# Update on clinicopathological assessment of renal health in non-racing greyhounds

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# Abstract

**Background:** Serum creatinine concentrations differ in greyhounds compared with non-sighthounds, but it is not known whether urine creatinine concentrations also differ and whether any difference would influence the interpretation of the urine protein to creatinine ratio (UPC). Additionally, there is some evidence for greyhounds having higher serum symmetric dimethylarginine (SDMA) than non-sighthounds, but this has yet to be confirmed in healthy non-racing greyhounds.

**Objectives:** The objectives of this study were fourfold: (1) to compare the urine creatinine concentrations in healthy greyhounds and a control group of healthy non-sighthounds, (2) to determine the UPC reference interval in healthy greyhounds and to compare this with the UPC reference interval in a control group of healthy non-sighthounds, (3) to determine the serum SDMA concentration reference interval in healthy greyhounds and to compare this with the serum SDMA concentration reference interval in a control group of healthy non-sighthounds and with a previously established canine serum SDMA concentration reference interval, and (4) to establish whether lean body mass is correlated with serum creatinine and urine creatinine concentrations in greyhounds.

**Methods:** The study used an observational cross-sectional design and included 98 clinically healthy non-racing greyhounds and 24 non-sighthound dogs with similar weight, age and sex distributions, as determined by t-test and chi-squared tests. SDMA, urine creatinine concentration and UPC values were measured from blood and urine samples. Linear regression was used to compare the greyhound and non-sighthound

groups. Greyhound reference intervals were determined for SDMA and UPC using non-parametric methods. These were compared with the reference intervals for the non-sighthound group and with current International Renal Interest Society guidelines. In the greyhound sample, the association of urine creatinine with thigh circumference, height and weight was estimated using Pearson correlation. Statistical significance was set at $P < 0.05$ for all analyses.

**Results:** Mean urine creatinine was approximately 22% higher in greyhounds than non-sighthounds after adjusting for urine concentration ($P < 0.05$). The upper limit of the greyhound UPC reference interval was 0.20 or 0.42, depending on whether strict or moderate exclusion criteria, respectively, were applied. The mean UPC was 29% lower in greyhounds than non-sighthounds, but this difference was not statistically significant ($P = 0.1$). The serum SDMA reference interval for greyhounds was 6.3–19.7 µg/dL (0.31–0.98 µmol/L). The upper end of this interval was higher than the upper limit of the published canine reference interval (6–13 µg/dL), and the mean concentration was statistically significantly higher in greyhounds (13.0 µg/dL) than non-sighthounds (10.2 µg/dL, $P < 0.001$). In greyhounds, there were weak correlations between the three morphometric measurements and both serum creatinine and urine creatinine after adjusting for urine concentration.

**Conclusions and clinical importance:** These findings provide further evidence that greyhounds require several breed-specific reference intervals when evaluating renal function. Apart from having higher serum creatinine, greyhounds also have higher SDMA and higher urine creatinine when compared to non-sighthounds. Although UPC trended slightly lower in greyhounds, this finding was not significant, and therefore the

threshold for non-proteinuria set by IRIS guidelines appears to be appropriate for greyhounds based on the calculated reference interval.

# Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the Master of Veterinary Science

(ii) due acknowledgement has been made in the text to all other material used

(iii) the thesis is fewer than 30,000 words in length, inclusive of footnotes, but exclusive of tables, maps, bibliographies and appendices.

Rebekah Liffman

# Preface

Part of this work (Chapter 5) has been published in the following paper:

- Liffman R, Johnstone T, Tennent-Brown B, Hepworth G, Courtman N. Establishment of reference intervals for serum symmetric dimethylarginine in adult nonracing Greyhounds. *Vet Clin Pathol*. 2018;00:1–6. https://doi.org/10.1111/vcp.12638

Parts of this work have also been presented at the following conferences:

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# Chapter 1. Review of Literature

## 1.1. Physiology of creatinine

Creatinine is a small molecule that is produced from creatine and creatinine phosphate. Creatine production begins in the kidney from the transamidination of arginine, glycine and methionine, to produce guanidinoacetate[1], although there is evidence that in dogs, some creatine is absorbed from ingested meat in the alimentary tract.[2] N-methylation of guanidinoacetate then occurs in the liver to produce creatine, which is then distributed to muscle cells.[1] The creatine undergoes a spontaneous, irreversible, non-enzymatic reaction and combines with a dephosphorylated form of creatine phosphate to produce creatinine in a temperature- and pH-dependent reaction.[3]

Approximately 95–98% of creatine is stored in muscle cells (with some also stored in the brain, kidney, liver, blood and urine).[4] Several studies have shown that in humans, if lean body mass remains stable, the turnover from creatine to creatinine is relatively constant and continuous at a rate of approximately 1.6–1.7% per day.[4,5] Daily endogenous production of creatinine in healthy Beagles has been reported at approximately 380 µmol/kg.[6]

Creatinine is commonly used as a marker of glomerular filtration rate (GFR) because it fits many of the criteria for the role: it is highly water soluble, not protein bound, freely filtered by the glomerulus and not re-absorbed in renal tubules.[6] Approximately 99% of exogenously administered creatinine is recovered in the urine of dogs within 24 hours.[6] Secretion of creatinine by active transport in the renal proximal tubule has been reported in humans and mice, and some studies have found weak

proximal tubule secretion in male, but not female, dogs.[7-11] The clinical relevance of this finding is not clear because these studies were performed under general anaesthesia, during which altered renal haemodynamics can occur.[12,13] Other studies have not found tubular secretion in dogs, or have found it to be of negligible significance in healthy animals.[6,14]

## 1.2. Creatinine as a diagnostic tool

Serum creatinine is frequently used in veterinary and human medicine to assess renal function.[15,16] Serum creatinine analysis is accessible to most general practice veterinary clinics and is relatively inexpensive to perform.[17,18] Urine creatinine is measured using methods that are similar to those used for the measurement of serum creatinine.[19] The most commonly used methods are the Jaffe and modified Jaffe spectrophotometric methods and the enzymatic method.[20] These techniques are widely used and therefore allow comparison of results between studies (see Table 1-1). However, accurate measurement of creatinine using the Jaffe and modified Jaffe methods is limited by several interferences.[20-22] Serum creatinine can be overestimated by as much as 15–25% due to the presence of Jaffe-like chromogens, including proteins, lipids, ketones, glucose, ascorbic acid and acetoacetic acid. Bilirubin is a notable interference, with creatinine underestimated by up to 50%.[20,23-25] The enzymatic method is reported to have fewer interferences than the Jaffe and modified Jaffe methods but is more expensive to run.[18,20,23,24,26,27]

Measurement of urine creatinine using the Jaffe method is more accurate than the measurement of serum creatinine; this is likely due to interfering substances being proportionately less abundant in urine than in plasma.[19] The production and excretion of

creatinine can be affected by many physiologic and pathologic processes. Some of the most important causes for variation in both serum and urine creatinine are described below.

### 1.2.1. Biological variation

In one study that measured serum creatinine in non-greyhounds every two weeks over a 6 month period, the biological variation of serum creatinine in dogs was found to be wide. When using the Jaffe method to calculate serum creatinine concentrations, the within-dog coefficient of variation (CV) was 9.0%, (which includes an analyser CV of 4.6%) and the critical difference was 23.3 µmol/L (i.e. the difference in a laboratory result that is likely due to a disease process rather than incidental biologic variation).[28] In fasted dogs, plasma creatinine is stable over 24 hours[6] and there are no significant differences in urine creatinine excretion between day and night samples[29]; therefore circadian factors are unlikely to be a clinically significant factor in creatinine measurement.

### 1.2.2. Diet

In dogs, plasma creatinine concentrations may increase[30] within 12 hours of ingesting pelleted food or decrease[31] after ingestion of commercial dog food (composition not specified). Another study found serum creatinine concentration increased within one hour of ingesting cooked meat, but showed no change after eating raw meat.[32] Urine creatinine excretion was significantly greater in dogs fed mixed or protein-rich diets, when compared with dogs that were fasted, or fed a low protein diet.[33]

### 1.2.3. Body weight and muscle mass

Serum and urine creatinine correlate strongly with body weight and muscle mass in humans.[34-39] In dogs, serum creatinine increases with increasing body weight[40-43] and strongly correlates with striated muscle mass.[39,44] To the author's knowledge, the relationship between urine creatinine and body weight has not yet been explored in dogs.

### 1.2.4. Age

It is unclear whether there is a correlation between age and creatinine. While two studies have demonstrated a decline in serum creatinine in middle aged to older dogs[41] (even when adjusted for body composition by dual-energy X-ray absorptiometry scanning[44]), another study showed no difference between three age groups of dog.[45] The relationship between urine creatinine and age has been assessed in 40 healthy dogs with no statistically significant association found; however, it should be noted that urine creatinine was not adjusted for urine concentration in this study.[14]

### 1.2.5. Gender and neutering status

The effect of gender and neutering status on creatinine concentration is minimal. There were no statistically significant differences in urine creatinine or serum creatinine between the sexes in one univariate study[29], and one multivariate study[43], or differences were small enough to be considered clinically insignificant when gender and neutering status were compared.[41,44]

### 1.2.6. Breed

Apart from studies focusing on sighthounds,[46-50] studies of other breed-specific differences in creatinine are scarce. Differences in serum creatinine have been found

between canine breed groups that included toy, working, mastiff-like, retriever/other, mastiff-like, terrier scent hound and spaniel/pointer groups, although creatinine concentrations were not stated in the study, serum creatinine appeared to be higher in larger breeds when extrapolated from the graphs.[41]

### 1.2.7. Drugs

When using the Jaffe method, positive interferences were observed with clinically achievable doses of cephalothin and cefoxitin,[51] therapeutic doses of acetaminophen and aspirin, and all doses of methimazole.[52] This was presumed to be due to these drugs acting as Jaffe-like chromogens. Cimetidine, salicylates and trimethoprim appear to inhibit secretion of creatinine by the proximal tubule.[53] Vitamin D derivatives cause increased serum creatinine via unknown mechanisms, while corticosteroids have been shown to increase serum creatinine concentration and urine creatinine excretion in both rats and humans, probably by accelerating muscle metabolism and increasing GFR.[54-56]

### 1.2.8. Sample storage

Creatinine is very stable with storage. When serum and plasma samples were stored at −20°C or −80°C for up to 8 months and underwent three freeze–thaw cycles over 72 hours, there were no clinically relevant changes in creatinine concentrations.[57,58]

Urine creatinine is also very stable during storage. At room temperature, mean values did not significantly change for up to 72 hours[59] and no clinically relevant changes were seen when urine samples were stored for 1 to 15 years at −20°C to −80°C in humans, chimpanzees and dogs.[60-63]

### 1.2.9. Disease

Plasma and serum creatinine both increase with dehydration. Initially, this is due to the depletion of total body water leading to extracellular hypernatraemia, which draws cellular fluid and creatinine into blood plasma.[64] However, with progressive dehydration, decreased GFR also causes reduced filtration.[65] Diseases leading to increased serum creatinine include primary and secondary renal disease, ureteral obstruction and uroperitoneum.[16] Low serum creatinine can be seen in portosystemic shunts due to diuresis and in animals with low muscle mass (e.g. cachexia and hyperthyroidism).[16,66,67] Urine creatinine concentration is decreased in dogs with urine bladder disease, liver disease, uterine disease, pancreatic disease, prostatic disease, blood parasites and mammary gland tumours, but not in dogs with neurological disease.[14] This is likely due to the production of poorly concentrated urine in these disease states.

## 1.3. Glomerular filtration rate estimation methods in dogs

Several methods can be used to estimate GFR, and these are used in both clinical and experimental settings. Measurement of endogenous markers are generally easiest to perform but suffer from lack of precision and sensitivity.[68-70] Endogenous markers include serum creatinine, cystatin C, symmetric dimethylarginine (SDMA) and endogenous urine creatinine clearance. Exogenous clearance tests involve the introduction of a marker and then measuring the rate at which it is cleared from the body via renal excretion. Such markers include inulin, iohexol, radionucleotides and creatinine clearance.[71]

### 1.3.1. Serum creatinine

There is a moderate correlation between serum creatinine concentration and GFR in dogs.[6,31,72] However, the use of serum creatinine as a marker of canine kidney function has limitations. In particular, serum creatinine can have low sensitivity in the diagnosis of renal disease, and serum creatinine can remain within the reference interval for healthy dogs until functional renal mass is reduced by approximately 75%.[6,11,73] In addition, serum creatinine reference intervals are wide for dogs (and are reported to range from 35 to 250 $\mu mol/L^1$), which can limit the use of population-based reference intervals.[74] Other non-renal factors can affect serum creatinine concentrations (see Section 1.2).[16,39,44]

In addition to the measurement of endogenous creatinine, exogenous creatinine can be administered in creatinine clearance studies so that serum creatinine concentrations become high enough that endogenous interferences become clinically insignificant.[19,75] Pharmacokinetic analysis of plasma concentration over designated time points can be performed to estimate GFR, or urine creatinine can be measured to determine renal clearance rate.[6,76]

### 1.3.2. Symmetric dimethylarginine

SDMA has recently been introduced as a marker of GFR in dogs. SDMA is released into the cell cytoplasm following the intranuclear methylation of the amino acid arginine.[77] Proteins carrying SDMA are involved in DNA repair, protein translocation and signal transduction; the degradation of these methylated proteins leads to free SDMA in the serum.[77] Like creatinine, SDMA appears to be predominantly eliminated in the kidneys[78], although this hasn't yet been unequivocally proven.[79] In humans, a meta-analysis concluded that SDMA shows strong correlation with GFR in people, but more research is

still required to determine whether there is any renal tubular absorption or whether SDMA production is affected by other diseases.[80] Indeed, increased SDMA has been shown to be risk factor for cardiovascular events[79], and is associated with hyperthyroidism[81], diabetes mellitus[82] and polycystic ovarian syndrome in people.[83] In animals, SDMA has been proposed as a superior marker of renal disease than creatinine,[44,70,72,84] as unlike creatinine, SDMA is minimally affected by lean body mass.[44,72,85,86] However, recent research suggests SDMA may not be superior[69], or may be more sensitive but less specific[87] when compared with serum creatinine in the assessment of renal function. Additionally, SDMA cannot be used to predict the severity of disease, or the likelihood of a poor outcome in critically ill dogs[88]. Thus, more investigation is required into the use of SDMA as a marker for GFR in veterinary medicine.

