Accurate Early Markers of Renal Damage for Veterinary and Research Use

Introduction
The assessment of renal function in small animals is of interest both to veterinary practitioners and research organisations, especially those investigating the possible nephrotoxic effects of drugs developed for humans or companion animals. For veterinarians, one primary concern is the early diagnosis of chronic kidney disease (CKD), defined as primary renal disease present for an extended period of time, especially in cats. Studies suggest that 10 % of cats over 10 years and nearly 50 % of cats over 15 years old will suffer from the disease, with a proportion of cats progressing to end-stage renal disease. In dogs, CKD is less common but progresses more rapidly, with survival times after diagnosis often less than a year. There is also a clinical benefit in earlier detection of acute kidney injury (AKI) as implementing early administration of supportive care can lead towards a more favourable outcome than for a later diagnosis.

For research organisations, safety studies for development compounds conducted by the human pharma industry commonly require that a drug be tested in two mammalian species. The dog is generally viewed to be a sensitive second species (to complement the rat) and provides a valuable non-rodent species for decision-making. Classically, these are terminal studies and the kidney histology is investigated for signs of drug-induced damage. Efforts for identifying a good safety biomarker are motivated by (i) the pressures to reduce the number of animals sacrificed in scientific experiments, imposed both by regulators and public opinion, (ii) the need for an assessment of the rate of damage and evaluation of functional alterations which are not possible with histological endpoint only. The use of different tests during a study may also enable the location and type of pathological changes to be identified, and provide information on the sequence in which such pathological changes occur. Finally, tests which are used in animal experiments on living animals may be of further use in early human clinical investigations, where it is not possible to obtain the same histological information.

In research and in veterinary practice then, there is a need for an accurate and sensitive way to assess renal function in the living animal, such that small changes in function can be detected, whether due to the effects of drugs or the onset of CKD. Much of the research into such tests has focused on their use as a diagnostic tool in veterinary practice or as a research tool, but there is no reason why a technique used for one may not also be useful for the other.

Several methods for evaluating the kidney function in the living animal are currently used or have been proposed, some of which are detailed below.

Circulating Markers of Renal Function Measured from Blood

The relationship between plasma creatinine and glomerular filtration rate (GFR)

In veterinary practice, the classic way to identify CKD is to measure plasma creatinine concentration. Due to the variation in normal serum creatinine concentration and the inverse exponential relationship of serum creatinine to glomerular filtration rate, serum creatinine increases only a small degree for a relatively large decrease in renal function in the early stages of CKD. This makes single measurements insensitive in detecting early kidney disease. Serial measurement may increase the sensitivity but the largest rises in serum creatinine occur in the later stages of CKD. As a result, the use of creatinine concentrations is really limited to assessing the extent of the damage in later stages of CKD. Similarly, in drug toxicity tests, a significant amount of damage (loss of 75 % of nephrons, the functional units of the kidney) will have already occurred before any changes in creatinine concentration are observed (Figure 1A). Besides, creatinine levels are also affected by several non-renal factors, such as muscle mass, diet and medication, making it a less reliable marker of renal status.

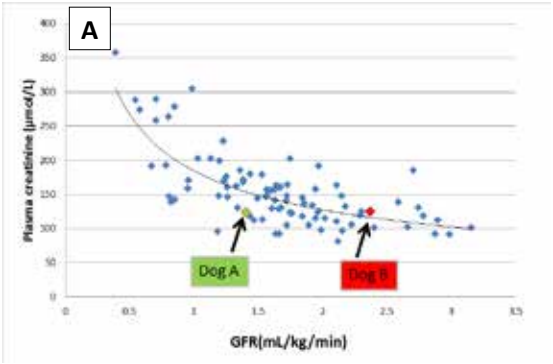

Figure 1 A: inverse exponential relationship between plasma creatinine and glomerular filtration rate. Renal function of dog A and B cannot be differentiated based on the plasma creatinine measurement alone.

