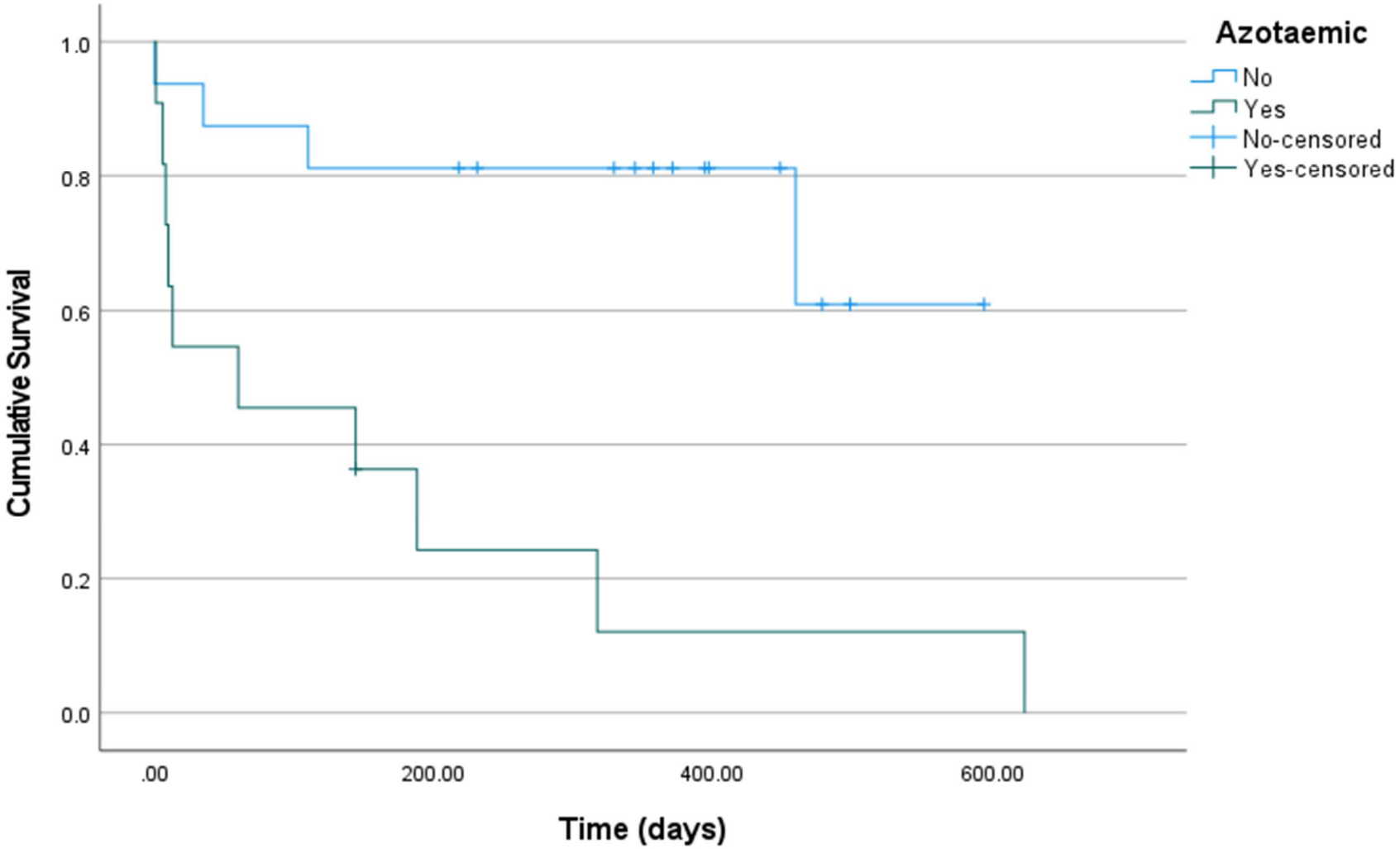


Figure 29: Kaplan-Meier survival plot for dogs with and without azotaemia (creatinine >125µmol/L). Log-rank (Mantel-cox) p=0.001

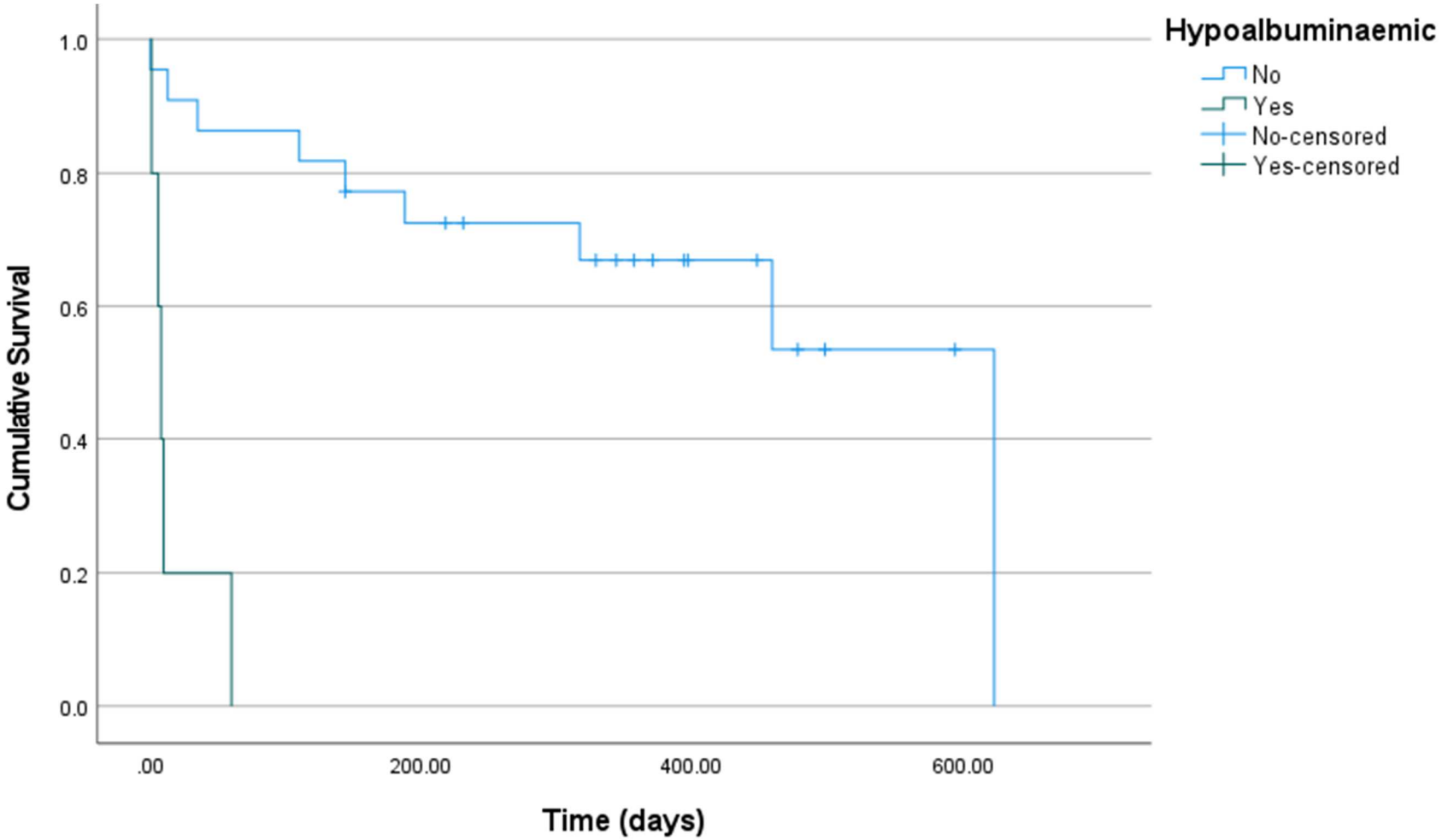


Figure 30: Kaplan-Meier survival plot for dogs with and without hypoalbuminaemia (serum albumin <25g/L). Log-rank (Mantel-cox) p=0.000013

	Clinicopathological abnormality	MST (95% CI) with abnormality present	MST (95% CI) without abnormality	Log-rank (Mantel Cox)
Overall population	Azotaemia	66 (0-150)	NR	**0.001**
	Increased SDMA (n=23)	60 (0-130)	459 (253-665)	0.163
	Hypoalbuminaemia	8 (3-12)	623 (NR)	**0.000013**
	Hypercholesterolaemia	623	317 (0-689)	0.293
	Severe proteinuria	459 (NR)	188 (0-399)	0.400
Trigger Cohort	Azotaemia	6 (0-146)	NR	**0.003**
	Hypoalbuminaemia	1 (NR)	NR	**0.002**
	Hypercholesterolaemia	NR	459 (168-750)	0.477
	Severe proteinuria	NR	35 (NR)	0.270
No Trigger Cohort	Azotaemia	66 (0-133)	NR	**0.040**
	Hypoalbuminaemia	10 (0-43)	623 (NR)	**0.002**
	Hypercholesterolaemia	623 (NR)	110 (47-174)	0.521
	Severe proteinuria	NR	188 (67-308)	0.968

Table 16: MST for samples with and without the presence of markers of disease progression/severity. Significant results in bold red. For some, the MST and/or 95% CI was not available due to the fact the 45th percentile was not reached for this subset of data and therefore, the MST was not reached (NR). 95% CI = 95% confidence interval; MST=median survival time.; P value=associated statistical significance; SDMA=symmetric dimethylarginine.