### 1.3.3. Other markers of glomerular filtration rate

Other markers of GFR include cystatin C, urine endogenous creatinine clearance and exogenous markers. Cystatin C is a low molecular weight protein that is expressed in many tissues, is produced at a constant rate and is secreted in urine.[89,90] A review of the veterinary use of cystatin C concluded that cystatin C has the potential to become a valuable biomarker in dogs, but more studies are required to look specifically at method validation, biological influences and direct comparisons with GFR.[91]

Urine creatinine clearance measurements are rarely performed in small animal practice because they requires urinary catheterisation or housing in metabolic cages over 24 hours to ensure complete urine collection.[1] Mean daily urine creatinine excretion in dogs is variable, ranging from 299–425 µmol/kg/day.[6,92] This marked variation may be due to variation in the analytical methods used and other factors that influence GFR such

as breed, normal biological variation, hydration status and the use of sedation during GFR measurement.[37] Other clearance study markers include inulin (which is considered to be the gold standard), iothalamate, iohexol and various radionuclides.[73,93] Disadvantages of these techniques include the requirement for frequent sampling, patient compliance, cost, and in the case of radionuclides, radiation exposure.[6] GFR markers of relevance to the current study are discussed further in the following sections.

## 1.4. Methods used as a standard for urine concentration and renal excretion of other substances

The renal clearance of protein, electrolytes, hormones and drugs can be assessed relative to urine concentration.[16,94-96] Urine concentration can be estimated by several methods, including USG, urine osmolality and urine creatinine.

### 1.4.1. Urine specific gravity and urine osmolality

The gold standard method for the determination of urine concentration is urine osmolality, which is a measure of the concentration of all analytes present in urine.[97] Osmolality is defined as the number of solute particles per kilogram of solvent. Urine solutes include urea, $Na^+$, $K^+$, ammonium ($NH4^+$), $Cl^-$ and other anions.[98] Urine osmolality is most commonly measured by freezing point depression osmometry, which relies on the principle that each mole of dissolved solute will decrease the freezing point of a liquid by 1.86°C.[16] Despite its accuracy, urine osmolality is not frequently measured because most laboratories do not have the equipment required and the analyser is expensive to purchase.[97] Instead, urine concentration is estimated by USG using a hand-

held refractometer. Measurement of USG by refractometry is highly correlated with osmolality in canine urine (r = 0.92–0.96).[97,99]

Urine osmolality in healthy dogs ranges from 160–2,800 mOsm/kg,[100] with the reference interval for healthy adults dogs recently determined to be 369–2,416 mOsm/kg.[99]

Several substances such as bilirubin and ketones can affect the correlation between USG and urine osmolality,[101] but this might not be clinically significant.[102] Substances such as haemoglobin, glucose and protein may[101] or may not[102] affect the correlation between USG and urine osmolality. Other factors influencing both USG and urine osmolality include age,[100] diurnal variation[100] and storage, with freezing at −20°C and −80°C for more than 7 days associated with a decrease in urine osmolality of up to 5% in canine samples.[103] The effect of storage on USG has not been assessed in canine samples, but in other species, USG is not affected by storage (up to 2 weeks at −20 to −93°C)[104] or freezing and thawing.[105]

### 1.4.2. Urine creatinine as a standard for urine concentration

Unlike serum creatinine, urine creatinine is rarely interpreted on its own. Urine creatinine is excreted at a constant rate that reflects GFR, and in humans, urine creatinine is positively correlated with urine concentration, as assessed by USG and osmolality.[106-109] In veterinary practice, urine creatinine is commonly used as a biomarker for urine concentration in the quantification of the renal excretion of several substrates.[16] There are very few studies of urine creatinine concentration in healthy dogs and none of these studies compared urine creatinine concentrations between breeds. Most of the studies of urine creatinine concentration did not exclude unhealthy dogs, and only one unpublished

thesis examined the correlation between urine creatinine and urine concentration in dogs; the conclusion being that USG was more highly correlated with urine osmolality ($r = 0.94$) than urine creatinine ($r = 0.65$).[110] These studies are summarised in Table 1-1.

**Table 1-1. Summary of canine studies of 24-hour urine creatinine excretion**

| Reference | Urine creatinine (μmol/L) | Disease exclusion | Number of subjects | Method | Breed | Did study correlate urine creatinine with urine concentration |
|---|---|---|---|---|---|---|
| Fojut-Palka, Winnicka 2008[14] | Mean ± SD 18,900 ± 8,100 | Yes | 40 | Modified Jaffe | Various | No |
| Vasconcelos and Pacheco 1999[111] | Mean ± SD 23,214 ± 9,969 Range 8,000–53,300 | Yes | 26 | N/S (abstract only) | German Shepherds | No |
| Rossi, Giori et al. 2012[59] | Mean 12,181 Range 397–36,950 | No | 50 | Modified Jaffe | N/S | No |
| Rossi, Bertazzolo et al. 2015[112] | Median 7,293 Range 654–28,465 | No | 30 | Modified Jaffe | Various | No |
| Rossi, Bertazzolo, Binnella, Scarpa, Paltrinieri 113 | Median 7,991 Range 1,785–51,361 | No | 391 | Modified Jaffe | Various | No |
| Surman, Couto et al. 2012[49] | Mean ± SD 37,800 ± 13,350 Range 8,660–82,960 | Yes | 48 | Enzymatic | Greyhounds | No |
| Moyen 110 | Mean 14,121 Range 1,037–38,693 | No | 170 | Enzymatic | Various | Yes |

Note: N/S, not stated; SD, standard deviation.

## 1.4.3. Assessment of proteinuria in dogs

### *1.4.3.1. Definition and significance of the urine protein to creatinine ratio*

In humans and animals, 24-hour urine collection is the gold standard for determining urine protein excretion. However, spot urine protein to creatinine ratios (UPCs) are frequently used to estimate daily protein excretion because of the method's simplicity and

convenience.[114] The UPC is a ratio whereby urine protein concentration (g/L) is divided by urine creatinine concentration (g/L). The UPC is highly correlated with urine protein excretion in dogs,[115-117] and if the UPC is less than 4, just one measurement provides good clinical precision.[118]

Another technique to account for urine concentration is to divide urine protein by USG or osmolality, which may lead to a more accurate indication of proteinuria.[119] This technique is rarely used, likely due to convention and the lack of canine reference intervals.[94,109,120]

#### *1.4.3.2. Measurement of proteinuria*

Several methods are used to analyse urine protein concentration. Dry reagent test strips (dipsticks) are rapid and inexpensive methods whereby urine protein concentration can be semi-quantitatively determined in the point-of-care setting.[121] Different protein concentrations result in different colour changes on the reagent pads. There are, however, several limitations to this method: interpretation is subjective,[121] alkaline urine can lead to false positive results[65] and protein levels vary with urine concentration.[16]

Benchtop analysers can also be used to measure protein concentration in urine. The urine sample is centrifuged and the supernatant is measured to avoid false positive results caused by proteinaceous material commonly found in urine sediment (e.g. mucous, cells and tubular casts).[122] Benchtop analysers use several methods. The trichloroacetic acid precipitation method relies on the precipitation of protein, which leads to increased solution turbidity and is temperature sensitive.[16] Dye binding methods include the Coomassie brilliant blue assay and the Pyrogallol red-molybdate method. The benzemonium chloride method relies on bound proteins leading to solution turbidity that

is proportional to the protein concentration.[16] Immunoassays can be used to quantify low concentrations of albumin (microalbuminuria) and electrophoresis can be used to separate proteins into subtypes by weight and charge.[123] The immunoassay is highly sensitive and does not differentiate between functional and pathologic proteinuria. Therefore, routine laboratory methods, such as UPC, may be more clinically useful in veterinary patients.[124,125]

#### *1.4.3.3. Urine protein and the urine protein to creatinine ratio in normal dogs*

The diagnosis of proteinuria helps to establish the presence and degree of tubular and glomerular disease.[123] In healthy dogs, urine protein excretion is minimal because most of the small proteins that pass through the glomerulus are reabsorbed in the proximal tubule.[16] Thus, protein concentrations in healthy animals are expected to be low, although small amounts of urine protein (0.04–0.95 g/L or 1+ on a urine dipstick) can be normal in dogs with concentrated urine.[16,126,127] The *IRIS Consensus Recommendations for Treatment of Canine Proteinuric Kidney Disease* state that in dogs, a UPC of less than 0.2 is non-proteinuric, 0.2–0.5 is borderline proteinuric and greater than 0.5 is proteinuric.[128] This consensus statement is based on several studies that assessed UPC or protein excretion in healthy dogs and in dogs with renal disease (see Table 1-2).[129]

**Table 1-2. Summary of studies evaluating urine protein excretion in healthy dogs**

| Reference | Urine protein (mg/kg/24hr) | Urine protein to creatinine ratio | Number of dogs | Method | Breed |
|---|---|---|---|---|---|
| White, Olivier, et al. 1984[115] | (1.9–11.7)<br>Mean 4.73 ± 3.7 | Median 0.095<br>(0.08–0.54) | 8 | Tricholoroacetic acid – ponceau S | Various |
| Center, Wilkinson et al. 1985[117] | (0.2–7.7)<br>Median 1.5 | Median 0.05<br>(0.01–0.38) | 19 | Tricholoroacetic acid precipitation | Various |
| Grauer, Thomas et al. 1985[116] | (0.6–5.1)<br>Mean 2.3 ± 1 | Median 0.07<br>(0.02–0.17) | 16 | Coomassie blue | Beagles |
| Tvedten, 2016[126] (letter to editor) | 179 ± 89 mg/L | (0.02–0.21) | 40 | Spectrophotometric, spectroscopy | N/S, unpublished data, |
| Wijayawardhane, Karunathilakee et al, 2017[130] | N/S | Mean 0.06 ± 0.05<br>All < 0.2 | 51 | Pyrogallol red-molybdate | N/S |

Note: N/S, not stated.