Direct measurement of GFR is considered the gold standard test of kidney function. Decreases in GFR indicate that kidney damage is occurring. Urinary clearance of inulin is accepted as the best measure of GFR, but the test is difficult to carry out. Other measures of GFR have been developed for cats and dogs, including clearance of filtrated markers such as exogenous creatinine and iohexol. These tests require repeated blood samples over a few hours to calculate the rate of disappearance of the marker which is equivalent to its rate of appearance in urine (equal to GFR). Although these techniques have been fully validated, they have not been widely used, due to historically a high number of blood samples being

required and a lack of commercial service to calculate clearance of the marker. An iohexol clearance assay of GFR requiring only three blood samples (2, 3 and 4h after injection of the bolus, see Figure 1B) is now available for veterinarians in Europe (deltaDot/Royal Veterinary College, UK) as well as in the USA (Michigan State University). It is anticipated that this will become more commonly used in the future. As a marker of renal damage in research animals, GFR may be more sensitive than alternatives; in an individual animal, GFR seems to vary naturally by about 10-20 %, so changes greater than this would indicate renal damage.

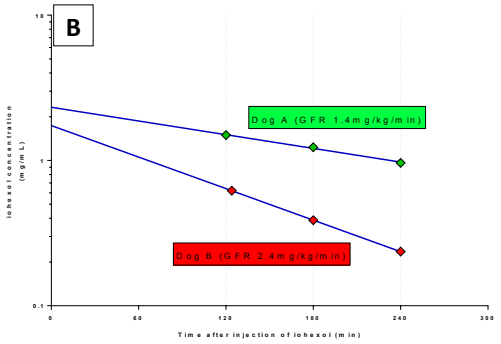

Figure 1B: plasma concentration time curve for iohexol after injection of a iohexol bolus (647mg/kg) for dog A and B. The steeper slope for dog B illustrates the higher iohexol clearance (ie higher GFR) of this dog. Ioxexol concentrations were measured by capillary electrophoresis by deltaDot / RVC.

New circulating markers that could predict GFR better than plasma creatinine

Cystatin C (CysC) - CysC is a 13 kilodalton (kDa) protein and proteinase inhibitor involved in intracellular protein catabolism that is produced at a constant rate. It is not bound to plasma proteins and is freely filtered by the glomerulus. It is not secreted by the proximal tubules, and although it is reabsorbed at this site, the reabsorbed fraction is subsequently catabolised. These features would seem to make it a good candidate marker for GFR. In addition, it has been reported that urinary CysC concentrations are much lower in healthy individuals compared with individuals with renal tubular damage, meaning that it can be used as a marker of proximal tubular damage. Some studies have found that dogs with CKD had significantly higher serum concentrations of CysC than healthy dogs or dogs with non-renal disease[1]. However, other studies have found an overlap in the range seen, which may cause problems with using CysC as a marker of kidney damage[2]. A further problem is that there is no current veterinary assay commercially available, so assays developed for use in humans must be used instead with a resulting loss of accuracy. Furthermore, higher CysC levels have been reported to be associated with greater height, weight and lean muscle mass, the same interferences seen with creatinine[3].

Symmetric dimethylarginine (SDMA) – Recent research has shown that serum concentrations of SDMA may have some advantages over creatinine as a surrogate marker of renal function. Both are well correlated with glomerular filtration rate in later-stage CKD, increasing as GFR falls. However, one study has found that SDMA is more sensitive as a marker of renal damage than creatinine, allowing CKD to be detected with only 40 % loss of kidney function, as opposed to the 75 % required for creatinine to be used. It has been claimed that use of SDMA concentrations enabled the onset of kidney disease to be detected in cats on average 17 months earlier, and in one case four years earlier, than was possible using creatinine concentration measurement alone[4]. Furthermore, unlike creatinine, SDMA concentrations are not affected by lean muscle mass, which declines in older cats. This can cause creatinine concentrations to fall, whereas SDMA concentrations continue to rise as GFR decreases. An assay of SDMA has recently been launched by IDEXX.