Parameter	Chi-squared	P-value	OR (95% CI)
Azotaemia	**5.632**	**0.018**	**7.589 (1.309-43.922)**
Increased SDMA (n=19)	2.358	0.125	-
Hypoalbuminaemia	**10.432**	**0.001**	**NA**
Hypercholesterolaemia	0.910	0.340	-
Severe proteinuria	0.564	0.453	-

Table 17: Association between the presence of markers of disease progression/severity (assessed categorically) and 6-month survival. Significant values in bold red. Study population n=27 unless otherwise indicated. NB. The OR for hypoalbuminaemia is not available since none of the dogs with hypoalbuminaemia were alive at 6-months. OR=odds ratio; P value=associated statistical significance

Parameter	Mann-Whitney U	P-value
Creatinine	**35.0**	**0.012**
SDMA (n=19)	23.0	0.083
Serum Albumin	**36.0**	**0.013**
Cholesterol	63.5	0.483
UPC	84.0	0.960

Table 18: Association between the presence of markers of disease progression/severity (assessed continually) and 6-month survival. Significant values in bold red. Study population n=27 unless otherwise indicated. SDMA=symmetric dimethylarginine; P value=associated statistical significance; UPC=urine protein creatinine ratio

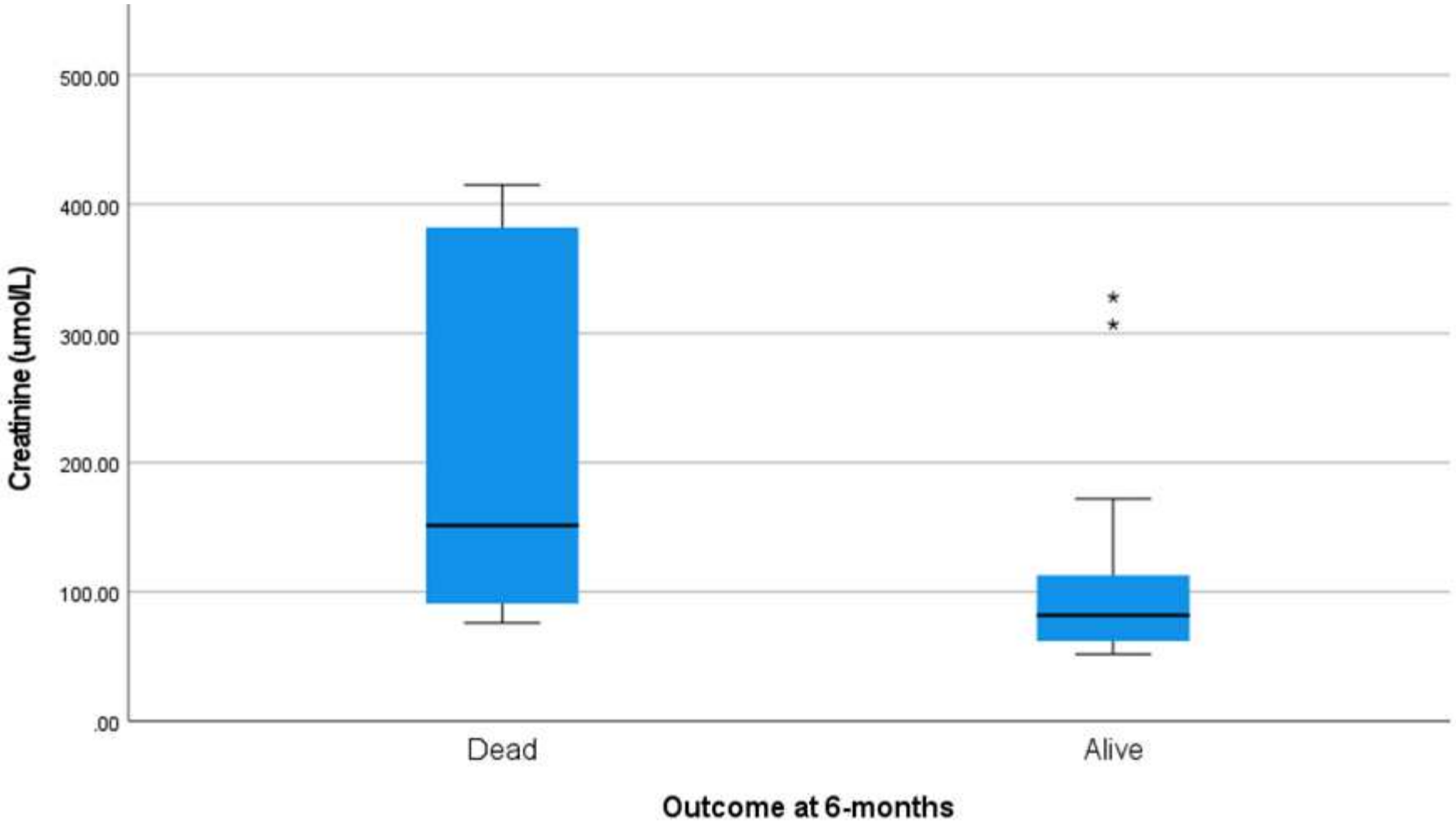


Figure 31: Box and Whisker Plot showing creatinine between dogs with a urine protein creatinine ratio >2.0 that were alive and dead at 6-month follow up. Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box. The asterix represents major outliers.

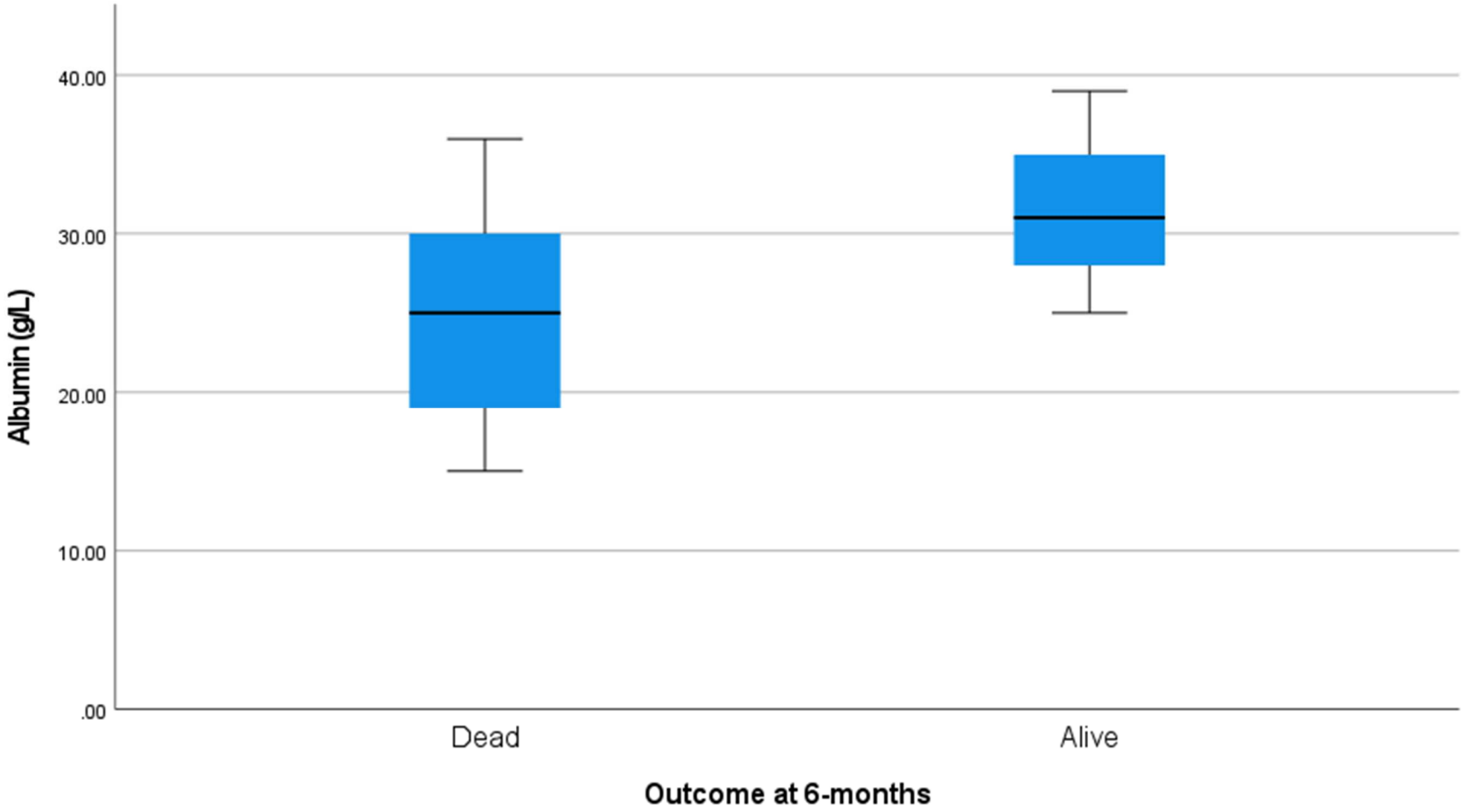


Figure 32: Box and Whisker Plot showing serum albumin between dogs with a urine protein creatinine ratio >2.0 that were alive and dead at 6-month follow up. Whiskers represent maximum (top) and minimal (bottom) values that are not outliers. The interquartile range is represented by the box and the median by the horizontal line within the box.

3.4 Discussion

3.4.1 Presence and significance of tubular damage in a cohort of dogs with UPC >2.0

The main aim of the prospective part of this master's project was to further characterise the urine protein profile of dogs with a UPC of >2.0 and to determine the frequency of tubular involvement in this population. Interestingly, while none of the dogs in our study population (UPC >2.0) had only tubular proteins present, 71.9% had a mixture of both glomerular and tubular proteins present suggesting combined damage. Albumin and transferrin were however the predominant proteins present in the vast majority (90.6%) of cases suggesting that although tubular damage is present most of the proteins in dogs with a UPC >2.0 are glomerular in origin. When the urinary tubular biomarker NGAL was measured, all but two dogs (94.3%) had an abnormal value again suggesting the presence of concurrent tubular damage in most of the study population.