### *1.4.3.4. Factors influencing urine protein and urine protein to creatinine ratio*

High urine protein concentrations occur for several reasons, including pathologic, physiologic and analytic causes. For example, urine protein concentrations may be higher in older[70,131] and entire dogs[117,127] when compared with younger and castrated dogs, respectively. Although many breed-specific glomerulopathies have been described, there is no clear breed effect on UPC in healthy dogs.[123,132] Exogenous or endogenous glucocorticoids, such as hydrocortisone, reversibly increase UPC, likely because these hormones increase GFR.[70,131] It is not clear whether physical activity affects urine protein concentrations; most canine studies show no significant changes in urine protein excretion after exercise,[133-137] but some show increased protein concentrations, particularly after swimming.[133,135,138]

Collection methods appear to have minimal impact on urine protein concentrations, with samples collected via free-catch showing no significant difference from those collected by cystocentesis.[59,112,113,139]

Urine samples showing a combination of haematuria, bacteriuria and pyuria were found to have a higher median UPC than urine samples showing just one.[140] Generally, higher urine protein concentration and higher UPC were associated with an active sediment,[59,112] but UPC was not correlated with degree of pyuria (> 5 white blood cells per high-power field) or bacteriuria. Most (81%) of the pyuric samples had a UPC of less than 0.5.[140] Haematuria influences UPC[141] but does not increase UPC unless haematuria visibly discolours urine.[140,142]

Urine protein concentrations fluctuate with storage. At room temperature, UPC was stable[143] or significantly increased[59] at 12 hours. At 4°C, urine protein was stable for 1 week, and UPC was either stable or transiently increased at 12 hours. Samples frozen at −20°C for up to 3 months were minimally affected.[59,143]

## 1.5. Renal health and greyhounds

Greyhounds are predisposed to several conditions in which markers of renal function are analysed. These conditions include hypertension, renal disease, ischaemic stroke, and cutaneous and renal glomerular vasculopathy.[49,144-148] Hypertension leads to proteinuria, and quantification of proteinuria is used to assess severity of disease and response to treatment.[149] Greyhounds appear to have a high incidence of microalbuminuria, which is more sensitive than UPC measurement in the diagnosis of proteinuria.[49,144,146,150] UPC, however, is still the most widely used method to assess proteinuria.[151] Given the lack of

breed-specific reference intervals, the generic International Renal Interest Society (IRIS) recommendations for classifying proteinuria are used for all dog breeds.[128]

Serum creatinine is the most extensively studied method for evaluating renal disease in greyhounds. Serum creatinine has been shown to be consistently higher in greyhounds compared with non-sighthounds, and a greyhound-specific serum creatinine reference interval of 99–174 µmol/L is now in common use (the reference interval in non-sighthounds is 20–150 µmol/L).[46-50] Several hypotheses have been proposed to explain the high serum creatinine concentration in greyhounds. These include the influence of dietary creatinine intake[152] and a lower GFR[46] and greater muscle mass in greyhounds than in non-sight-hounds.[47,153] Increased dietary intake of creatinine is unlikely to explain breed-related differences in serum creatinine because serum creatinine concentrations were significantly different between greyhounds and non-greyhounds that were fed the same diet for 6 weeks.[46] GFR correlates negatively with serum creatinine and the influence of GFR on serum creatinine in greyhounds is unclear, with conflicting findings in the literature. Previous studies have found that GFR in greyhounds may be higher than,[153] similar to[154] or lower than[155] that of other breeds. The different methods used to determine GFR and small study samples may explain these discordant results; further study is needed to investigate the relationship between GFR and serum creatinine in greyhounds.

Greyhounds have been shown to have approximately 30% more muscle and 28% higher daily serum creatinine production than other dog breeds,[155,156] and this may, at least in part, explain the approximately 50% higher serum creatinine concentration seen in greyhounds.[155,156] In humans, serum creatinine and urine creatinine correlate with

muscle mass,[37-39] and various formulae are routinely used to predict GFR based on serum creatinine and other variables such as age, race and sex that act as surrogate markers for muscle mass.[157] Although there is less evidence in dogs, two studies have demonstrated a strong correlation between muscle mass and plasma creatinine in dogs,[39,44] although no greyhounds were included in these studies. One study investigated USG, urine creatinine concentrations and UPC in greyhounds but did not measure osmolality, adjust urine creatinine for urine concentration or compare results with other breeds.[49] Therefore, further studies are required to determine if the analytes tested differ between greyhounds and other breeds.

SDMA holds promise as a marker of renal disease in greyhounds because unlike creatinine, this analyte shouldn't be affected by muscle mass.[44,72,85,86] However, a recent study found that mean SDMA concentrations were significantly higher in greyhounds (16.2 µg/dL) compared with other breeds (12.2 µg/dL, $P < 0.001$) and 13 of 19 greyhounds had SDMA concentrations above the recommended upper reference limit, compared with only one non-greyhound.[158] Given the small sample size, the authors recommended further studies to confirm this finding. SDMA is being more frequently utilised in the diagnosis of canine renal disease, and appropriate reference intervals are needed to avoid misdiagnosing renal disease in greyhounds.

## 1.6. Summary of the previous findings and study objectives

Despite urine concentration being routinely assessed in renal disease investigations, the author is unaware of any literature in which greyhound urine concentration has been specifically assessed for differences with other breeds. In veterinary medicine, urine concentration is most commonly estimated using a

refractometer, due to its ease of use, affordability and high correlation with osmolality.[97,99] Osmolality testing of urine samples was performed in this study and compared with USG to assess whether USG is a satisfactory measure of urine concentration for this purpose.

Thus, the objectives of the study shown in chapter 3 were to:

- establish a sample of healthy non-racing greyhounds and a control group comprising healthy non-sighthounds of similar age, weight, gender and neutering status to allow comparison of clinicopathologic parameters of renal health
- compare urine concentration, as measured by USG and urine osmolality, in healthy greyhounds with a control group containing healthy non-sighthounds.

Serum creatinine has consistently been shown to be higher in greyhounds than non-sighthounds.[46-50] Despite this, no studies have investigated whether greyhounds also have higher urine creatinine than non-sighthounds. Because urine creatinine is affected by urine concentration, it must be adjusted for USG or urine osmolality for accurate determination of urine creatinine concentration.[106-109] If greyhound urine contains more creatinine at a given urine concentration than non-sighthound urine, this could affect UPC reference intervals in greyhounds. Currently, the guidelines from IRIS for classifying proteinuria are used in greyhounds.[49] These generic canine recommendations may not be appropriate for greyhounds if their urine creatinine is significantly higher than other breeds and the use of generic reference intervals for greyhounds could lead to flawed clinical decision-making and prognostication.

Thus, the objectives of the study shown in chapter 4 were to:

- compare urine creatinine concentration, relative to urine concentration, in healthy greyhounds with the urine creatinine concentration, relative to urine concentration, in a control group comprising healthy non-sighthounds
- determine the UPC reference interval in greyhounds and compare this with the reference interval in a control group comprising healthy non-sighthounds and the current IRIS guidelines.

SDMA is becoming a frequently used assay in the assessment of renal function, with many studies demonstrating a strong correlation with GFR.[44,70,72,84,159] A commercial immunoassay released by IDEXX is now commonly used to measure serum SDMA, and an adult canine reference interval of ≤ 14 ug/dL (≤ 0.63 µmol/L) is used.[160] There are contradictory studies regarding the effect of breed on SDMA, with no significant difference in SDMA concentrations between three non-sighthound breeds,[161] yet SDMA concentrations have been shown to be significantly higher in greyhounds than other breeds in one small study.[158] Larger studies are required to confirm this difference.

Thus, the objective of the study shown in chapter 5 was to:

- establish a reference interval for serum SDMA concentration in non-racing greyhounds and compare this reference interval with serum SDMA concentrations in non-sighthound dogs and with previously established canine reference intervals.

Greyhounds have high muscle mass, which is likely an adaptation from decades of selective breeding for sprinting.[48,50,152,162,163] Greyhounds have been found to have greater relative muscle mass and a lower percentage of body fat compared with non-sighthounds,

even when total body weight is taken into account.[156,162-166] Serum creatinine has been shown to be positively correlated with muscle mass in both humans and dogs,[37-39 39,44] but to the author's knowledge, the correlation between urine creatinine and muscle mass has only been investigated in humans. Serum creatinine is predominantly eliminated by the kidneys, and one would assume that the higher serum creatinine in greyhounds would lead to higher urine creatinine clearance and that this would be positively correlated with muscle mass, but this remains to be determined.

Thus, the objective of the study shown in chapter 6 was to:

- establish whether lean body mass is correlated with serum creatinine and urine creatinine, relative to urine concentration, in greyhounds.

# Chapter 2. Materials and Methods

## 2.1. Subjects

This study was conducted at the University of Melbourne and participating dogs were enrolled from September 2016 to July 2017. Ethical approval for this study was granted by the University of Melbourne Animal Experimentation Ethics Committee (ID: 1613906) and signed owner consent was obtained for each dog. Dogs were potentially eligible for enrolment into one of two study groups: (1) greyhounds and (2) non-sighthounds. The non-racing greyhound group (n = 149) were retired racing greyhounds (identified through the Greyhound Adoption Program Victoria), dogs at training kennels that were not racing or in full training, and privately owned pet greyhounds. Non-sighthound dogs (n = 35) were sourced from staff, students and clients at U-Vet Werribee Animal Hospital and the University of Melbourne Faculty of Veterinary and Agricultural Sciences and from shelters. All dogs lived in Victoria, Australia.

Dogs were included if they were aged 1–12 years, of any gender or neutering status and deemed healthy after assessment of medical history, performance of a physical examination and assessment of selected laboratory measures (see sections 2.3 and 2.4).

Owners provided information on the racing status and health of each dog within the previous 14 days, including any surgical procedures or medical conditions. Greyhounds that were not in active race training (i.e. had not raced or trained for at least 7 days) were eligible for enrolment into the greyhound group. Dogs in the non-sighthound group had to weigh 24–42 kg and included any breed other than sighthounds.

All dogs were fasted for at least 8 hours but free access to water was permitted. Dogs were excluded if they had been administered topical or oral corticosteroids, stilboestrol or antibiotics within the previous 14 days, if they were sedated or anaesthetised, or if free catch urine could not be collected.

## 2.2. Measurements and sampling

Measurements and sampling took place from 7 – 11am where the dogs were housed, in public spaces when the dog was being walked or at the U-Vet Werribee Animal Hospital. Weight and body condition score for each dog were recorded according to American Animal Hospital Association guidelines.[167] Each dog was placed in a normal standing posture and a flexible tape measure was used to measure thigh circumference. The point of measurement was where the leg met the flank. Dog height was measured from the ground to the top of the withers (palpated as top of scapula). A single measurement was taken from each location.

Each dog was walked on a leash and a midstream urine sample was collected in a clean container during voluntary urination. The urine was poured into new sterile container and kept on ice during transport to the laboratory.

If urine collection was successful, 3 mL of blood was collected from either the jugular vein using a 21G needle (NIPRO Corporation, Osaka, Japan) and 3 mL syringe (Becton Dickinson, Singapore) or during the placement of a cephalic intravenous catheter (22 gauge, Smith's Medical, Kent, UK) if an elective medical or surgical procedure had been planned following collection. The immediate blood draw was placed into a 2.5 mL serum separation tube (Vacuette tube®, Greiner Bio One Frickenhausen, Germany) and a 0.5 mL EDTA microtube (MiniCollect®, Greiner Bio One, Frickenhausen, Germany).

## 2.3. Blood testing

Laboratory measurements included packed cell volume, total solids, serum creatinine and SDMA (Figure 2-1). Packed cell volume was determined by centrifuging a plain microhaematocrit tube (Fronine Pty Ltd, NSW, Australia) filled with EDTA anticoagulated whole blood at 14,800 G for 5 minutes (Orbital 260 centrifuge; Clements, NSW, Australia). Total solids was determined by refractometry using plasma from the centrifuged microhaematocrit tubes.

Chilled serum tubes were centrifuged within 4 hours of sample collection and approximately 0.5 mL of serum was placed into two Eppendorf tubes® (Eppendorf AG, Hamburg, Germany). One of these samples was either immediately analysed for serum creatinine or refrigerated at 4°C and analysed at the U-Vet Werribee Animal Hospital clinical pathology laboratory within 36 hours of collection. The second serum sample was immediately frozen at −80°C for storage for up to 3 months. This sample was later thawed and sent to an external laboratory for batch analysis of SDMA using the IDEXX enzyme immunoassay for SDMA™. This assay has previously been validated for canine use, and has had analytical performance characteristics and precision studies published.[72]

Serum creatinine was measured with the COBAS INTEGRA® 400 plus (Roche Diagnostics Ltd, Rotkreuz, Switzerland)[168] using a kinetic colorimetric assay (modified Jaffe). Quality control was performed daily as directed by the manufacturers with calibrator solutions (Calibrator for Automated Systems, Roche Diagnostics Ltd, Rotkreuz, Switzerland) and verified using two control solutions (PreciControl ClinChem Multi 1 and PreciControl ClinChem Multi 2, Roche Diagnostics Ltd, Rotkreuz, Switzerland).