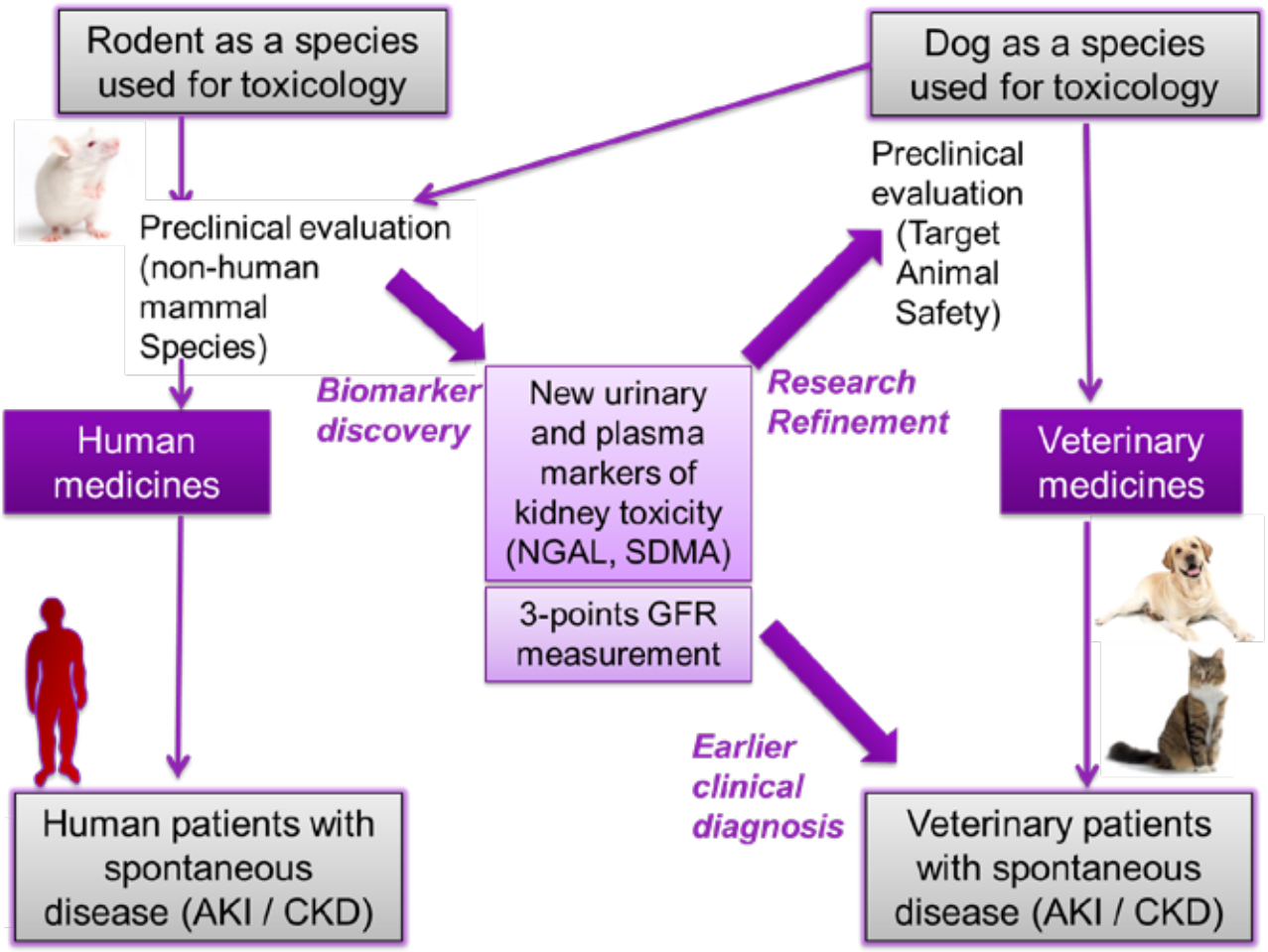

Figure 2: new markers of renal of renal function or toxicity at the interface between veterinary and human pharma and preclinical and clinical studies.