Despite the frequent presence of both tubular and glomerular proteins, our results showed that glomerular proteins, specifically albumin and transferrin appeared to predominate in most samples. In dogs that had a G-UPE profile, only albumin and transferrin were detected. Albumin and transferrin were present in all dogs with a M-UPE profile (in addition to a variable combination of other glomerular and tubular proteins) and were also the only proteins found in the healthy control samples albeit to a much lesser extent as these urine samples required precipitation prior to the detection of any protein. The predominance of these glomerular proteins in our samples suggest that dogs with a UPC of >2.0 are less likely to have primary tubulointerstitial disease. Recently, some authors have suggested that utilising a UPC cut-off of >2.0 is inaccurate for the detection of glomerular proteinuria as dogs with tubulointerstitial disease have been shown to have a UPC exceeding this magnitude (Hokamp et al., 2016, Schneider et al., 2013), however, our results suggest the use of this UPC cut-off appears appropriate. Further studies assessing a larger cohort of dogs are perhaps required to further interrogate this cut-off.

Pre-existing studies have assessed the UPE profile of proteinuric CKD dogs. Although these dogs do not provide an identical population to that enrolled into our study, several authors have demonstrated CKD dogs with a UPC >2.0 to have both high and low molecular weight protein bands suggesting concomitant glomerular and tubular damage (Ferlizza et al., 2020, Hokamp et al., 2018, Chacar et al., 2017). As covered in the introduction, previous literature has documented dogs with histopathological confirmation of glomerular disease to have a UPC of >2.0, however, these studies did not detail the nature of the urinary proteins present

(i.e. glomerular or tubular). Characterisation of the urine profile present in dogs with glomerular disease has been attempted by other studies, however, the criteria of a UPC of >2.0 has not always been applied. For example, Lavoué *et al.* (2015) studied a population of Dogue de Bordeaux's (in which familial glomerulopathy is reported) and found that 13/36 dogs with a UPC of >0.5 had a mixture of tubular and glomerular proteins present on UPE analysis. Our results suggest that, in keeping with prior studies assessing proteinuric CKD dogs, a UPC of >2.0 is usually attributed to a mixture of tubular and glomerular proteins. Our study also allowed for quantification of the proteins present and results suggest that although a mixture of glomerular and tubular proteins may be present, glomerular proteins tend to predominate.

Albumin and transferrin are HMW proteins that are lost into the urine due to the loss of permselectivity of the glomerulus. Considering albumin and transferrin were present in all samples it is possible that these proteins are lost first followed by other glomerular/tubular proteins. Therefore, it is possible that the subgroup of dogs with a G-UPE pattern (albumin and transferrin) are at an earlier stage in their disease process compared to the dogs that have a M-UPE pattern. The idea that dogs with both tubular and glomerular proteins present are at a more advanced stage of disease is supported by our results considering UPE pattern and markers of disease progression (creatinine and SDMA). Although the results failed to reach statistical significance, dogs with a M-UPE pattern were more commonly azotaemic compared to those with a G-UPE pattern (47.8% versus 22.2%). Similarly, more dogs had an increased SDMA in the population with a M-UPE pattern (53.3% versus 25% in dogs with a G-UPE pattern). The development of hypoalbuminaemia can also occur with more severe disease and again, although statistical significance was not reached, hypoalbuminaemia was seen more commonly in dogs with a M-UPE pattern compared to dogs with a G-UPE pattern (no dog with a G-UPE pattern was hypoalbuminaemic whilst 26.1% of those with a M-UPE pattern had hypoalbuminaemia). Further evidence to suggest that the presence of tubular damage is associated with more advanced disease is provided by our survival analysis.

Dogs with a G-UPE pattern had a longer median survival time compared to those with a M-UPE pattern (623 days versus 317 days), however, this did not reach statistical significance. When 6-month survival was assessed no dog with a G-UPE pattern present was dead compared to the 45.5% of dogs with a M-UPE pattern that were dead at by 6-months. Although again, statistical significance was not reached, the P-value for Chi-squared analysis was approaching significance (p=0.057) and hence it is possible if a larger study population

was assessed a statistical difference would have been seen in 6-month survival between the two groups. Similarly, it is possible that had a larger group been assessed the overall median survival times between the groups would have reached significance.

Our study population all had a UPC of >2.0 and therefore, what would have historically been referred to a glomerular disease, however, approximately 75% of these dogs had tubular involvement documented on UPE analysis. Several human studies have demonstrated the presence of LMW 'tubular' proteins in people with glomerular disease and there are multiple hypotheses as to why these tubular proteins may be detected in the urine. It has been suggested that chronic renal failure can lead to increased serum levels of LMW proteins, such as alpha-1 microprotein, and these increased levels simply exceed the load a single nephron can re-absorb (Maack et al., 1979). Along a similar line, both human and canine studies have shown that proteinuria associated with glomerular disease can lead to competition for tubular reabsorption between HMW and LMW proteins and hence fewer LMW proteins being absorbed (Thielemans et al., 1994, Bernard et al., 1987, Vinge et al., 2010). Other studies have described various mechanisms by which the tubules can become directly damaged due to glomerular disease for example, glomerular disease has been associated with loss of the post-glomerular peritubular capillary network, a consequence of which is tubular cell ischaemia (Bohle et al., 1981). In murine models, tubular enzymatic damage can occur due to rupture of lysosomes following excessive tubular lysosomal processing of proteins filtered at the glomerulus, additionally, direct damage to the tubules may occur due to their obstruction with proteinaceous casts (Bertani et al., 1986, Olbricht et al., 1986). Finally, as noted in our study, increased glomerular permeability can lead to increased loss of transferrin; transferrin leads to an increased uptake of iron via the epithelial cells, and once inside the cell the formation of reactive oxygen species is catalysed by this iron leading to peroxidative damage (Alfrey et al., 1989). Figure 33 shows a proposed sequence of events that demonstrate how tubulointerstitial injury can arise from primary glomerular disease.

In line with our results, in humans the presence of tubular proteins in cases of glomerular disease has been suggested to be associated with more advanced disease. For example, in humans with IgA nephropathy, the presence of LMW proteins has been shown to be significantly associated with increased creatinine and a higher incidence of chronic renal failure (Nagy et al., 1987, Woo et al., 1991). Meanwhile, in patients with lupus nephritis, mixed proteinuric patterns including LMW proteins were most noted and were significantly associated with the degree of infiltration of the interstitium with leukocytes and glomerular

sclerosis (Bazzi et al., 1995). Similarly in children with glomerular disease, the presence of tubulointerstitial abnormalities on renal biopsies are associated with a poorer prognosis; additionally, a review of earlier studies of paediatric proteinuria suggested that the presence and degree of tubulointerstitial changes were a major factor in determining outcome (Cameron, 1992, Portman et al., 1986).

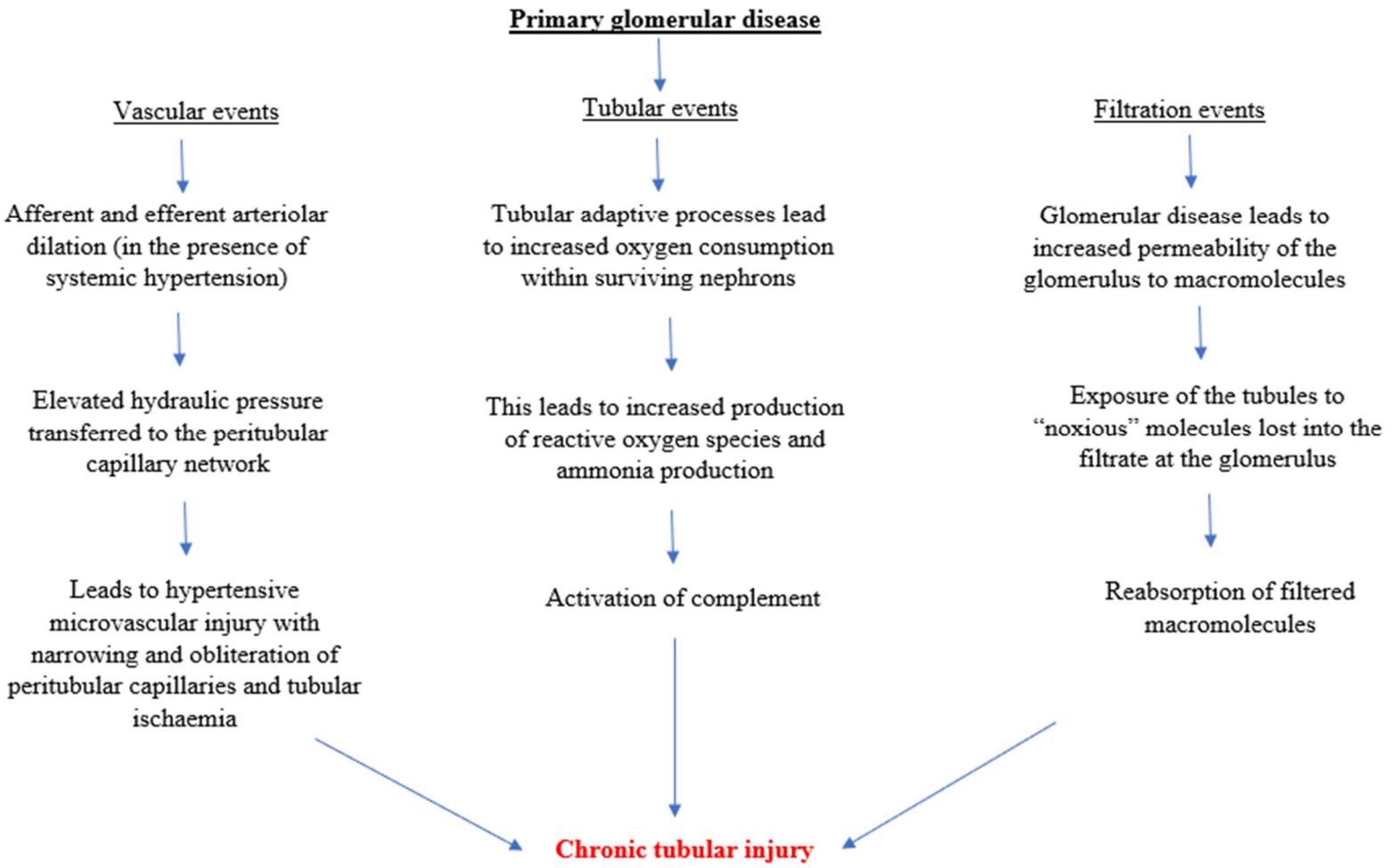


Figure 33: Proposed sequence of events regarding development of chronic tubular injury secondary to primary glomerular disease. Adapted from (Fine et al., 1993)

Although tubular proteins were noted in approximately 75% of the study population, the NGAL results were abnormal for almost all dogs suggesting that NGAL perhaps provides an earlier method for screening for tubular damage compared to presence of tubular proteins on UPE. However, it is also possible that the variability noted was simply due to the different methodology utilised; for example, for the tubular proteins to be detected on UPE profiling they may need to reach a higher level compared to that required for NGAL to be detected via ELISA. NGAL was found to be significantly lower in dogs with a G-UPE pattern, additionally, the only two dogs that had a normal NGAL result also only had glomerular proteins present on UPE analysis. These results add further weight to the theory that tubular damage occurs as a consequence of protracted glomerular proteinuria. Dogs with a G-UPE pattern have an increased NGAL result although to a significantly lesser extent than those with a M-UPE pattern suggesting that early tubular damage is present in dogs with only glomerular proteinuria present and that as disease progression occurs, these dogs go onto lose tubular proteins which is detected on UPE analysis and NGAL levels also further increase.