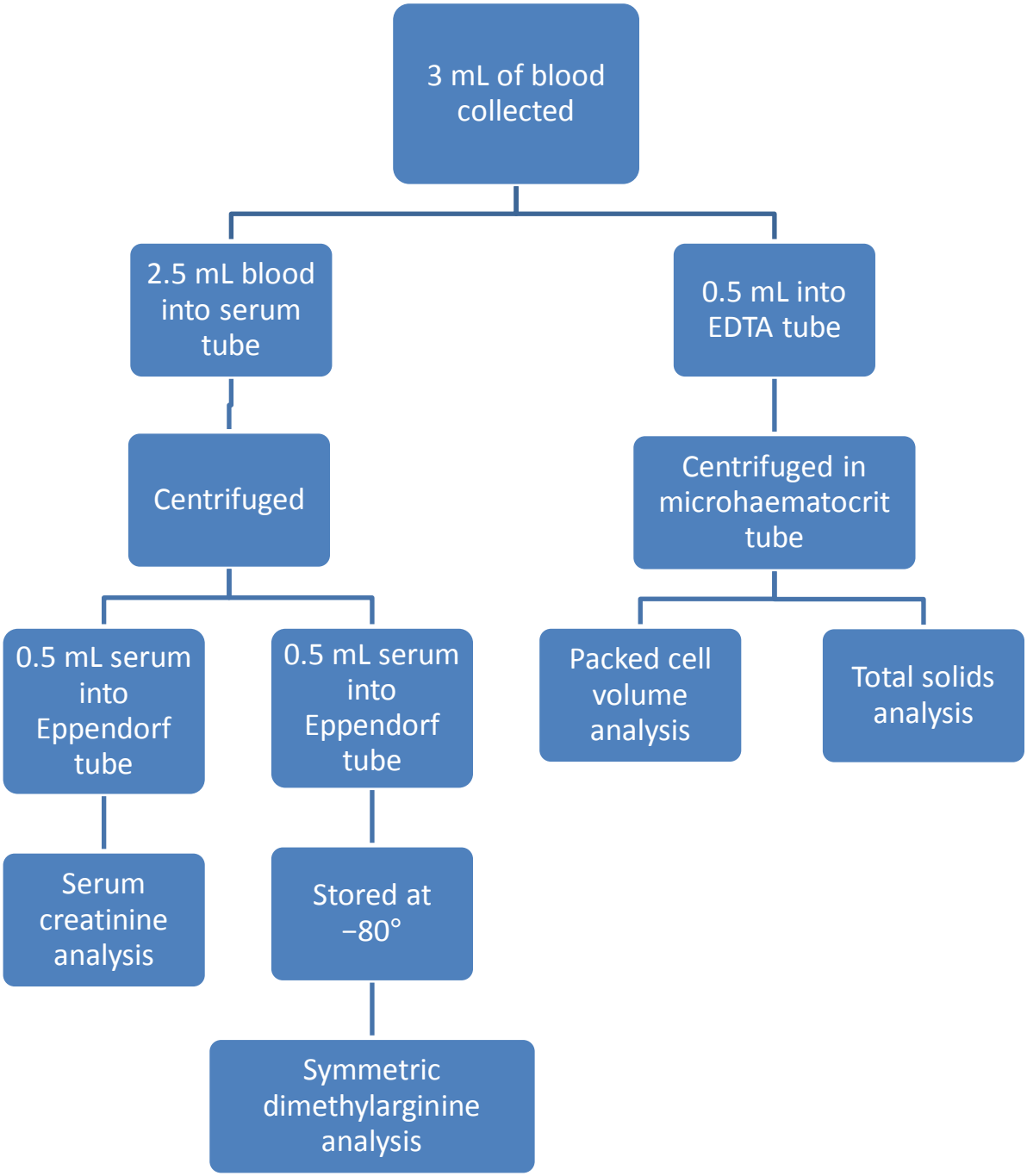


**Figure 2-1. Flow chart of the blood collection and analysis in this study.**

## 2.4. Urine testing

Urinalysis was performed within 6 hours of collection; 5 mL of urine was centrifuged at 2,100 G for 3 minutes and 3 mL of the supernatant was equally divided into two separate Eppendorf tubes® (Figure 2-2). The remaining sediment was re-suspended with 0.5 mL of supernatant, and was microscopically examined to count the number of red blood cells, white blood cells and epithelial cells per 40× field. The presence of casts, crystals, bacteriuria and spermaturia were also recorded. An air dried sediment smear was also examined after staining with Wright's Giemsa to confirm the findings noted on the wet preparation.

The supernatant was used for dipstick analysis, USG measurement, osmolality testing and UPC determination. Dipstick (Multistix, Siemens Healthcare Diagnostics Inc. NY, USA) analysis was performed manually according to the manufacturer's instructions. USG was measured within 4 hours of collection. A drop of supernatant was placed onto a hand-held refractometer (Atago, Tokyo, Japan) to obtain a single measurement. The refractometer was calibrated daily with distilled water.

Because no osmometer was available on site, aliquots of urine supernatant were stored at −80°C for up to 120 days before analysis at the Bio21 Institute of Molecular Science and Biotechnology, Parkville, Australia. Before each series of measurements, the analyser was calibrated with three control solutions: 50 mOsm/kg, 850 mOsm/kg and 2,000 mOsm/kg (Advanced Instruments Inc., MA, USA). Once thawed at room temperature (approximately 20°C), samples were gently inverted 3–4 times and then measured according to the manufacturer's instructions. Because this analyser has a reported range from 0 to 2,000 mOsm/kg $H_2O$, samples with greater than 2,000 mOsm

had a 1:2 dilution performed with deionised water and the analyser result was doubled to obtain the result. Any samples with a result of less than 2,000 mOsm/kg $H_2O$ had a single measurement taken.

Osmolality samples underwent either one or two freeze–thaw cycles, the latter due to an analyser malfunction. The effect of freezing at −80°C and thawing of urine on osmolality was assessed using five urine samples from which two aliquots were made from each sample. The first aliquot underwent one freeze–thaw cycle before measurement and the second aliquot underwent two freeze–thaw cycles before measurement and the results were compared.

Urine protein and urine creatinine were either analysed immediately or supernatants were refrigerated after centrifugation and then analysed at the U-Vet Werribee Animal Hospital laboratory within 50 hours of collection. Urine creatinine was determined using the same analyser, calibrators and control solutions as for serum creatinine. Urine protein concentrations were measured using the turbidometric method, and urine creatinine concentrations were measured using the modified Jaffe method.

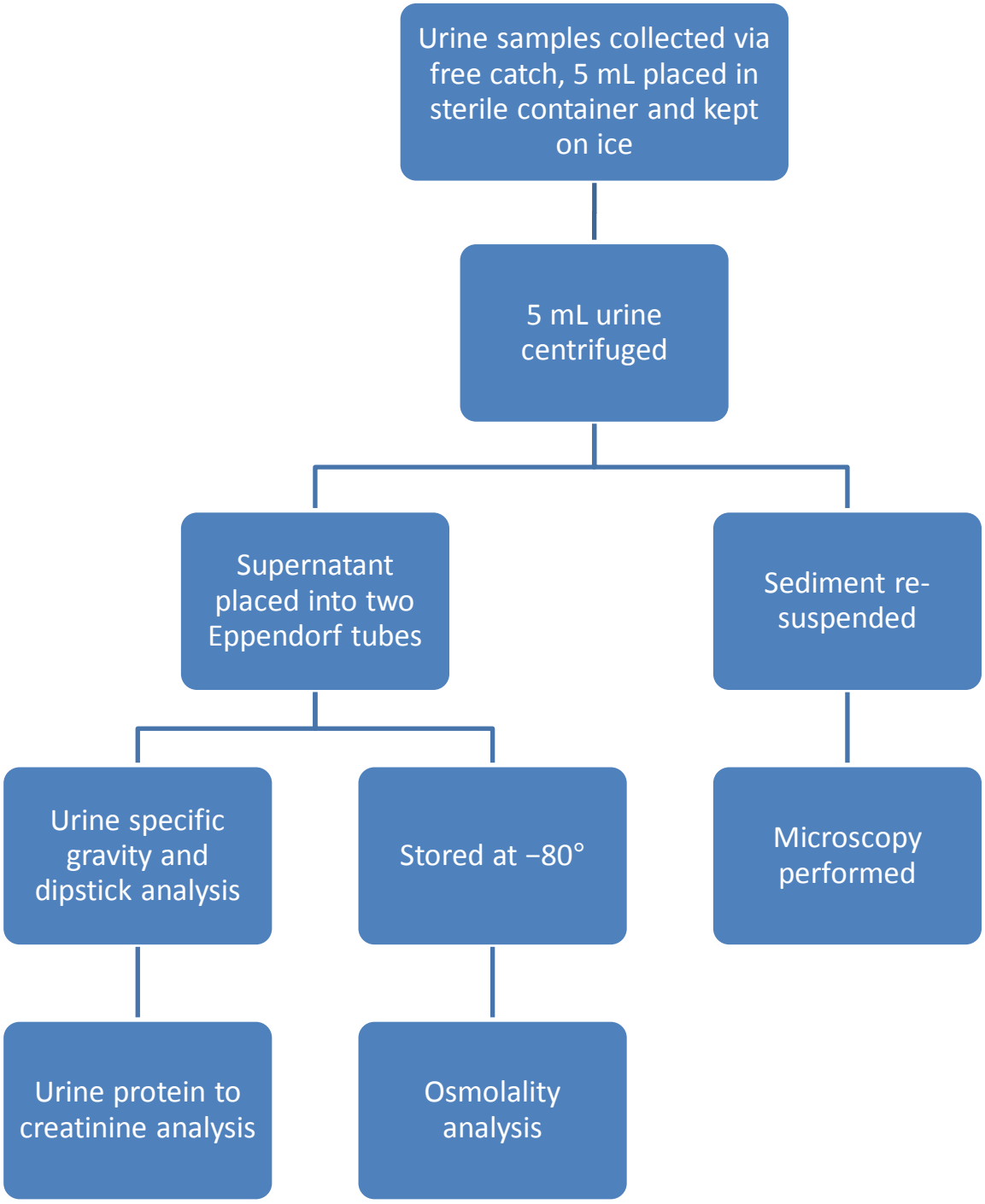


**Figure 2-2. Flow chart of the urine sample analysis in this study.**

## 2.5. Laboratory-based exclusion criteria

Greyhounds were excluded if they had one or more of the following values outside the breed standard reference intervals:

- serum creatinine > 170 µmol/L and USG < 1.030
- packed cell volume < 0.36 L/L
- total solids < 48 g/L.

For the non-sighthound group, dogs were excluded if they had one or more of the following values:

- serum creatinine > 140 µmol/L and USG < 1.030
- packed cell volume < 0.37 L/L
- total solids < 60 g/L.

Dogs from both groups were excluded if they had USG < 1.025, to ensure dogs with subclinical renal disease were not inadvertently included in the study. Additionally, dogs from both groups were excluded if urine samples showed gross haematuria, significant pyuria, ≥5 white blood cells per high-power field or bacteriuria, to prevent inclusion of dogs with urinary tract pathology.

## 2.6. Coefficient of variation determination

The CV was calculated according to the following formula:

$$\text{CV (\%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100$$

The intra-assay precision for urine osmolality was determined over 10 measurements of deionised water and three control solutions, which included concentration of 300 mOsm/kg (Genotec, Baden-Wurttemberg, Germany), 500 mOsm/kg and 1,500 mOsm/kg

$H_2O$ (Con-trol™, Precision Systems Inc, Natick, MA, USA). The inter-assay precision was determined with four to six measurements taken in the morning and afternoon over three non-consecutive days using the following solutions: deionised water, one control solution (300 mOsm/kg $H_2O$ Genotec, Baden-Wurttemberg, Germany) and three frozen aliquots of two urine samples, one which was moderately concentrated and the other highly concentrated. Aliquots of urine samples were utilised in inter-assay precision assessment instead of only commercial control solutions, as the control solutions would require several freeze-thaw cycles to be performed over several days, which could affect results.

For UPC CV determination, two urine samples that most closely approximated clinically relevant IRIS cut-offs were chosen (UPC= 0.2 and 0.5), as imprecision around these values is most likely to lead to flawed clinical decision making. Only one urine sample was chosen to evaluate each precision estimate due to limited sample volume. The intra-assay precision for urine creatinine, urine protein and UPC was determined over 10 consecutive measurements of a single sample. The inter-assay precision was determined with a morning and afternoon measurement of a urine sample over five consecutive days.

## 2.7. Statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Melbourne.[169] Statistical analysis was carried out with Minitab 17 Statistical Software (State College, PA, USA) and Microsoft Excel 2013 for Windows with the Reference Value Advisor v2.1 add-in.[170]

The number of greyhounds enrolled was based on the American Society for Veterinary Clinical Pathology and Clinical & Laboratory Standards Institute guidelines, which recommend at least 120 reference samples to determine reference intervals using the non-parametric method with 90% confidence limits.[171,172] The number of animals enrolled in the non-sighthound group was based on previously published studies that measured urine creatinine in healthy non-greyhound and greyhound dogs, using a two-sample t-test with power and alpha-error set at 80% and 5%, respectively.[14,49] Based on this method, seven animals were necessary in each group. To increase precision and to control for confounding factors that were apparent in previous studies, it was decided to enrol 35 animals. Laboratory data were assessed for compatibility with a normal distribution by probability plots. Greyhounds and non-sighthound dogs were compared for mean age, weight and laboratory results using two-sample t-tests and were compared for gender and neutering status using a chi-square test.

The difference in osmolality between samples that underwent one freeze–thaw cycle versus two–freeze thaw cycles was calculated using a paired t-test.

Urine creatinine and urine protein concentrations were converted into the same units – g/L (creatinine was converted from µmol/L to g/L by multiplying by 0.113 and then dividing by 1,000[173]). UPC was then calculated using the following formula:

$$\text{UPC} = \frac{\text{urine protein concentration}}{\text{urine creatinine concentration}}$$

Regression lines were calculated to compare the relationships between groups for urine concentration versus urine protein and urine creatinine. Frequency histograms were used to assess the distributions of the UPC data. The Dixon method was used to detect outliers.

The reference interval for UPC in greyhounds was calculated using the non-parametric method and comprises the central 95% of the fitted distribution with 90% CIs calculated around the lower (2.5%) and upper (97.5%) limits. For variables that were log transformed, the means for the two groups were compared using two-sample t-tests.