Histopathology:

For chronic toxicology studies in dogs, serial renal biopsies under ultrasound guidance would be perfectly feasible to undertake alongside GFR measurements if cumulative renal toxicity was a real concern. Clinically, renal biopsies may be carried out when cancer or other infiltrative disease is suspected. Although they could lead to an accurate diagnosis of early-stage CKD, the need for an anaesthetic and the potential complications related to the procedure (intra-renal haemorrhage) mean that the risks of the procedure need to be balanced against the value of the information obtained. In cats with small, shrunken kidneys in particular, the information obtained from a renal biopsy is unlikely to suggest a specific treatment of the cause of the kidney disease, since all that is detected is chronic interstitial nephritis and the underlying cause that has initiated this process is not evident.

Markers of Renal Function Excreted and Measurable in Urine:

Urinalysis:

Urine specific gravity can help identify early-stage renal damage. The normally concentrated urine in healthy animals has a urinary specific gravity (USG) higher than 1.030 in dogs and 1.035 in cats. USGs greater than those cut-off values indicate that a significant amount of glomerular filtrate is being reabsorbed by the renal tubules. In the case of loss of 60 % or more of the functional nephrons, urine become isosthenuric (1.008 < USG < 1.012). Conversely, if USG is less than 1.008, it indicates that the kidneys are capable of producing hypotonic urine and therefore partially functional, although they may be insensitive to vasopressin (diabetes insipidus) or demonstrate tubular impermeability to water.

Proteinuria, or the presence of protein in the urine, is also an indicator of kidney disease, although other diseases can also have the same effect. A recent review of the value of screening for proteinuria concluded that "Persistent proteinuria, in the absence of lower urinary tract disease or reproductive tract disease, is usually an indication of renal damage or dysfunction"[5]. Proteinuria is a result of one of two mechanisms being impaired; either a breakdown in the selectivity of glomerular filtration, resulting in an increased amount of protein in the filtrate, or the failure of the tubules to reabsorb the protein after filtration. Tests for proteinuria generally measure the albumin concentration of urine, but the most used methods for testing, the dipstick colourmetric test and the sulfosalicylic acid (SSA) turbidimetric test,

are not particularly sensitive or specific. Higher quality ELISAs are available which can provide more accurate results.

New urinary biomarkers:
A number of urine protein markers have been identified from the urinary proteome excreted in preclinical models of toxicity in the rat. In humans, some of them are used clinically for the identification of renal damage, either from acute kidney injury or chronic kidney disease. The translation of these markers to dogs for safety studies in experimental animals or to detect early changes in renal function (AKI onset or CKD progression) is in its infancy.

These new markers include:

- Neutrophil Gelatinase-associated Lipocalin (NGAL) is potentially useful for the diagnosis of both acute and chronic renal disease in human and dog. Urinary and serum NGAL levels have been reported to be raised in several different kidney diseases, including IgA nephropathy, autosomal polycystic kidney disease, and diabetic nephropathy.
- Kidney Injury Molecule-1 (KIM-1). Sustained KIM-1 expression has been proposed to promote kidney fibrosis and provide a way in which acute and recurrent injury could be linked with progressive chronic kidney disease. In a recent study of human patients with type-1 diabetes and proteinuria, serum KIM-1 level at the start of the study accurately predicted the rate of estimated GFR loss of the next 5-15 years[6]. KIM-1 has been approved by the Food and Drug Administration and European Medicines Agency as a urinary biomarker for monitoring preclinical nephrotoxicity in rats. However, more research needs to be done if it is to be used in studies involving dogs.
- Liver-type Fatty Acid–Binding Protein (L-FABP). In a study of 50 patients with CKD, the urinary concentration of L-FABP was well correlated with the extent of tubulointerstitial damage and urinary protein excretion[7].