NGAL did not correlate with creatinine or SDMA whilst it did weakly correlate with albumin and UPC. The lack of association between NGAL and creatinine is perhaps not surprising considering that NGAL is a marker of tubular damage rather than tubular dysfunction whilst creatinine and SDMA are used as surrogate markers for renal function. We have postulated that tubular damage is associated with more advanced disease in which an increased creatinine may be expected (due to decreased GFR). As tubular damage increases the level of NGAL may also be expected to increase. However, the relationship between this increase in tubular damage and decrease in function is unknown and direct correlation may not exist. Although in humans, NGAL has been reported to correlate well with GFR in both people with CKD and healthy controls (Bolignano et al., 2008, Gharishvandi et al., 2015), the relationship between NGAL and GFR in dogs is yet to be determined. It is known that the relationship between creatinine and GFR is non-linear, hence we also assessed the reciprocal of creatinine and NGAL for an association to see if the reciprocal was a more appropriate option, however, this generated a similar result with no statistical association found between NGAL and creatinine. Limited previous work exists in this area in dogs; one previous study failed to detect a correlation between GFR or creatinine and NGAL in healthy dogs, whilst in the overall study population (which included healthy dogs and those with CKD and AKI) a weak positive correlation was reported between creatinine and NGAL (Steinbach et al., 2014).

Increasing NGAL was significantly associated with decreasing serum albumin (Pearson Correlation Score -0.533, p=0.002) and the presence of hypoalbuminaemia (Mann-Whitney U 29.0, p=0.002). Increasing NGAL was also associated with increasing magnitude of UPC (when UPC was assessed on a continuous basis only, Pearson Correlation Score 0.536, p=0.003). Although there was a statistical association between NGAL and serum albumin (as the NGAL level increased, the serum albumin decreased), the Pearson Correlation Score suggests that this is only a weak correlation, similarly, only a weak correlation appears to be present between NGAL and UPC (increasing NGAL was associated with increasing UPC). Hypoalbuminaemia and increased UPC are often taken to represent the presence of more severe disease and therefore it is possible that the higher the NGAL result the more severe disease is present, however, due to the weak correlations detected here and the small sample numbers included in our study, further work is required to corroborate this finding.

Based on the results generated by this part of our study, several areas for further work have been identified. Firstly, work is required to evaluate NGAL in more detail and determine if the lack of association with creatinine and SDMA is persistent in a larger population of dogs and to determine the nature of the relationship shared between NGAL and GFR. Additionally, it would be interesting to see if the relationship reported here between NGAL and serum albumin and UPC is persistent in a larger population of patients. The potential for NGAL to represent an early marker of tubular damage is an interesting area for further work. Our ROC curve analysis suggested that using a NGAL of 74.2ng/mL allowed for a sensitivity of 68.2% and a specificity of 100% for the detection of a mixed protein pattern. Further interrogation of this cut-off in a wider population is required. Additionally, serial NGAL measurements in dogs would be of great interest to determine whether NGAL reduces in response to treatment. Currently, changes in UPC are monitored to determine response to treatment, however, when end-stage disease is reached is it possible that UPC may drop simply due to the reduction in functioning nephrons and hence a drop in UPC at this stage of disease may not be representative of a positive response to treatment. Although NGAL is a marker of tubular damage it may represent a better parameter to indirectly monitor response to treatment in more advanced stages of disease and further work is required to explore this possibility. Ideally, any further work would also be carried out in combination with renal biopsies so that histopathology could be used to corroborate findings.

3.4.2 Assessment of the presence of individual proteins on UPE analysis as potential markers of disease progression/severity and prognosis

We also evaluated the significance of individual tubular proteins. The presence of each protein was assessed as a potential marker for more advanced or severe disease and for prognostic significance.

The significant results from this evaluation were that the presence of alpha-1 microprotein was associated with increased creatinine and a negative outcome at 6-month follow-up. Furthermore, although it did not reach statistical significance, it is interesting to note that no dog without a1 microprotein protein present was hypoalbuminaemic compared to 27.3% (n=6/22) dogs with a1 microprotein protein present that had low albumin. Additionally, presence of IgG was statistically associated with a lower serum albumin. Although there was a moderate amount of overlap on assessment of the box and whisker plots suggesting that we should interpret these results with caution, these findings suggest that the presence of alpha-1 microprotein and IgG may be associated with more advanced and/or severe disease.

Little previous work has been assessing urinary alpha-1microprotein in canine literature. In humans, the use of alpha-1 microprotein has been reported to assist in the estimation of GFR in various renal diseases and has also been proven to be useful for screening for tubular abnormalities and detection of chronic asymptomatic renal tubular dysfunction (Kusano et al., 1985, Yu et al., 1983). Specifically, alpha-1 microprotein has been reported to be of use in the diagnosis of renal tubular dysfunction secondary to cadmium intoxication, Balkan nephropathy, diabetic nephropathy and pyelonephritis (Kido et al., 1985, Everaert et al., 1998, Cvoriscec, 2000, Hong et al., 2003, Holmquist et al., 1993). In line with our investigations, alpha-1 microprotein has been associated with progression and higher mortality in people with CKD and there are early suggestions that levels may help identify patients at increased risk of AKI following cardiac surgery (Robles et al., 2021, Amatruda et al., 2021).

IgG is found in the urine of dogs with X-linked hereditary nephropathy and is reported to increase in the urine prior to the onset of azotaemia in this cohort of dogs (Vinge et al., 2010, Nabity et al., 2012). IgG has been reported in association with renal damage secondary to the presence of an underlying infectious disease process such as leishmania, leptospirosis and pyometra (Zaragoza et al., 2003a, Solano-Gallego et al., 2003, Zaragoza et al., 2004), and has also been reported in dogs with hyperadrenocorticism and chronic renal failure secondary to juvenile nephropathy in a Bernese Mountain dog (Raila et al., 2007, Smets et

al., 2012b). Most of the previous studies relating to IgG simply assess its presence to try to confirm glomerular damage in certain situations rather than assessing the prognostic or clinical implications of its presence.

In humans, not only has IgG been shown to be significantly associated with disease severity in patients with idiopathic membranous nephropathy but it has also been reported to be a useful biomarker in both predicting the course of disease and response to treatment in certain nephropathies (Hou et al., 2019, Bazzi et al., 2001, Bazzi et al., 2009, Bazzi et al., 2014, Bazzi et al., 2013). In a study assessing both IgG and alpha-1 microprotein it was found that urinary IgG levels were significantly linked with the extent of tubulointerstitial damage present *and* alpha-1 microproteins; this finding led to the authors of this paper postulating that IgG may be the protein driving further pathological changes (Bazzi et al., 2001). This postulation supports our earlier hypothesis that primary glomerular damage and the loss of glomerular proteins leads to tubular damage over time and hence the loss of tubular proteins.

Considering the substantial overlap on the box and whisker plots, in addition to the fact that neither alpha 1 microprotein nor IgG showed statistical significance when creatinine or serum albumin were assessed categorically, further work is needed to confirm the suggestion that the presence of these proteins may be a marker of more advanced/severe disease. The possibility that alpha 1 microprotein could be of use prognostically also warrants further investigation; if this result proved to be a consistent finding in a larger cohort of dogs this would be clinically useful as it would provide a non-invasive marker of poor prognosis.

The amount of urine albumin as a percentage of the total protein content was determined and analysed. It was found that the percentage of urine albumin was associated with the presence of azotaemia and hypoalbuminaemia; dogs with azotaemia or hypoalbuminaemia were found to have a lower percentage of urine albumin present. When creatinine and serum albumin were assessed as continuous parameters, they were still found to be statistically associated with the percentage of urine albumin. This result is perhaps to be expected considering the previous discussion regarding the presence of tubular proteins suggesting more advanced disease. As the percentage of albumin decreases in the urine it follows that the percentage of other proteins in the urine must increase and therefore, it is likely that in the samples with a lower percentage of urine albumin present there is a higher percentage of tubular proteins which may suggest more advanced disease hence fitting with the increased creatinine. As the percentage of urine albumin falls it may also be being replaced by other glomerular proteins perhaps suggesting more severe damage to the glomerulus and hence the loss of enough albumin to make dogs become hypoalbuminaemic. Although it is also

possible that in fact, the converse is true, and that the drop in urine albumin arises simply because the dogs are hypoalbuminaemic and therefore there is less albumin available to be lost into the urine. Further work is again required to interrogate these suggestions and determine if the percentage of urine albumin as a total of total protein content lost could be clinically useful.

3.4.3 Difference between UPE analysis for dogs with and without an underlying trigger for proteinuria

We hypothesised that dogs without an identifiable trigger would have characteristic UPE patterns that would enable them to be distinguished from dogs with a trigger. If this were the case, it would be of great clinical benefit as it would enable us to screen dogs thought not to have a trigger to see if one had been missed. This is of importance as dogs with an underlying trigger will often respond better to treatment if this trigger is also addressed.