SDMA measurements were assessed for normality using frequency histograms and probability plots (or Q-Q plots). The SDMA and serum creatinine measurements for greyhounds and non-sighthound dogs were compared using a two sample t-test. The association between SDMA and serum creatinine was assessed using Pearson correlation and a scatterplot. Reference limits and their 90% confidence intervals were determined parametrically.

The association between creatinine and body mass was assessed using Pearson correlation. Urine creatinine was corrected for urine concentration using the formula:

$$\frac{\mathrm{UCr}}{(\mathrm{USG}-1)} \times 100$$

where UCr is urine creatinine and USG is urine specific gravity.[109] Statistical significance was set at $P < 0.05$ for all analyses.

# Chapter 3. Establishment of sample groups and comparison of urine concentration in greyhounds and non-sighthounds

## 3.1. Results

### 3.1.1. Study groups

The final analysis included 98 greyhounds and 24 non-sighthounds. A total of 51 greyhounds and 11 non-sighthound dogs were excluded from the study for reasons outlined in Figure 3-1.

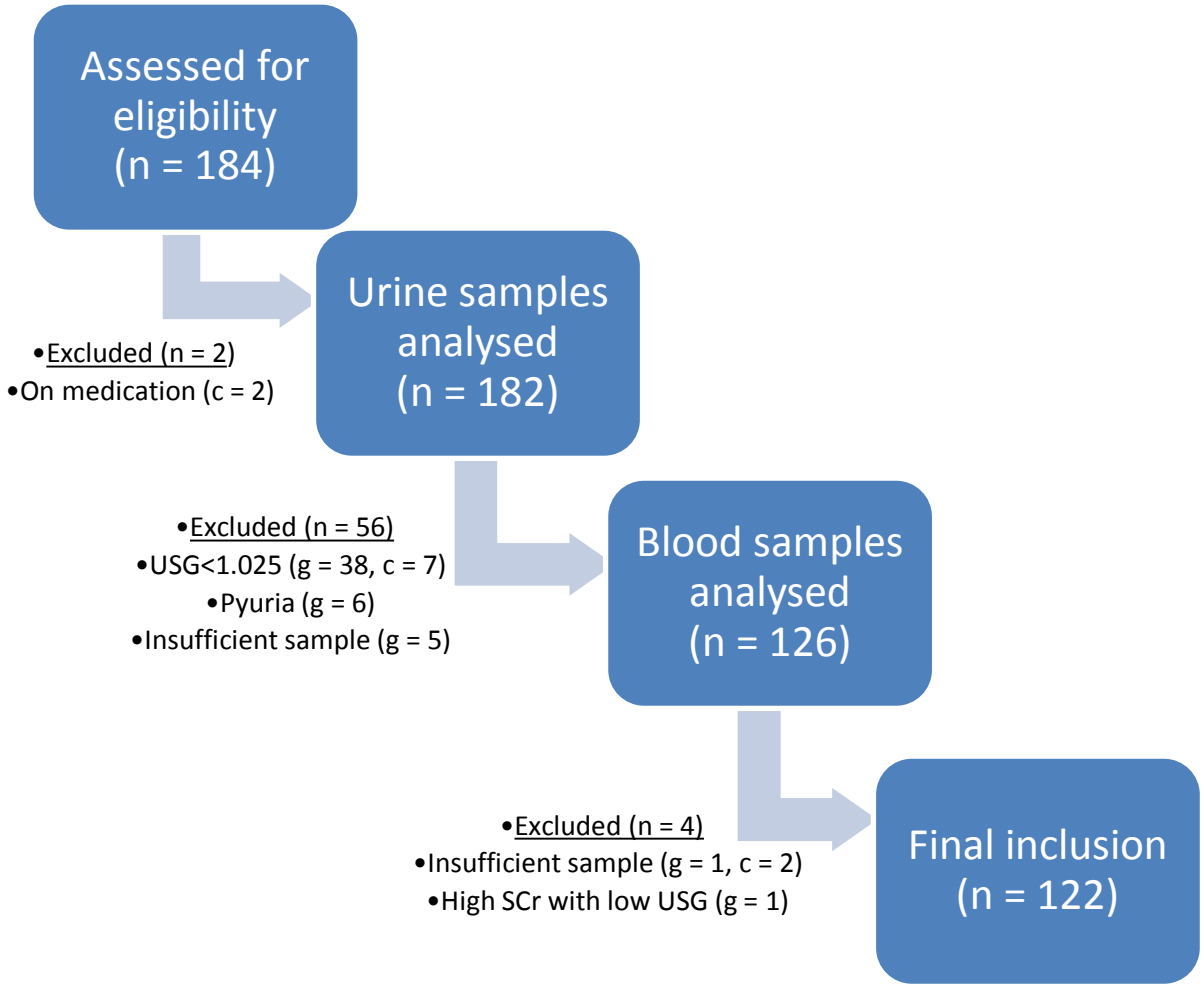


**Figure 3-1. Flow chart of the exclusions in this study.**

Note: n, total number of dogs; g, greyhounds; c, non-sighthounds; SCr, serum creatinine; USG, urine specific gravity

The final analysis included 38 greyhounds sourced from the adoption program, 25 greyhounds sourced from three trainers and breeders, two greyhounds from a shelter and 33 greyhounds from private dog owners. Five dogs from the non-sighthound group were sourced from a shelter and 19 were privately owned dogs. The non-sighthound group consisted of the following breeds: mixed breed (n = 8), Labrador retriever (n = 8), golden retriever (n = 1), wirehaired pointer (n = 1), German shepherd (n = 1), koolie (n = 1),

Belgian shepherd (n = 1), mastiff (n = 1), kelpie (n = 1) and a setter (n = 1). None of the dogs included in the study received any medications other than routine anthelmintics. There was no statistically significant difference in gender (chi-squared test; P=0.65), neutering status (chi-squared test; P=0.72), age (t-test; P= 0.47) or weight (t test; P=0.73) between the greyhound and non-sighthound groups (Table 3-1).

**Table 3-1. Summary of gender, neutering status, age and weight in greyhound and non-sighthound groups**

| Category | Greyhound n = 98 | Non-sighthound n = 24 |
|---|---|---|
| Male intact, n (% of group) | 11 (11.2%) | 2 (8.3%) |
| Male neutered, n (% of group) | 43 (43.9%) | 10 (41.7%) |
| Female intact, n (% of group) | 14 (14.3%) | 5 (20.8%) |
| Female neutered, n (% of group) | 30 (30.6%) | 7 (29.2%) |
| Age (years) mean ± SD | 4.1 ± 2.3 | 4.5 ± 2.4 |
| range (years) | 1–10 | 1–8 |
| Weight (kg) mean ± SD | 31.6 ± 3.8 | 31.2 ± 5.2 |

Note: SD, standard deviation; n, number

### 3.1.2. Blood results

The mean concentrations of packed cell volume and serum creatinine were significantly higher in greyhounds than in non-sighthounds (Table 3-2). Mean packed cell volume was 7.6 L/L higher and mean serum creatinine was 38.1 µmol/L higher in greyhounds than non-sighthounds, while total solids was significantly lower in greyhounds than non-sighthounds.

**Table 3-2. Blood results for greyhounds and non-sighthounds**

| Category | Greyhound (n=98) | Non-sighthound (n=24) | P value |
|---|---|---|---|
| Serum creatinine (μmol/L) mean ± SD | 125.6 ± 13.8 | 87.5 ±19.2 | <0.001 |
| range | 86.0–161.0 | 48.0–119.0 | N/A |
| Packed cell volume (L/L) mean ± SD | 52.0 ± 5.8 | 44.4 ± 4.8 | <0.001 |
| Total solids (g/L) mean ± SD | 6.1 ± 0.5 | 6.7 ± 0.6 | <0.001 |

Note: N/A, not applicable; SD, standard deviation

### 3.1.3. Urine coefficient of variation

There was no statistically significant difference in urine osmolality between the samples that underwent one freeze–thaw cycle and those that underwent two freeze–thaw cycles (paired t-test; P = 0.66) (see Appendix 1). CV results were all less than 3%, with lower concentrations showing higher CV than the higher concentration solutions (see Tables 3-3 and 3-4).

**Table 3-3. Intra-assay variability results for urine osmolality analysis**

| | 0 mOsm/kg (deionised water) | 300 mOsm/kg solution | 500 mOsm/kg solution | 1,500 mOsm/kg solution |
|---|---|---|---|---|
| Samples run | 10 | 10 | 10 | 10 |
| Mean (mOsm/kg) | 0.1 | 299.6 | 510.4 | 1,500.5 |
| SD (mOsm/kg) | 0.57 | 4.2 | 3.4 | 5.9 |
| CV (%) | N/A* | 1.4 | 0.66 | 0.39 |
| Minimum (mOsm/kg) | 0 | 289 | 506 | 1489 |
| Maximum (mOsm/kg) | 1 | 304 | 518 | 1507 |

Note: SD, standard deviation; CV, coefficient of variation; *CV cannot be calculated accurately when mean is close to zero.

**Table 3-4. Inter-assay variability results for urine osmolality analysis**

| | 0 mOsm/kg (de-ionised water) | 300 mOsm/kg | Urine Sample #1 | Urine Sample #2 |
|---|---|---|---|---|
| Samples run | 6 | 6 | 4 | 4 |
| Mean (mOsm/kg) | 6.33 | 301.83 | 498.25 | 1933.25 |
| SD (mOsm/kg) | 5.57 | 8.38 | 5.32 | 7.50 |
| CV (%) | N/A* | 2.8 | 1.1 | 0.38 |
| Minimum (mOsm/kg) | 0 | 289 | 494 | 1923 |
| Maximum (mOsm/kg) | 9 | 314 | 505 | 1941 |

Note: SD, standard deviation; CV= coefficient of variation; *CV cannot be calculated accurately when mean is close to zero.

### 3.1.4. Urine concentration

There was no significant difference in mean USG (t-test; P = 0.21) or urine osmolality (t-test; P = 0.11) between groups (see Table 3-5).

**Table 3-5. Summary of urine specific gravity and urine osmolality results for both groups**

| Category | Greyhound (n=98) | Non-sighthound (n=24) | P value |
|---|---|---|---|
| USG | | | |
| mean ±SD | 1.039 ±0.01 | 1.041 ±0.007 | 0.21 |
| range | 1.025–1.050 | 1.028–1.051 | |
| Osmolality (mOsm/kg) | | | |
| mean ±SD | 1,526 ± 295^ | 1,649 ± 330 | 0.11 |
| range | 926–2,127^ | 1,048–2,254 | |

Note: SD, standard deviation; ^only 97 results available

The correlation between urine osmolality and USG was assessed (Figure 3-2). The greyhound and non-sighthound groups showed similar strong correlations between urine osmolality and USG (r = 0.94 for greyhounds, 0.91 for non-sighthounds). There was no statistically significant difference between the slopes (P = 0.49). The urine osmolality in greyhounds was 39 mOsm/kg lower than the osmolality in non-sighthounds (P = 0.09).

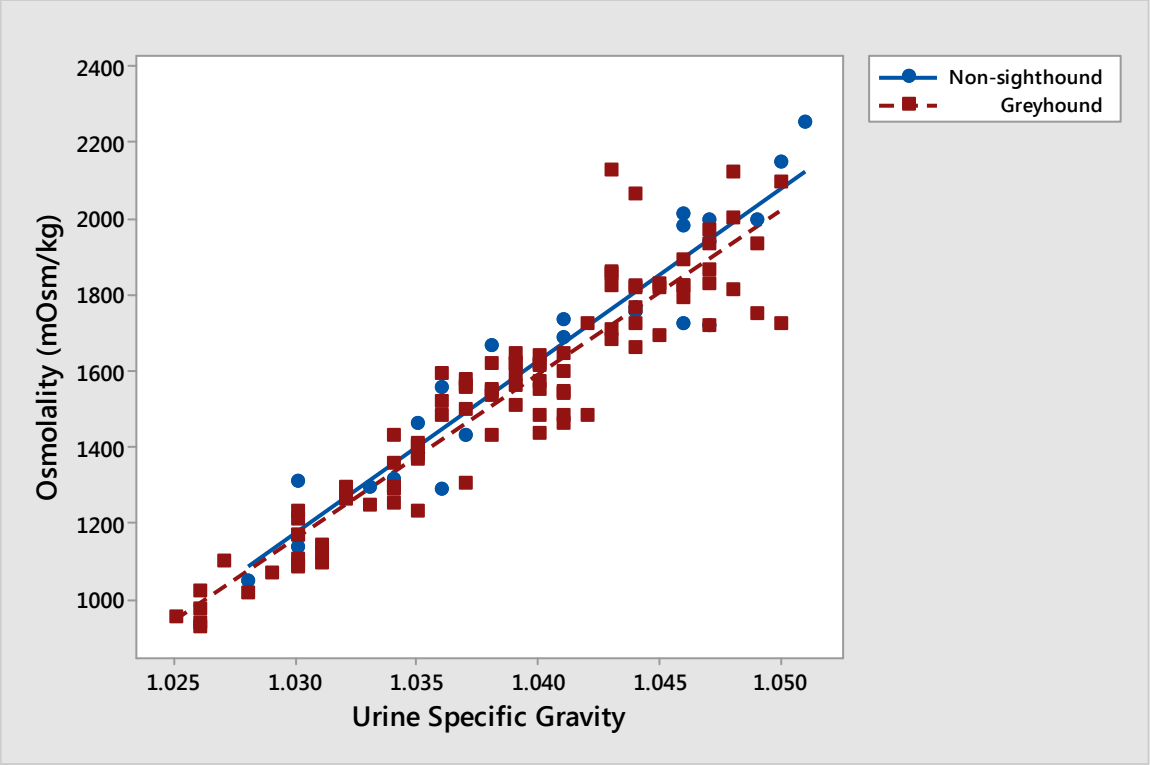


**Figure 3-2. Scatterplot with regression lines for the association between urine osmolality and urine specific gravity for greyhounds and non-sighthounds.**

## 3.2. Discussion

By collecting samples from greyhounds that were sourced from several locations, with a wide age range and different neutering status, the aim was to assemble a sample group that was characteristic of non-racing greyhounds in Australia. It was reassuring that there were no significant differences in gender, neutering status, age and weight between the greyhound and non-sighthound groups, which should minimise the effects of confounding variables on the study results.