Although these markers may prove useful in animal studies as well in the future, more experimental evidence is still needed to demonstrate their suitability for identifying renal damage in studies involving rodents and dogs and to develop them as commercially available assays.

Conclusion
There is a need for both veterinary practices and research organisations to be able to measure kidney damage in living small animals, both for diagnosis of disease and to obtain information on the toxicity of drugs being tested in pre-clinical trials. In the past, the crude measurement of plasma creatinine was the standard, but this marker is not very sensitive and may be affected by non-renal factors (muscle mass, diet). Newer tests, including serum SDMA concentration and direct measurements of GFR, are more sensitive, enabling earlier detection of kidney damage. They also have the advantage of being applicable in humans, allowing the same tests to be used in clinical trials when the nephrotoxicity of a drug needs to be monitored. Other biomarkers of renal disease have been proposed and some seem promising, but more work is still needed to demonstrate their effectiveness. The future development of a multiplex ELISA for measuring several urinary biomarkers at the same time in companion animals, associated with increased acceptance of GFR testing, could greatly improve functional renal explorations in research animals or clinical patients.

References

1. Almy FS, Christopher MM, King DP et al. Evaluation of cystatin C as an endogenous marker of glomerular filtration rate in dogs. J Vet Intern Med 16, 45–51 (2002)
2. Braun JP, Perxachs A, Péchereau D *et al*. Plasma cystatin C in the dog: Reference values and variations with renal failure. Comp Clin Pathol 11, 44–49 (2002)
3. Knight EL, Verhave JC, Spiegelman D, Hillege HL, de Zeeuw D, Curhan GC *et al*. Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. Kidney Int. 65(4), 1416-21 (2004)
4. Hall JA, Yerramilli M, Obare E, Yerramilli M, Jewell DE. Comparison of serum concentrations of symmetric dimethylarginine and creatinine as kidney function biomarkers in cats with chronic kidney disease. J Vet Intern Med 28(6), 1676–1683 (2014)
5. Harley, Leyenda, and Cathy Langston. "Proteinuria in Dogs and Cats." The Canadian Veterinary Journal 53(6), 631–638 (2012)
6. Sabbisetti VS, Waikar SS, Antoine DJ *et al*. Blood kidney injury molecule-1 is a biomarker of acute and chronic kidney injury and predicts progression to ESRD in type I diabetes. J Am Soc Nephrol. 25(10), 2177–2186 (2014)
7. Kamijo A, Sugaya T, Hikawa A *et al*. Urinary excretion of fatty acid-binding protein reflects stress overload on the proximal tubules. Am J Pathol 165, 1243–1255 (2004)

Dr Sam Williams: Analytical biochemist, his PhD was on the detection of recombinant erythropoietin (EPO) in anti-doping samples. Currently working on a collaboration between the Royal Veterinary College, London and deltaDOT, looking at drug metabolism in veterinary applications and their transfer to humans, and the development of veterinary therapeutic drug monitoring applications. Recently launched an on-demand assay of glomerular filtration rate for cats and dogs
Email: samwilliams@rvc.ac.uk

Dr Ludovic Pelligand: Graduated from the École Nationale Vétérinaire d'Alfort (Paris, France) in 2001, where he completed a two-year small animal internal medicine internship. He then completed a three-year anaesthesia residency at the Royal Veterinary College where he gained his European Diploma of Veterinary Anaesthesia and Anaesthesia in 2006. Has been employed as a clinical pharmacology research fellow and is now a lecturer in clinical pharmacology and anaesthesia.
Email: lpelligand@rvc.ac.uk