In human medicine, most of the work on identifying urinary protein profiles relating to specific diseases has focused on full urinary proteome analysis mainly using 2D gel electrophoresis/capillary electrophoresis (CE) and mass spectrometry (MS) or matrix-associated laser desorption/ionisation-time of flight MS (MALDI-TOF MS). For example, analysis of the proteome has suggested groups of urinary proteins that can be used to differentiate between FGFS and MCD (Perez et al., 2017). Work has also been performed in relation to diabetic nephropathy where results from CE-MS have been used for early detection of diabetic renal damage (Meier et al., 2005, Mischak et al., 2004).

From our results we did not find any clear patterns that could distinguish between dogs with and without a trigger for their proteinuria. However, interestingly, no dog with either IgA or haptoglobin present had a trigger detected. IgA nephropathy is one of the most common forms of glomerulonephritis in people, (D'Amico, 1987, Levy and Berger, 1988), however, it is infrequently reported in dogs. In canine literature, Harris *et al.* (1993) reported three clinical cases of IgA nephropathy whilst a review of 100 dogs undergoing post mortem noted marked glomerular deposition of IgA in 6% of cases (Miyauchi et al., 1992). In humans a substantial amount of work has been based on trying to identify the urine protein pattern associated with IgA nephropathy and it has been shown that the amount of urinary IgA (and IgG) levels are significantly higher in the urine of patients with IgA nephropathy compared to healthy controls or patients with other non-IgA nephropathy (Matousovic et al., 2006). Although validation is still required, a urine proteome map has been discovered for IgA

nephropathy in people whilst further studies have utilised proteomic analysis to differentiate between humans with severe and mild forms of the disease; distinguish between IgA nephropathy and membranous nephropathy and predict disease progression and response to treatment with ACEi (Park et al., 2006, He et al., 2012, Rudnicki et al., 2021, Rocchetti et al., 2008, Haubitz et al., 2005).

Interestingly, in humans with IgA nephropathy levels of a1 microprotein are reported to be the same as compared to healthy controls and significantly lower than in urine samples of patients with diabetic nephropathy (Yokota et al., 2007). In contrast, all our dogs with IgA detected on UPE also had a1 microprotein present. It has previously been suggested that there may be differences between canine and human IgA; the studies of canine IgA referenced previously all document different histopathological findings in dogs compared to humans and there is just one case report documenting a dog with IgA nephropathy in which histopathology findings were similar to those seen in humans (Yabuki et al., 2016). Regardless of these possible differences between human and canine IgA the fact that urine protein analysis can increase suspicion of IgA nephropathy in humans combined with the fact that all dogs with IgA detected in our study lacked a clear trigger for their proteinuria suggests that the presence of IgA in canine urine samples may be indicative of primary glomerular disease (i.e. that without a trigger). A larger cohort of dogs with IgA present in their urine and renal histopathology available would be required to explore the significance of urine IgA further. The fact that urine protein analysis can also predict response to human patients with IgA nephropathy is also of note. As we discussed in the retrospective study, a large proportion of dogs failed to respond to ACEi therapy. If urine biomarkers predictive of response could be discovered in dogs this would be an exciting development as it may help to identify patients in which alternative methods for RAAS inhibition, such as ARBs may be a more appropriate first line treatment.

No dog with haptoglobin present had a trigger detected. As with IgA, we only had small numbers of dogs with haptoglobin present but this finding is interesting as it is perhaps unexpected when human literature is considered. Most of the research in human literature regarding haptoglobin has reported an association with type 2 diabetes mellitus and it is suggested that urine haptoglobin can predict risk of mortality and allow for early identification of patients at risk of diabetic nephropathy (Liu et al., 2020, Bhensdadia et al., 2013). Haptoglobin in canine renal disease has been minimally researched and therefore further work is required to determine the possible significance of our findings that dogs with haptoglobin present do not have an underlying trigger for their proteinuria.

Perhaps the most similar human study to ours is represented by the work performed by Varghese *et al.* (2007) who utilised results from UPE analysis to generate a prediction algorithm (utilising an artificial neural network) that could distinguish between FSGS, lupus nephritis, membranous nephropathy and diabetic nephropathy. We did not perform histopathology on any of the dogs enrolled in our study and therefore association with a specific UPE pattern and histopathological changes was not evaluated. We instead assessed whether specific UPE patterns were attributable to dogs without a trigger for their proteinuria. Our results did not support our hypothesis that dogs without a trigger would have a specific set of UPE patterns present, additionally, we found that the UPE pattern could not be used to determine between the various trigger present in our population. Although we identified three UPE patterns that were seen in several dogs, these patterns could be seen in dogs with or without a known trigger and therefore did not help differentiate between these two cohorts of dogs. On further consideration of the literature these results are perhaps not surprising.

For example, previous studies assessing dogs with hyperadrenocorticism have reported the presence of either glomerulosclerosis, glomerulonephritis or amyloidosis (Waters et al., 1997, Ortega et al., 1996, Center et al., 1987, Van Winkle, 1993, Littman et al., 1988). Similarly, in human patients with hyperadrenocorticism and nephrotic syndrome a range of histopathological changes have been reported including the presence of FSGS and membranoproliferative glomerulonephritis (Chan and Michelis, 1992, Hiraiwa et al., 1988, Hsieh et al., 2007, Stuart and Chandran, 2001, Tatsumi et al., 1995).

The lack of consistency with dogs with neoplasia as a trigger is also perhaps not surprising. As previously discussed, there are numerous ways in which neoplasia is hypothesised to cause proteinuria. For example, proteinuria has been postulated to arise due to the abnormal products of the neoplastic cells or development of immune complexes; immune complexes can form *in situ* against tumour antigens or circulating immune complexes against tumour antigens can become lodged in the glomerulus (Jhaveri et al., 2013, Eagen, 1977, Bacchetta et al., 2009). It is possible that the UPE results of dogs with neoplasia depends on which of these potential mechanisms occur.

We only assessed a relatively small cohort of dogs meaning that the numbers within each subgroup were low. It was also unknown as to what extent the possible triggers we identified had on the UPE profiles generated, for example, in the dogs with hyperadrenocorticism it was not confirmed whether hyperadrenocorticism was the only cause of proteinuria in these cases or if it was just a contributing factor. Further assessment of a larger group of dogs with and without possible triggers is required to screen for possible patterns that may be of clinical

use. It may also be of interest to serially follow dogs with identified triggers to document how their UPE patterns may change in response to treatment for the trigger.

3.4.4 Association between markers of disease progression/severity and survival

To evaluate prognostic markers in dogs with a UPC of >2.0 we assessed whether creatinine, SDMA, serum albumin, cholesterol or UPC were associated with overall and 6-month survival. One of the key purposes of this aspect of the study was to assess whether the results of the prospective study concurred with those reported in our retrospective study.

As with the retrospective study, parameters were assessed on both a continual and categorical basis. The main finding was that the presence of azotaemia and hypoalbuminaemia were associated with overall survival and that creatinine and serum albumin (assessed on both a continuous and categorical basis) were associated with 6-month survival. SDMA was also evaluated and was not found to have a statistical association with survival, however, considering the findings associated with creatinine and the fact that the sub-population of dogs with SDMA results was smaller it is suspected that lack of significance between SDMA and survival in this study is due to the small sample population. In contrast to the retrospective study, the prospective results did not find UPC to be associated with survival either overall or at 6-months.

The association between increased creatinine and survival is unsurprising as creatinine is a marker of GFR with increased creatinine representing decreased GFR and therefore more advanced disease being inferred. Previous literature has assessed creatinine and survival in various populations of dogs with proteinuria and results are in accordance with our findings (O'Neill et al., 2013, Hokamp et al., 2016). These previous studies have focused on dogs with proteinuric CKD and hence our study adds valuable data confirming, as expected, increased creatinine to be a negative prognostic indicator in a cohort of dogs with a UPC of >2.0.

Serum albumin was also found to be associated with survival in our study with both the presence of hypoalbuminaemia and decreasing serum albumin at time of enrolment being associated with a poorer prognosis. Again, this finding is not unexpected and is fitting with previous literature reporting hypoalbuminaemia to be a negative prognostic indicator (Lorbach et al., 2020, Parker and Freeman, 2011). Original literature assumed that severe proteinuria was responsible for the development of hypoalbuminaemia (Aresu et al., 2017), however, recent literature suggests that proteinuria alone may not be solely responsible for

the presence of hypoalbuminaemia. For example, Lorbach *et al.* (2020) found that a significant correlation between decreased albumin and increased UPC was absent in the cohort of dogs they assessed with FSGS, a similar finding has also been reported in some human literature where normal albumin levels were found despite patients' proteinuria being classed within nephrotic range (De Vriese et al., 2018, Praga et al., 1999). In our prospective study, we also found that despite all dogs having a UPC of >2.0 only 18.8% (n=6/32) were hypoalbuminaemic. One possible hypothesis for the lack of hypoalbuminaemia in all patients with significant proteinuria is that the development of marked protein loss is usually slow in onset hence possibly allowing for compensation to occur (Praga et al., 1999).

In our prospective study, we did not find that the magnitude of UPC was associated with survival. Although one previous study evaluating a cohort of dogs with FSGS also found UPC not to be associated with survival (Lorbach et al., 2020), there are several studies that suggest the opposite and which have demonstrated marked proteinuria to be a negative prognostic indicator (Jacob et al., 2005, Wehner et al., 2008). In humans and dogs there are several studies showing that a reduction in proteinuria following ACEi treatment is associated with a slowed progression of disease (Grauer et al., 2000, Grodecki et al., 1997, Maschio et al., 1996, Lewis et al., 1993) and therefore this implies that an increased UPC is a negative prognostic indicator. Lorbach *et al* (2020) discussed that the lack of association in their study with UPC and survival may have arisen as proteinuria was not the ideal biomarker to identify glomerular scarring in their cohort of dogs, additionally, they also discuss the variability of UPC as having a possible effect. In our study, histopathology was not available, however, it was presumed that not all dogs will have had FSGS, therefore, the hypothesis proposed by Lorbach *et al.* cannot be applied to all our cases. It is, however, possible that small study numbers may have contributed to the lack of statistical significance seen in our prospective study.