Intra-assay and inter-assay CV results for the osmometer used in this study were less than 2% (mean 0.81%) and less than 3% (mean 1.4%), respectively, over the full range of samples tested, which is similar to those reported in another study.[103] The

number of freeze–thaw cycles appears to have little impact on urine osmolality results, although larger studies are required to confirm this.

There were no statistically significant differences noted between urine osmolality and USG as measurements of urine concentration in greyhounds. Urine osmolality is not commonly used in clinical practice, and therefore it is encouraging that USG is an accurate estimation of urine concentration in greyhounds.

The aim of this chapter was not to establish reference intervals of USG for the healthy greyhound population, and therefore previously published USG reference intervals were used to determine exclusion criteria.[174] Many greyhounds (26%) and non-sighthounds (20%) that appeared clinically normal had a USG less than 1.025, and this led to many dogs being excluded from the study. Thus, these results should not be extrapolated to reflect normal urine concentration in these groups. The reason why so many dogs were excluded for insufficient urine concentration is unclear, and it is possible that many healthy dogs were unnecessarily excluded due to these strict criteria. The author felt it was important to apply strict criteria to avoid the inadvertent inclusion of dogs with subclinical renal disease. The incidence and causes of low USG in apparently healthy young dogs requires further study.

The results demonstrated that there was no significant difference in USG or urine osmolality between the two groups and therefore adequate and comparable greyhound and non-sighthound groups were established. USG and urine osmolality were highly correlated for both groups in this study. This agrees with previous studies and suggests that USG is an adequate estimation of urine concentration in greyhounds, when USG $\geq$ 1.025. Because USG is more commonly used in clinical practice, and since it is not

inferior to urine osmolality, it was decided to correlate renal markers assessed in subsequent chapters to USG rather than urine osmolality.

# Chapter 4. Assessment of urine creatinine, urine protein and the urine protein to creatinine ratio in greyhounds and non-sighthounds

## 4.1. Results

### 4.1.1. Coefficient of variation results

The intra-assay and inter-assay CV for urine creatinine, urine protein and UPC are shown in Table 4-1. All CV results were less than 6%, with inter-assay urine protein CV having the highest value, and urine creatinine intra-assay CV being the lowest.

**Table 4-1. Intra-assay and inter-assay results for urine creatinine, urine protein and urine protein to creatinine ratio**

| | Intra-assay | | | Inter-assay | | |
|---|---|---|---|---|---|---|
| | **UCr** | **UPr** | **UPC** | **UCr** | **UPr** | **UPC** |
| Sample run (times) | 10 | 10 | 10 | 10 | 10 | 10 |
| Mean | 41443 | 1.17 | 0.25 | 21714 | 1.31 | 0.53 |
| SD | 252.1 | 0.010 | 0.003 | 481.1 | 0.070 | 0.024 |
| CV (%) | 0.60 | 0.86 | 1.0 | 2.2 | 5.3 | 4.5 |
| Minimum | 41105 | 1.15 | 0.25 | 21413 | 1.24 | 0.50 |
| Maximum | 41869 | 1.19 | 0.26 | 22692 | 1.26 | 0.56 |

Note: CV, coefficient of variation; SD, standard deviation; UCr, urine creatinine; UPr, urine protein.

### 4.1.2. Urine results

Urine creatinine and urine protein results without adjustment for urine concentration are shown in Table 4-2. The distributions of both analytes were clearly skewed and therefore both non-transformed and log-transformed variables were analysed (see Appendix 2 and

3, respectively). There was some evidence for higher mean urine creatinine in greyhounds when compared to non-sighthounds (t-test, P = 0.07 for both the log-transformed and the non-transformed variables). There was no significant difference in mean urine protein concentration between groups (t-test, P = 0.46 for the log-transformed variable and P = 0.83 for the non-transformed variable). The urine creatinine and urine protein ranges were wider and the standard deviation was higher in the greyhounds than the non-sighthounds.

**Table 4-2. Urine laboratory results for greyhounds and non-sighthounds**

| Category | Greyhound (n = 98) | Non-sighthound (n = 24) | P value |
|---|---|---|---|
| UCr mean ± SD (µmol/L) | 27,562 ± 10,903 | 23,773 ± 8,237 | 0.07 |
| UCr median (µmol/L) (Q1-Q3) | 25,377 (21,303–32,018) | 23,945 (17,404–26,302) | 0.65 |
| UCr range (µmol/L) | 11,510–86,200 | 13,276–49,740 | N/A |
| UPr mean ± SD (g/L) | 0.29 ± 0.32 | 0.31 ± 0.30 | 0.83 |
| UPr median (g/L) (Q1–Q3) | 0.20 (0.13–0.31) | 0.21 (0.16–0.33) | 1.0 |
| UPr range (g/L) | 0.05–2.49 | 0.10–1.28 | N/A |

N/A, not applicable; SD, standard deviation; UCr, urine creatinine; UPr, urine protein.

### 4.1.3. Urine creatinine concentration

Urine creatinine was then plotted against USG to investigate whether greyhounds have higher urine creatinine concentration, relative to urine concentration (Figure 4-1). Urine creatinine in greyhounds showed greater variation at higher USG than non-sighthounds. For urine creatinine vs USG, greyhounds showed a correlation of r = 0.59,

whilst non-sighthounds had a correlation of r = 0.41. There was some difference between slopes, albeit not significant (P = 0.08).

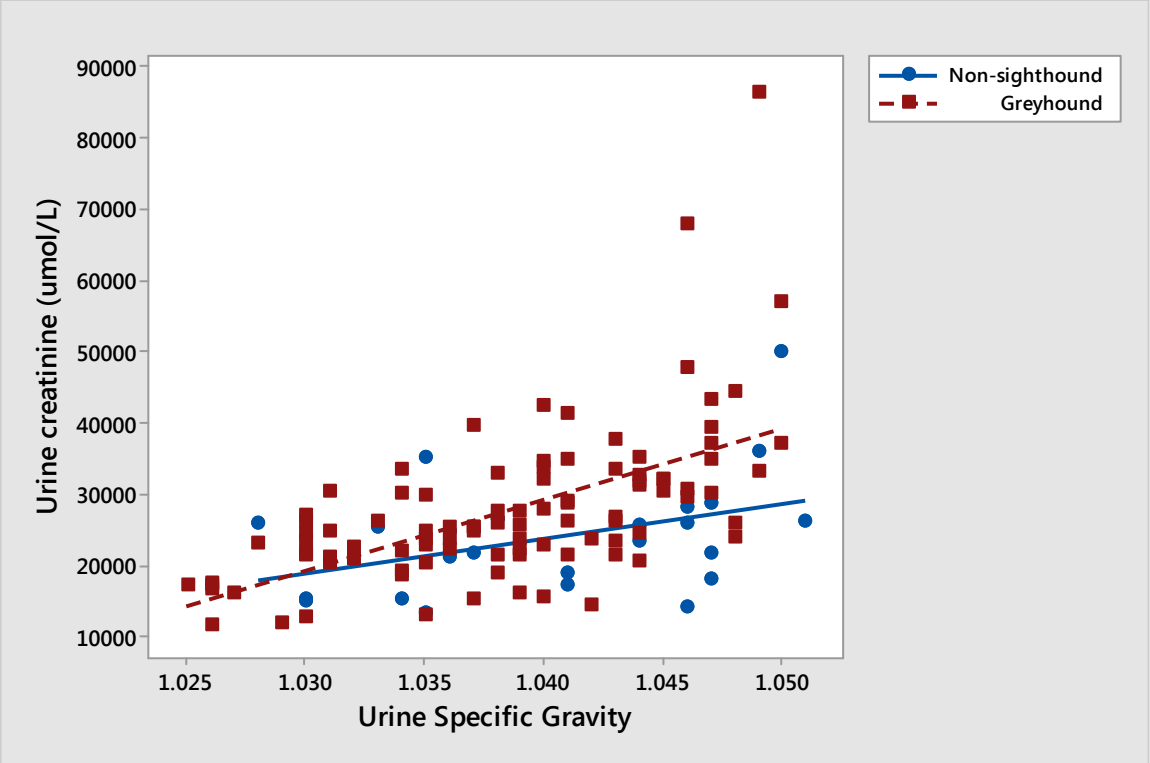


**Figure 4-1. Scatterplot with regression lines for the association between the urine creatinine and urine specific gravity for greyhounds and non-sighthounds.**

The urine creatinine values were clearly skewed to the right, thus a log transformation was applied to the data from both groups (Figure 4-2). The transformed variables were more highly correlated than the non-transformed variables in greyhounds (r = 0.64) and was the same in non-sighthounds (r = 0.41). There was no statistically significant difference between the slopes (P = 0.11). The equation for greyhounds was logUCr = -22.1 + 30.88x USG + 0.1989 (P=0.002). The estimated difference between the greyhound and non-sighthound group was 0.1989 on the log scale, or $e^{0.189}$ = 1.22 on the original

scale. This means that greyhounds were estimated to have 22% higher urine creatinine than non-sighthounds, when adjusted for urine concentration.

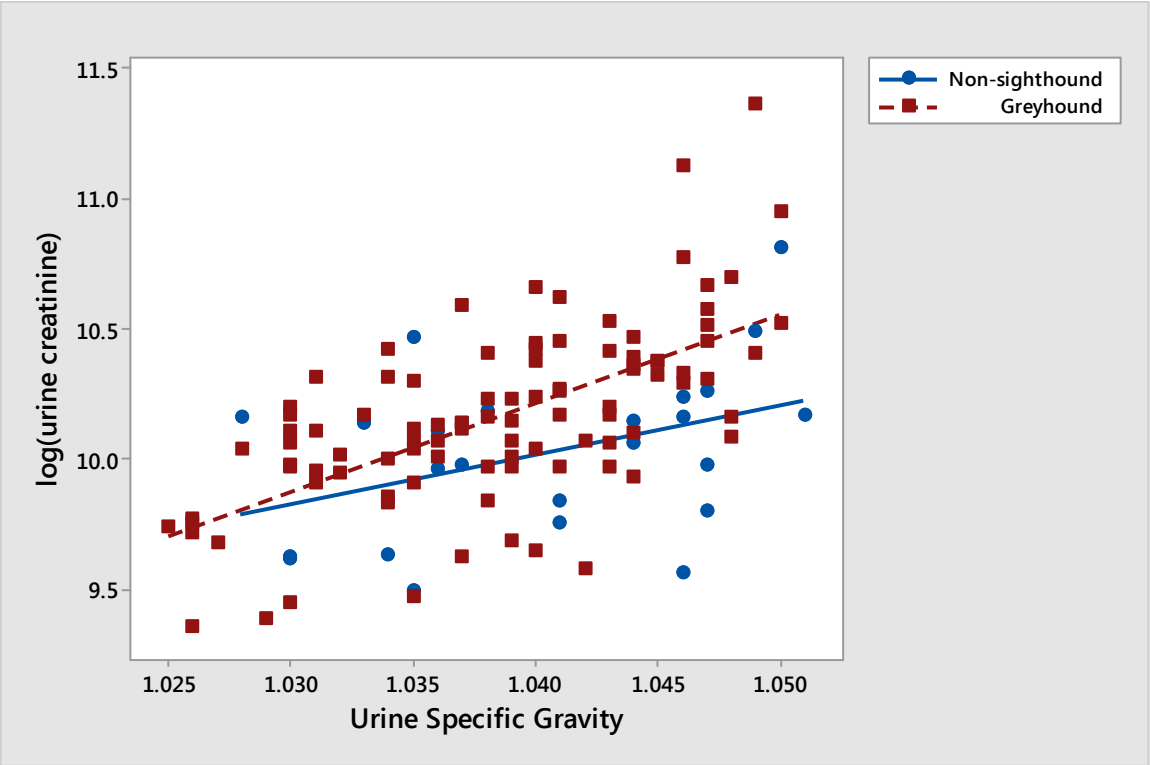


**Figure 4-2. Scatterplot with regression lines for the association between the log-transformed urine creatinine and urine specific gravity for greyhounds and non-sighthounds.**

### 4.1.4. Urine protein concentration

For both greyhounds and non-sighthounds, urine protein was not correlated with USG (greyhounds r = 0.08, non-sighthounds r = 0.21) (Figure 4-3). There was no statistically significant difference between slopes (P = 0.94) or between groups (P = 0.63) when comparing regression lines. Thus, there was no statistically significant difference between greyhounds and non-sighthounds for urine protein, relative to urine concentration. The

extreme outlier noted in Figure 4.3 was one of several outliers detected in the UPC analysis (see section 4.1.5).