3.4.5 Stability of Urinary biomarkers – GGT and NGAL

In addition to NGAL, we planned to further evaluate the presence of tubular damage in dogs with a UPC of >2.0 via measurement of GGT. Unfortunately, the planned work regarding GGT was not performed since urine samples were stored under conditions at which it was discovered GGT was unstable. This study has proven that urinary GGT is unstable when stored at -20 degrees although appeared stable when frozen at -80°C degrees for up to 30 days. Additionally, GGT also appeared stable when kept either at room temperature or within the fridge for up to 7 days. The addition of a PI to the urine samples prior to freezing did not appear to impact GGT levels and did not prohibit GGT degradation when urine was frozen

at -20°C degrees. Although several prior studies have assessed the effect of urinary pH on GGT (Ilchyshyn et al., 2019, Brunker et al., 2009, Jung et al., 1982), only one earlier study has evaluated the stability of GGT under different storage conditions. The results of this earlier study also suggested that storage of urine for measurement of GGT at -20°C degrees is inappropriate as a mean reduction of GGT of 72% was reported in samples frozen at -20°C degrees for 24-hours or longer (Ilchyshyn et al., 2019). Conflictingly, previous literature reports that storing GGT at -80°C degrees for three weeks leads to an unacceptable reduction which was not the case in the current study (Ilchyshyn et al., 2019). The fact urine GGT appears unstable at -20°C degrees is of clinical significance as urine samples are often first frozen at this temperature before analysis or being transferred to -80°C for prolonged storage. Interestingly, the addition of a urine protein inhibitor does not appear to prevent this degradation and therefore, pending future work to confirm the stability of GGT under different conditions, it is advised that urine samples are either immediately processed for urine GGT measurement or frozen directly at -80°C degrees.

Although the NGAL ELISA required preliminary work to determine the adequate dilutions to bring our samples on scale, once these dilutions had been established the ELISA overall worked well. Several samples required marked dilution and ideally repeat ELISAs would have been run to ensure accuracy at such high dilutions, however, this was not possible for this master's project due to cost and time constraints. We used a cut-off of 13ng/mL to determine an abnormal NGAL result. This cut-off was chosen based on pre-existing literature and consideration of the values of NGAL reported in the healthy control dogs for various previous studies. A recent study has reported a much lower reference range for NGAL in dogs; Davis *et al.* (2021) assessed 110 healthy dogs and reported a reference range of 0.04-3.67ng/mL. These results were considerably lower than the other previous study that has also looked at a relatively large cohort of healthy dogs in which 42 healthy dogs were analysed and a reference range of 0.1-12.9ng/mL reported (Segev et al., 2013b). It is possible that the discrepancy between these studies is due to the difference in methodology of urine collection (Davis *et al.* analysed urine samples that were freely voided whilst Segev *et al.* utilised samples collected via cystocentesis). The majority of our NGAL results were abnormal, therefore, we did not stratify the NGAL results categorically. As a result, although the exact cut-off to determine an abnormal NGAL is debatable, altering the value used to define normal would not have impacted the statistical analysis performed with respect to NGAL.

NGAL values were available for just 7 healthy dogs in our study with a median value of 11.1ng/mL (range: 0.74-16.8). Two of the control dogs had an NGAL result above the cut-

off that we used to define normal (13.4ng/mL and 16.8ng/mL). The variability of NGAL results reported for healthy dogs between the current study and the pre-existing literature suggests that further evaluation is required to determine a reliable reference range. Of interest, previous literature has suggested that NGAL is impacted by pyuria (Davis et al., 2021, Proverbio et al., 2015), one control sample with an active sediment was run in error and generated an NGAL result of 143.1ng/mL in fitting with this previous literature and highlighting the importance of consideration of sediment examination when interpreting NGAL readings.

Originally, we had planned to perform proteomic analysis in addition to UPE and therefore, we stored urine samples with and without PI. Currently, there is no substantial evidence to support the use of PI for gel-based protein analysis and therefore we opted to perform some comparisons between plain and PI containing urine during the preliminary work for this thesis; a clear advantage to the inclusion of PI was not found. This is potentially an important consideration for future studies in this area as the use of PI comes with cost implications. Most of the veterinary research regarding urinary protein assessment/quantification appears to be done without the use of a PI although comparative studies utilising samples with and without PI are lacking; a recent systematic evaluation of the human literature suggested that there is no requirement for the use of a PI for gel-based urinary proteomics which is in fitting with the findings of our current study (Havanapan and Thongboonkerd, 2009).

3.4.6 Study limitations

This prospective study had several limitations. To begin with, the study lacked concurrent renal biopsies to accompany urinalysis and protein profiling. Whilst lack of renal biopsies meant that the protein patterns observed could not be associated with a histopathological diagnosis it did not impact on the interpretation of results that the study generated and the lack of histopathological diagnosis did not limit the ability to draw conclusions from this study. Although every attempt was made to accurately classify dogs with and without a trigger it is possible that a small proportion of dogs may have been incorrectly classified as having or not having a trigger for their proteinuria. Despite the prospective nature of the study a variety of factors influenced the extent of trigger screening, meaning an exhaustive screening process was possibly not performed in every case. However, each case underwent a specifically tailored work-up and therefore it is presumed that each dog will have been exposed to the appropriate level of screening to determine as accurately as possible whether a trigger was present. We used gels specifically formatted for urine electrophoresis, however, there were some limitations associated with the gels/our methodology. Modifications to the

gel running conditions needed to be made due to equipment available in our laboratory and it is possible this led to mild changes in the results we obtained. We used the manufacturers guidelines for interpretation of the protein bands on the gels, however, confirmation of the protein we expected to be present was not performed. Techniques such as western blotting could have been utilised to confirm the identity of individual proteins, however due to time and financial constraints this analysis was not performed for this master's study. Finally, the gels were not loaded with an equal amount of protein (instead, as per manufacturer guidelines, a *maximal* concentration of 2mg/mL was used). As each lane was not loaded with the same protein concentration direct comparisons could not be made between levels of individual proteins between lanes. However, this study was, in the majority, a qualitative study and hence accurate quantitative data was not required to achieve the study aims, hence the lack of standardisation between protein content in each lane was not thought to have significantly impacted the results.

Typically, documentation of persistent proteinuria is recommended to exclude a transient aetiology. However, we allowed enrolment of cases based on only one UPC measurement of >2.0. On retrospective analysis of the cases enrolled, over three quarters of our study population had persistent proteinuria documented. In the remaining cases although persistency was not proven considering the magnitude of UPC required for inclusion into this study it was thought that a transient aetiology for proteinuria would be much less likely to be present.

The urine sediment in cases of glomerular disease is not always completely inactive, for example, some cases of glomerulonephropathy can have inflammatory changes present within the urine (Borys et al., 2019, Grauer et al., 1988). Whilst the exclusion of urine samples with an active sediment may have led to the exclusion of some such cases of glomerular disease we excluded these samples to try to eradicate the inclusion of dogs with post-renal proteinuria. By adopting this approach, we may have missed some cases that were eligible for inclusion but we were less likely to wrongly include a dog with post-renal proteinuria. Additionally, measurement of NGAL has been shown to be impacted by the presence of pyuria and urinary tract infection (Nabity et al., 2012, Daure et al., 2013) adding further reasoning to the exclusion of samples with an active sediment. Whilst the exclusion of dogs with a globulin of >47g/L was performed to try to avoid inclusion of dogs with pre-renal proteinuria it may have led to the inadvertent exclusion of dogs with infectious diseases such as Leishmania or Ehrlichiosis. However, the prevalence of these disease in the UK is thought to be low and hence it was hoped few cases would have been potentially wrongly excluded based on the cut-off for globulin selected.

Urine aliquots for UPE analysis and NGAL ELISA were stored at -80°C for a variable amount of time and while protracted storage has the potential to impact results due to degradation of protein previous literature suggests the degree to which this occurs is not of concern. For example, previous studies (Lavoue et al., 2015) have referenced unpublished data suggesting that UPE profiles are unaffected by canine urine being stored at -80°C for up to 6 months whilst in human medicine, several studies have also shown urine gel electrophoresis to be unaffected by samples being stored at -70°C (Lee et al., 2008, Kania et al., 2010). It has also been reported that NGAL is stable in urine samples for up to 8 years and that it is unaffected by freeze-thaw cycles. Therefore, we do not feel the way the urine was handled will have had any significant impact on our results.

Another important limitation to this study was the small sample size resulting in relatively low numbers of dogs being present for sub-group analysis. Additionally, the gels utilised for this study detected a given number of specific proteins only and hence a full analysis of all the proteins present within each sample was not performed. However, the gels have been specifically formulated for the analysis of canine proteinuria and therefore were deemed to evaluate the most important tubular and glomerular proteins. ImageJ was utilised to perform part of the analysis in this study, the cut-offs drawn to generate values using this software are relatively subjective and hence it is possible that subtle differences in values could arise between operators. To avoid intra-operator variability, analysis was performed by only one person, however, it remains the case that should this analysis be repeated slightly different results may be yielded. Additionally, interpretation of the banding patterns on the gels was slightly subjective because the bands did not always perfectly align with the marker. Again, to avoid intra-operator variability analysis was performed by one person but it is possible that misinterpretation of sporadic bands occurred. These differences in interpretation are thought to be subtle and therefore the chance of variation significantly impacting on results is thought to be low.

Finally, as this was a clinical research project, it is possible that some of the clinicopathological information was influenced by recent medical intervention.

CHAPTER FOUR

Conclusions

4.1 Conclusion

This thesis was performed in two parts with an initial retrospective study followed by a prospective study.

The retrospective study aimed to determine the proportion of dogs with presumed glomerular proteinuria that respond to ACEi therapy and if this response conveys survival benefit. The results of our study demonstrated that although less than half of our study population responded to ACEi treatment, response (as defined by a ≥50% reduction from baseline UPC or achieving a UPC of <0.5) was associated with a significant survival benefit in dogs with presumed glomerular proteinuria. The presence of baseline azotaemia, hypoalbuminaemia and the magnitude of proteinuria were found to be negative prognostic indicators in this retrospective study.

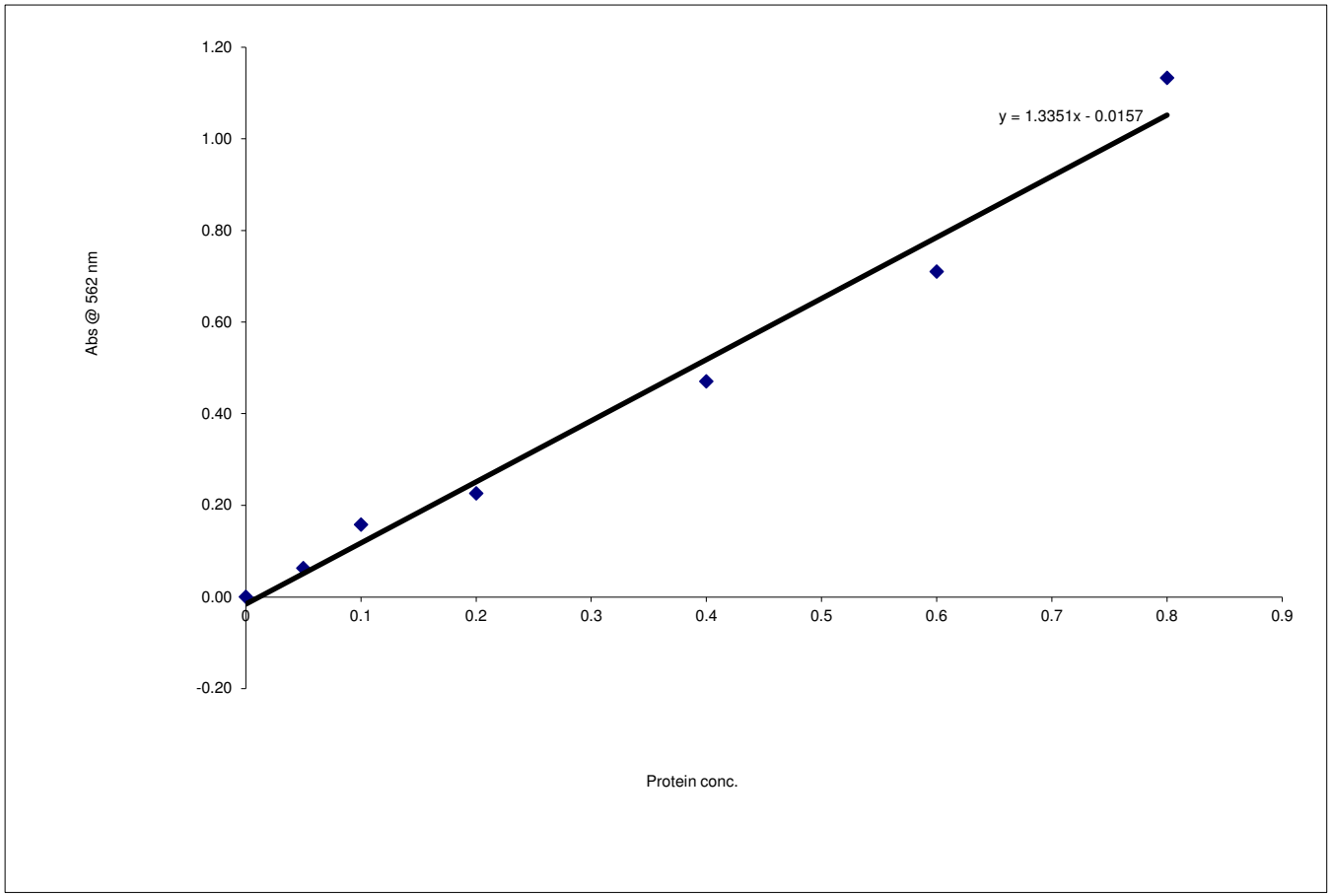
The prospective part of this thesis aimed to further explore the urinary protein pattern of dogs with what, historically, would have been presumed to be glomerular proteinuria (UPC >2.0). The main findings of interest were that most of our study population had evidence of tubular involvement and that detection of tubular proteins on UPE analysis suggested the presence of more advanced disease. The urinary biomarker NGAL was increased in almost all dogs and our work suggests that NGAL may be an earlier or more sensitive marker of tubular damage than UPE profiles. Our results also suggest that certain proteins (such as alpha 1 microglobulin and IgG) may be important biomarkers of disease progression/severity and hence prognosis. Importantly, our prospective study largely corroborates the results of the retrospective study regarding the prognostic implications of currently used markers of disease progression/severity. Results from both the retrospective and prospective work performed for this thesis have shown creatinine and serum albumin have important prognostic implications with increased creatinine and lower serum albumin being associated with a worse prognosis in dogs with a UPC >2.0. The retrospective study also highlighted UPC as a prognostic indicator and while this was not documented in the prospective study this may reflect the small sample size. On balance, although Lorbach *et al.* (2020) and Hokamp *et al.* (2016) previously also did not find UPC to be associated with survival, the majority of the pre-existing literature supports the findings of the retrospective study that an increased UPC is likely to be a poor prognostic indicator. This is further inferred by studies documenting that reduction of proteinuria leads to slowed progression of disease (Jacob et al., 2005, Wehner et al., 2008). It is possible that UPC was not identified as a prognostic marker by Lorback *et al.* (2020) as they assessed a very specific population of dogs (i.e. only those with FSGS) and UPC simply may not be an ideal prognostic marker

in this cohort of dogs. Further work is required to assess the prognostic implications of UPC and to corroborate the findings of the retrospective study.

Overall, it is felt that the work generated from this thesis adds substantively to the field and has highlighted interesting areas for further research. The retrospective study suggests that clinically, pursuing a response to ACEi therapy is beneficial as dogs that respond appear to have prolonged survival. However, further work is required to determine if dose escalations of ACEi help to increase the numbers of responders (or if we should instead look to trialling alternative therapy), assess the benefit and tolerability of ACEi treatment in dogs with higher IRIS stages and to further understand the apparently variable response to ACEi therapy in proteinuric dogs. The prospective study demonstrated that most dogs with a UPC of >2.0 have a mixed protein pattern on UPE and that the presence of tubular proteins in dogs with presumed glomerular disease may have prognostic significance. Further work is required to continue this research and to further analyse the urinary protein profile in dogs with variable magnitudes of UPC. Longitudinal studies are required not only to investigate the prognostic implications of the presence of certain proteins but also to evaluate the impact of/response to standard anti-proteinuric therapies. To further explore the relationship between UPE patterns and specific glomerular pathology, future studies would ideally assess the urine protein profile of dogs with a histopathological diagnosis.

CHAPTER FIVE

Appendices



Appendix 1: Graph of absorbance (abs) at 562nm against protein concentration for calculation of protein content via BCA assay. BCA= Bicinchoninic protein assay

Sample	Baseline (within 24 h)	Fridge within 48h	Room temperature within 7 days	Fridge within 7days	Freezer -20°C within 7days	Freezer -20°C within 7days + PI	Freezer -80°C within 7days	Freezer -80°C within 14 days	Freezer -80°C within one month
GGT-1	47	50	NA	45	9	NA	44	50	NA
GGT-2	98	96	NA	90	8	NA	93	94	NA
GGT-3	33	33	NA	30	8	NA	30	32	NA
GGT-4	72	80	70	80	5	NA	79	79	NA
GGT-5	121	112	112	113	6	NA	113	112	NA
GGT-6	20	16	10	16	7	NA	16	17	NA
GGT-7	58	57	NA	54	8	8	50	NA	54
GGT-8	24	36	32	34	6	5	28	NA	30
GGT-9	42	39	37	37	35	36	37	NA	39
GGT-10	41	38	39	37	7	8	38	NA	36
GGT-11	17	16	9	15	12	17	18	NA	19
GGT-12	30	30	28	29	5	6	29	NA	31

Appendix 2: Urine GGT levels of the 12 urine samples stored under various conditions. GGT = Gamma glutamyl transferase.

Well No.	Sample no.	Initial pg/mL	pg/mL corrected for dilution factor	ng/mL	Notes
1	002	NA	-	-	
2	003	329.14	32914.00	32.914	
3	004	NA	-	-	
4	007	418.78	41878.00	41.878	PI
5	008	NA	-	-	PI
6	012	210.49	21049.00	21.049	
7	017	NA	-	-	
8	027	76.39	7639.00	7.639	
9	031	55.36	5536.00	5.536	
10	039	NA	-	-	
11	040(3)	NA	-	-	
12	046	NA	-	-	
13	051(3)	364.13	36413.00	36.413	
14	054	NA	-	-	PI
15	058	NA	-	-	
16	033(2)	NA	-	-	
17	066	NA	-	-	
18	077	267.49	26749.00	26.749	PI
19	079	489.99	48999.00	48.999	
20	085	NA	-	-	
21	088	NA	-	-	
22	101	NA	-	-	
23	105	125.28	12528.00	12.528	
24	108	NA	-	-	
25	115	NA	-	-	
26	116	208.90	20890.00	20.89	
27	126	NA	-	-	
28	146	NA	-	-	
29	148	NA	-	-	
30	162	NA	-	-	
31	167	NA	-	-	
32	171	262.78	26278.00	26.278	

33	178	NA	-	-	
34	187	232.66	23266.00	23.266	
35	C04	0.75	75.00	0.075	
36	C05	3.09	309.00	0.309	
37	C06	16.80	1680.00	1.68	
38	C08	11.14	1114.00	1.114	
39	007	NA	-	-	Plain
40	077	345.48	34548.00	34.548	Plain

Appendix 3: Initial results from the initial 96-well NGAL ELISA. PI= urine sample run included a protease inhibitor. ELISA=Enzyme-linked immunosorbent assay, NGAL=neutrophil gelatinase associated lipocalin.