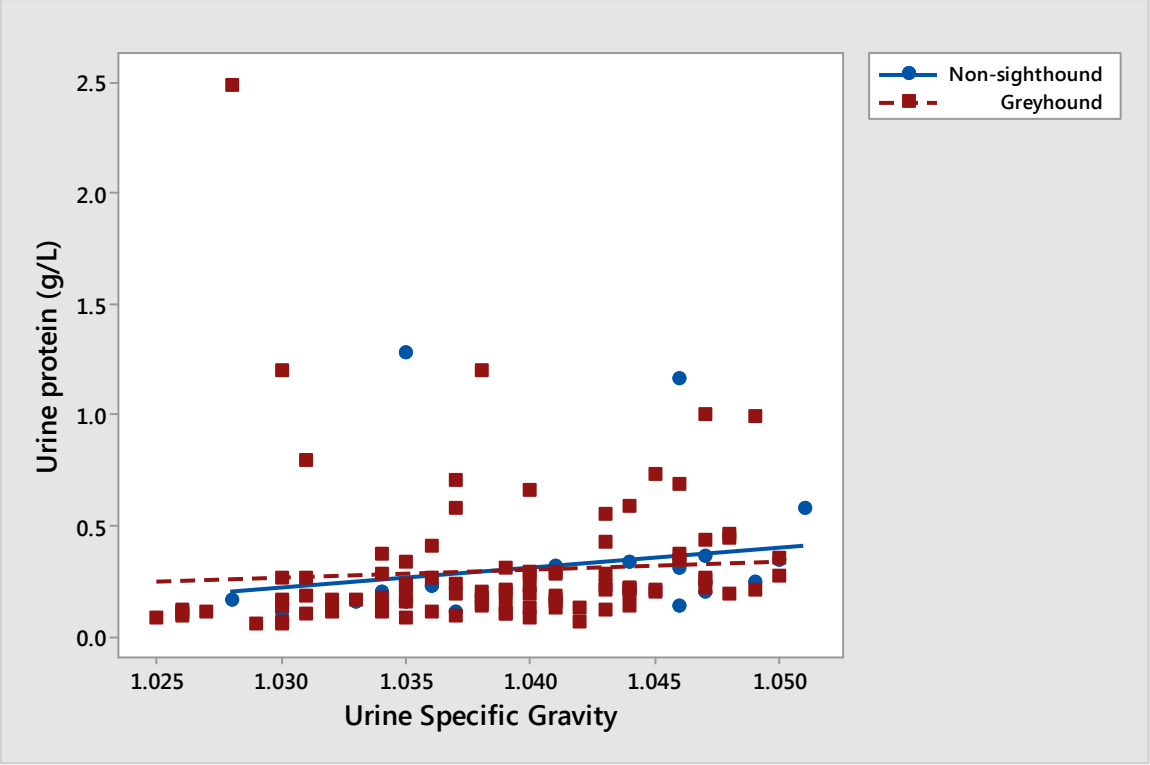


**Figure 4-3. Scatterplot with regression lines for the association between urine protein and urine specific gravity for both greyhounds and non-sighthounds.**

### 4.1.5. Urine protein to creatinine ratio

The UPC results were plotted for greyhounds and non-sighthounds (Figure 4-4). The UPC was heavily skewed to the right in both groups. A log transformation did not obtain an approximately Gaussian distribution for either group (Anderson-Darling test, both $P < 0.05$). Thus, the estimated lower 2.5% and upper 97.5% limit was determined using non-parametric methods and was found to be 0.037–0.42 in greyhounds. There were too few non-sighthounds to calculate an accurate reference interval for this group.

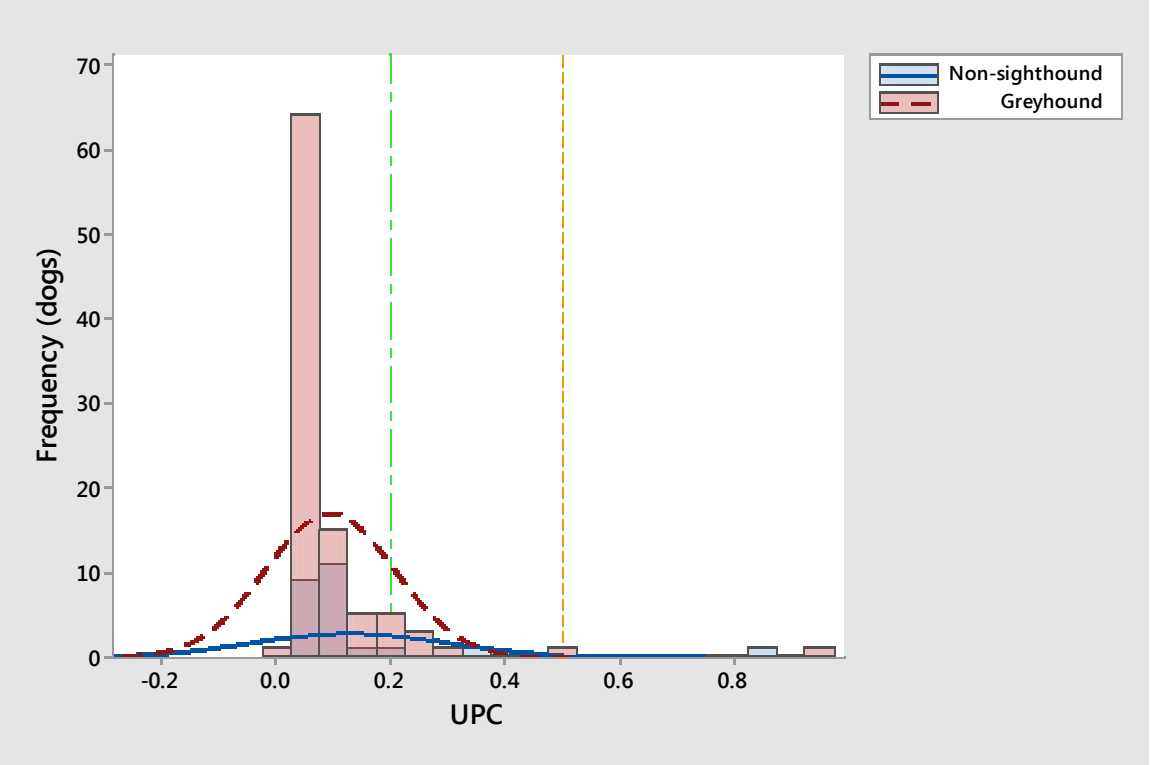


**Figure 4-4. Histogram of urine protein to creatinine for greyhounds and non-sighthounds with a normal distribution overlaid**

The green dashed line represents the IRIS cut-off for borderline proteinuria, and the orange dashed line represents the IRIS cut-off for overt proteinuria.

The Dixon and Tukey methods are recommended in ASVCP guidelines to detect outliers.[171] The Tukey method is not appropriate for non-Gaussian data, and therefore the Dixon method was used to further classify outliers. Three greyhounds and two non-sighthounds were identified as outliers using the Dixon method. When the outliers were excluded, the median UPC for greyhounds and non-sighthounds changed minimally or not at all, respectively. After exclusion of outliers, the greyhound UPC reference interval changed to 0.036–0.23 (Table 4-3).

**Table 4-3. Summary of urine protein to creatinine in greyhounds and non-sighthounds**

| Category | Greyhound (n=98) | Greyhounds with outliers removed (n= 95) | Non-sighthound (n= 24) | Non-sighthounds with outliers removed (n=22) |
|---|---|---|---|---|
| UPC median (Q1 to Q3) | 0.061 (0.05 to 0.09) | 0.060 (0.05 to 0.08) | 0.081 (0.06 to 0.12) | 0.081 (0.08 to 0.11) |
| Range | 0.02–0.96 | 0.023–0.25 | 0.04–0.85 | 0.038–0.20 |
| Estimated lower and upper limits | 0.037–0.42 | 0.036–0.23 | N/A | N/A |
| 90% CI for lower limit | 0.023–0.038 | 0.023–0.039 | | N/A |
| 90% CI for upper limit | 0.23–0.96 | 0.21–0.25 | N/A | N/A |

UPC was then compared between greyhounds and non-sighthounds, without exclusion of outliers. Because UPC was skewed to the right in both groups (Appendix 4), a log transformation was used for this analysis (Figure 4-5).

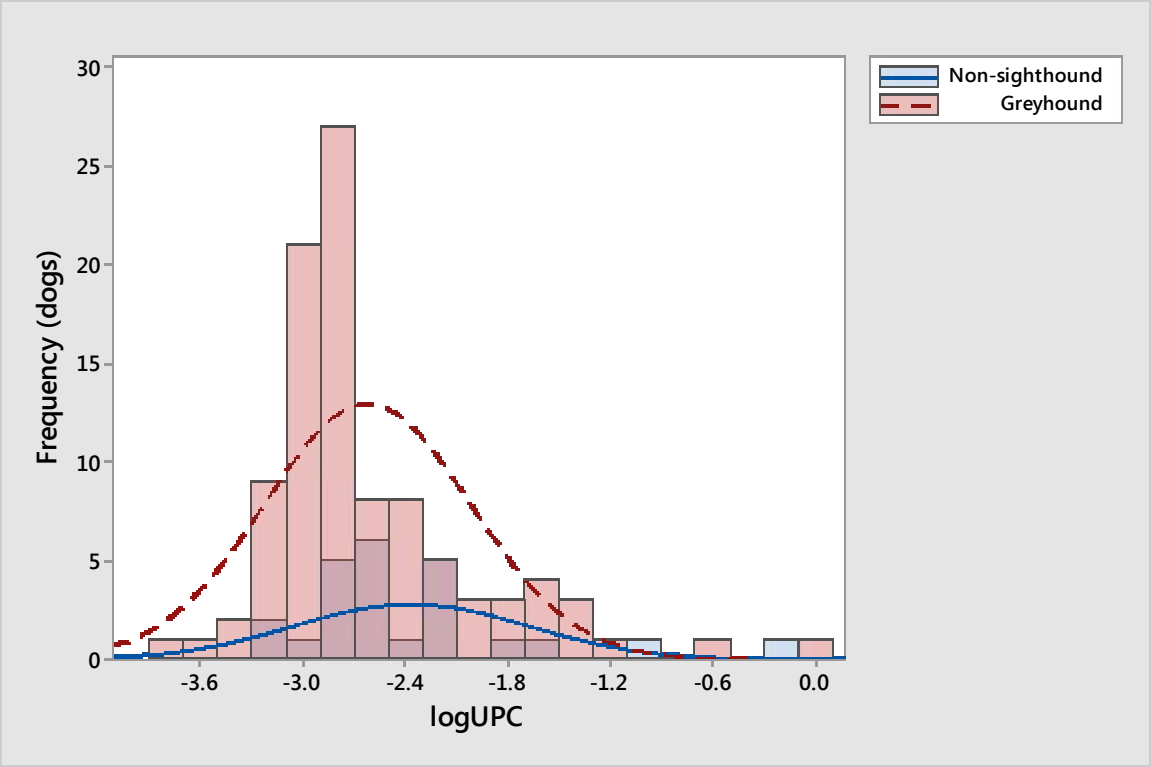


**Figure 4-5. Histogram of log transformed urine protein to creatinine for non-sighthounds and greyhounds with overlaid normal distribution.**

The log of the mean of UPC was −2.62 in greyhounds and −2.36 in non-sighthounds. The difference between these means was 0.26 on the log scale (or $e^{0.26}$ = 1.29 on the original scale) but was not statistically significant (P = 0.10). Therefore, the mean urine protein to creatinine ratio in greyhounds was estimated to be 29% lower than in non-sighthounds.

## 4.2. Discussion

The urine creatinine and protein assay in this study demonstrated good precision and confirmed that analytic variability was higher at lower urine protein and creatinine concentrations, which is a common finding in many analytes.[16] These results compare well to those of a previous study that reported median intra-assay CVs for urine

creatinine, urine protein and UPC of 2%, 8.3% and 8.6%, respectively.[59] Because recommendations for analytical precision have not yet been published for urine analytes in dogs, comparisons with the literature are not possible; however, a CV of less than 10% is often considered desirable,[175] and our results therefore show adequate precision at the measured concentrations.