Study no.	Sex	Age (days)	Weight (kg)	Breed
002	FN	2881	17.4	Crossbreed
003	FN	3914	7.8	Miniature Schnauzer
004	FN	3985	5.5	Yorkshire Terrier
007	MN	1608	65.0	Great Dane
008	ME	2577	7.1	Yorkshire Terrier
012	MN	3449	15.7	Cocker Spaniel
027	MN	2452	16.4	Crossbreed
031	FE	1507	18.1	Border Collie
033	FN	3872	11.6	Miniature Schnauzer
039	ME	548	20.0	Border Collie
040	FN	2247	15.3	English Springer Spaniel
046	FN	2653	20.4	Bearded Collie
051	FN	3826	6.9	Crossbreed
054	FN	3603	8.6	Lhasa Apso
058	FN	2952	5.2	Crossbreed
066	MN	2384	4.3	Yorkshire Terrier
067	MN	4634	26.7	Border Collie
077	ME	2132	12.0	Cocker Spaniel

079	FN	3631	16.5	Cocker Spaniel
085	MN	4725	7.2	Jack Russell Terrier
101	MN	4005	26.4	Border Collie
108	MN	1110	24.7	Airedale Terrier
115	FN	4337	26.4	English Pointer
116	ME	2767	10.2	Crossbreed
146	FN	3655	8.1	Lhasa Apso
148	ME	2558	10.4	Crossbreed
162	ME	2964	24.2	Shar Pei
167	FE	3999	17.5	Crossbreed
171	ME	2964	34.0	Weimaraner
178	FN	3683	25.1	Crossbreed
187	MN	1998	8.2	Cavalier King Charles Spaniel
226	FN	4760	13.9	Cockapoo

Appendix 4: Table demonstrating basic demographic information for the study population.

Study no.	Simplified diagnosis	Creatinine (µmol/L)	Albumin (g/L)	Cholesterol (mmol/L)	UPC	SDMA (µg/dL)	NGAL (ng/mL)	UPE pattern
002	Inflammatory	415	21	7.96	26.26	29.7	919.25	M
003	No trigger	125	28	10.52	2.87	30	33.8	G
004	Endocrine	172	35	6.08	10.82	15.3	84.91	M
007	No trigger	328	25	13.45	2.86	NA	57.05	M
008	Endocrine	58	36	6.84	12.50	NA	109.93	M
012	Neoplasia	52	39	6.1	6.23	6.2	22.61	G
027	Neoplasia	70	31	11.41	2.24	13.0	7.32	G
031	Endocrine	87	30	11.17	4.31	9.7	5.88	G
033	Hyperlipidaemia	82	32	8.55	5.42	11.5	214.90	M
039	Inflammatory	58	26	4.89	13.21	NA	169.10	M
040	No trigger	142	15	5.57	11.54	21.8	553.22	M
046	No trigger	94	28	10.97	3.71	14.2	75.26	M
051	Hyperlipidaemia	67	35	11.10	2.34	7.4	32.64	M
054	Endocrine	83	30	11.67	3.50	15.3	92.31	M
058	Neoplasia	60	31	17.70	5.85	18.8	219.19	M
066	No trigger	111	30	7.96	8.15	NA	257.69	M
067	Inflammatory	138	19	6.55	2.03	NA	668.01	M

077	No trigger	382	23	7.14	15.80	57.4	30.71	M
079	No trigger	62	39	7.63	6.78	NA	52.32	M
085	Endocrine	76	36	12.25	2.33	13	153.23	M
101	Inflammatory	113	28	11.99	6.16	NA	NA	M
108	No trigger	307	31	5.50	2.89	26.1	52.3	M
115	No trigger	91	30	6.47	2.19	10.6	106.41	M
116	Endocrine	63	37	11.18	2.88	11.0	29.22	G
146	No trigger	290	18	10.95	15.45	27.0	109.37	M
148	No trigger	400	27	6.11	3.45	22.2	54.11	M
162	No trigger	372	20	7.63	11.05	32.9	122.81	M
167	No trigger	109	30	6.88	3.15	NA	73029	G
171	No trigger	65	26	12.06	3.91	9.4	25.59	G
178	Neoplasia	161	31	NA	3.77	NA	59.67	M
187	No trigger	202	31	7.74	5.80	32.1	24.55	G
226	No trigger	110	35	8.99	6.05	14.9	NA	G

Appendix 5: Table demonstrating the simplified diagnosis and baseline biochemical parameters for the dogs included in this study. M= mixed protein profile on UPE, G = glomerular proteins only on UPE. SDMA=S , NGAL=neutrophil gelatinase associated lipocalin, UPE=urine protein electrophoresis.

Study number	% RBP	% Free light chain	% a1 microprotein	% Dimer of free light chains	% Albumin	% Transferrin	% IgG	% IgA	% Haptoglobin	% Alpha2 macroglobulins
002	0.00	0.00	6.40	0.00	67.11	22.85	3.64	0.00	0.00	0.00
003	0.00	0.00	0.00	0.00	79.62	20.38	0.00	0.00	0.00	0.00
004	5.93	12.88	6.35	0.00	51.41	23.43	0.00	0.00	0.00	0.00
007	0.00	0.00	3.85	0.00	42.46	26.00	13.81	8.23	5.64	0.00
008	0.00	0.00	19.35	0.00	66.05	14.60	0.00	0.00	0.00	0.00
012	0.00	0.00	0.00	0.00	80.87	19.13	0.00	0.00	0.00	0.00
027	0.00	0.00	0.00	0.00	65.87	34.32	0.00	0.00	0.00	0.00
031	0.00	0.00	0.00	0.00	82.19	17.81	0.00	0.00	0.00	0.00
039	2.33	0.00	0.00	0.00	78.78	18.88	0.00	0.00	0.00	0.00
040	0.00	0.00	3.74	0.00	54.79	34.16	7.32	0.00	0.00	0.00
046	0.00	0.00	5.62	0.00	61.25	27.84	5.28	0.00	0.00	0.00
051	0.00	0.00	1.96	0.00	78.72	19.32	0.00	0.00	0.00	0.00
054	0.00	0.00	11.64	0.00	67.58	20.78	0.00	0.00	0.00	0.00
058	0.00	0.00	2.22	0.00	67.15	24.90	5.74	0.00	0.00	0.00
033	4.30	0.00	9.45	0.00	50.41	25.72	10.12	0.00	0.00	0.00
066	0.00	0.00	5.93	0.00	58.24	28.73	7.10	0.00	0.00	0.00

067	14.57	0.00	18.88	0.00	50.95	15.60	0.00	0.00	0.00	0.00
077	7.85	0.00	19.52	0.00	54.85	17.78	0.00	0.00	0.00	0.00
079	0.00	0.00	4.74	0.00	69.66	25.60	0.00	0.00	0.00	0.00
085	6.07	0.00	14.85	0.00	67.86	11.22	0.00	0.00	0.00	0.00
101	6.61	0.00	10.70	0.00	63.60	19.09	0.00	0.00	0.00	0.00
108	4.27	6.84	7.35	0.00	54.80	16.35	0.00	6.28	4.12	0.00
115	0.00	0.00	6.79	0.00	55.71	31.84	5.67	0.00	0.00	0.00
116	0.00	0.00	0.00	0.00	83.61	16.39	0.00	0.00	0.00	0.00
146	0.00	0.00	6.77	0.00	54.31	26.95	7.85	4.12	0.00	0.00
148	6.13	0.00	7.15	0.00	59.20	27.53	0.00	0.00	0.00	0.00
162	0.00	0.00	16.44	0.00	50.23	24.34	8.99	0.00	0.00	0.00
167	0.00	0.00	0.00	0.00	90.30	9.70	0.00	0.00	0.00	0.00
171	0.00	0.00	0.00	0.00	78.00	22.00	0.00	0.00	0.00	0.00
178	0.00	0.00	3.01	0.00	61.46	31.22	4.31	0.00	0.00	0.00
187	0.00	0.00	0.00	0.00	77.84	22.16	0.00	0.00	0.00	0.00
226	0.00	0.00	0.00	0.00	83.29	16.71	0.00	0.00	0.00	0.00

Appendix 6: Individual protein contribution (%) to the total protein present in individual urine samples. IgA=Immunoglobulin A, IgG=Immunoglobulin G, RBP=Retinol binding protein

Positive if Greater than or equal to[a]	Sensitivity	1-Specificity
4.8800	1.000	1.00
6.6000	1.000	0.875
14.9650	1.000	0.750
23.5800	1.000	0.625
25.0700	1.000	0.500
27.4060	1.000	0.375
29.9650	1.000	0.250
31.6750	0.955	0.250
33.2200	0.909	0.250
43.0500	0.909	0.125
52.3100	0.864	0.125
53.2150	0.818	0.125
55.5800	0.773	0.125
58.3600	0.727	0.125
66.4800	0.682	0.125
74.2750	0.682	0.000
80.0850	0.636	0.000
88.6100	0.591	0.000
99.3600	0.545	0.000
107.8900	0.500	0.000
109.6500	0.455	0.000
116.3700	0.409	0.000
138.0200	0.364	0.000
161.1650	0.318	0.000
192.0000	0.273	0.000
217.0450	0.227	0.000
238.4400	0.182	0.000
405.4550	0.136	0.000
610.6150	0.091	0.000
793.6300	0.045	0.000
920.2500	0.000	0.000

Appendix 7: Co-ordinates of the curve from receiver operating characteristics (ROC) curve analysis assessing the cut-off of neutrophil gelatinase associated lipocalin (NGAL) to predict the presence of a mixed protein pattern on UPE. [a]The smallest cut-off values is the minimum observed test value minus 1, and the largest cut-off value is the maximum observed test value plus 1. All the other cut-off values are the averages of two consecutive ordered observed test values.

CHAPTER SIX

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