Urine creatinine was approximately 22% higher in greyhounds than non-sighthounds, when adjusted for urine concentration. Because creatinine is largely excreted in the kidneys and not reabsorbed, the most likely cause for the higher urine creatinine in greyhounds is the filtration of higher concentrations of serum creatinine in greyhounds compared with non-sighthounds. Other potential causes or contributing factors for the high urine creatinine in greyhounds include active renal tubular secretion and high GFR. Tubular secretion is an unlikely cause because previous studies have found this to be of negligible significance in dogs.[6,14] GFR in the greyhound has been assessed previously; one study reported a higher GFR in greyhounds compared with non-greyhounds,[153] but this was not supported by two other studies.[154,155]

There was no statistically significant difference in urine protein concentrations between greyhounds and non-sighthounds. This finding was not unexpected because excretion of urine protein depends very little on urine concentration and GFR, and unlike creatinine, is not affected by muscle mass. Instead, urine protein excretion depends on pre-renal, renal and post renal factors.[16]

The mean UPC in greyhounds was 29% lower than the mean UPC in non-sighthounds, however the difference was not statistically significant. It is interesting to note that the magnitude of the difference is similar to the difference in urine creatinine

between groups (22%). Given that urine protein was not statistically significantly different between groups, it is likely that the trend towards a lower UPC in greyhounds is due to higher urine creatinine concentration in this breed.

A UPC reference interval was established for greyhounds but the result depended on which data set was analysed. When outliers were excluded, the upper reference limit was 0.23, whereas when the outliers were included, the upper reference limit was 0.42. Identification and elimination of outliers are important in the evaluation of reference data, particularly in the accurate determination of reference intervals. However, the outliers identified in this study were not automatically excluded for the following reasons:

- According to the American Society for Veterinary Clinical Pathology reference interval guidelines, if 'individuals are selected randomly from well-defined populations and health is confidently established, retention of all reference values is favored'.[171]
- The dogs identified as outliers were not evidently dehydrated or unwell on physical examination.
- The outliers sat in the tails of the heavily skewed data, and the most extreme data points had two dogs with similar values, which makes them more likely to be true values than anomalies

Dogs identified as outliers were examined for potential causes for the increased UPC. Two greyhounds had trace or 1+ positive haemoglobin results on dipstick, but urine samples with grossly indistinguishable blood contamination were not excluded because several studies have shown that mild blood contamination (that is not evident grossly) has no significant effect on UPC.[140,142] Dogs with pyuria were excluded because this has been

shown to influence UPC.[59,112] No other abnormalities were identified that would constitute a pathologic cause for the high UPC in the outliers.

Other potential causes of variation in UPC were considered, including collection method, gender and neutering status. Samples were collected via free catch rather than cystocentesis due to welfare and ethical considerations; previous studies have shown no significant difference in UPC measurements in samples collected via free catch or cystocentesis.[59,139] In this study, all three dogs with a UPC greater than 0.5 were entire. UPCs have been reported to be higher in intact male dogs, although not greater than 0.5.[127] Collection method, gender and neutering status are unlikely to be a cause of for lower UPCs in greyhounds because there was no significant difference in these variables between greyhounds and non-sighthounds.

Dogs with USG < 1.025 were excluded from the analysis so that dogs with subclinical renal disease would be unlikely to affect the reference interval. However, as healthy dogs can show marked variability in urine concentration[100], analysis of a dataset whereby dogs were excluded based on USG <1.015 was also performed. As noted from the results in Appendix 5, this did not change the overall conclusion, with the upper reference interval for greyhounds with USG ≥ 1.015 being only slightly higher (UPC = 0.5) when compared to the dataset including dogs with a USG ≥ 1.025 (UPC = 0.42).

The results from this study suggest that greyhounds with UPC greater than 0.42 should be considered proteinuric, while a more conservative approach (e.g. ongoing monitoring) should be used when greyhounds have a UPC of 0.23–0.42. Interestingly, this recommendation aligns fairly well with the Algorithm for Substaging by Proteinuria proposed by IRIS for dogs.[128] Thus, our findings show that greyhounds have higher urine

creatinine and may have slightly lower UPC that non-sighthounds. The UPC difference is small enough to be considered clinically insignificant and the generic IRIS UPC guidelines can be used in greyhounds.

# Chapter 5. Symmetric dimethylarginine in greyhounds

## 5.1. Results

The mean SDMA concentration for the greyhounds was significantly higher than the mean for non-sighthounds, with a difference between the means of 2.8 µg/dL ($P < 0.001$) (Table 5-1).

**Table 5-1. Summary of symmetric dimethylarginine for greyhounds, non-sighthounds and generic canine reference intervals**

| Category | Greyhound (n=98) | Non-sighthound (n=24) | Published canine reference intervals (n= 122) |
|---|---|---|---|
| SDMA (µg/dL) mean ±SD | 13.0 ± 3.4 | 10.2 ± 2.9 | N/R |
| range | 6.0–21.0 | 4.0–14.0 | 5–17 |
| Estimated lower (2.5%) and upper (97.5%) limits (µg/dL) | 6.3–19.7 | N/A^ | 6–13 |
| 90% CI for lower limit (µg/dL) | 5.4–7.2 | N/A | N/R |
| 90% CI for upper limit (µg/dL) | 18.8–20.6 | N/A | N/R |

Note: CI, confidence interval; N/A, not assessed; N/R, not reported; ^The non-sighthound group was too small to accurately calculate the reference interval. Published canine reference intervals from V Rentko [176]

The SDMA for both groups showed no significant deviation from normality (Anderson-Darling test, P = 0.09 for greyhounds, P = 0.12 for non-sighthounds) (Figure 5-1). There were no outliers detected using either the Dixon or the Tukey methods. The serum SDMA reference interval for greyhounds was 6.3–19.7 µg/dL (0.31–0.98 µmol/L). The upper end of this interval was higher than the upper limit of the published canine reference interval (6–13 µg/dL).[176] There were 40 greyhounds and four non-sighthounds with SDMA concentrations higher than the published canine reference interval.[176]

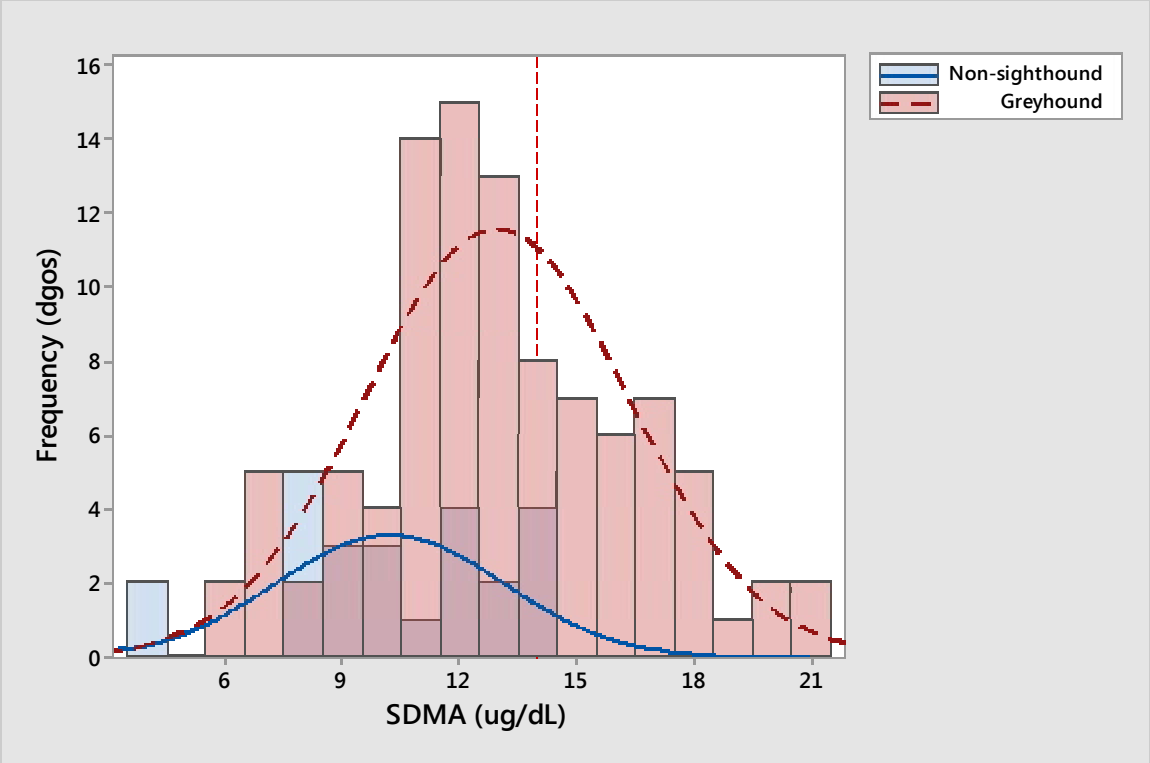


**Figure 5-1. Histogram of symmetric dimethylarginine concentrations for greyhounds and non-sighthounds with fitted standard curve.**

The orange dashed line represents the IDEXX recommended upper reference limit

There was a significant but weak correlation between SDMA and serum creatinine concentration (r = 0.22, P = 0.03 in the greyhound group; r = 0.36 P = 0.08 in the non-sighthound group; see Figure 5-2).

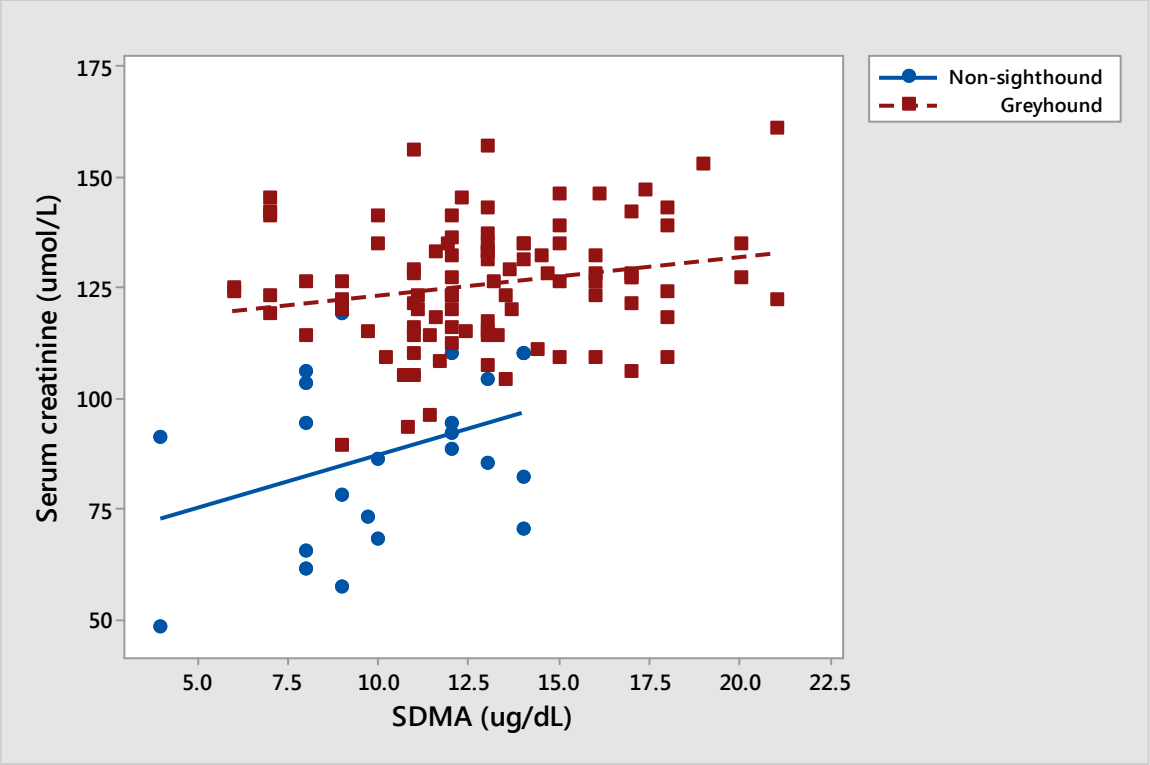


**Figure 5-2. Scatterplot with regression lines for the association between serum creatinine and symmetric dimethylarginine in greyhounds and non-sighthounds.**

## 5.2. Discussion

The reference interval for the serum SDMA concentration in greyhounds was 6.3–19.7 µg/dL (0.31–0.98 µmol/L) and the mean was statistically significantly higher ($P < 0.001$) than that of a group of non-sighthounds of similar weight, age and sex. The upper end of the greyhound reference interval is higher than the reported canine reference interval of 6–13 µg/dL (0.30–0.64 µmol/L),[176] suggesting that greyhounds require a wider serum SDMA reference interval than dogs of other breeds.

The cause of higher SDMA concentrations in greyhounds is unclear. Increases in serum SDMA and serum creatinine concentrations have been shown to predict a lower

GFR.[72,80] It is possible that the GFR of greyhounds is physiologically lower than that of other breeds and that this contributes to higher SDMA and serum creatinine concentrations. Yet, as previously discussed, other studies have reported contradictory results, with greyhounds having higher, comparable or possibly lower GFR than other dog breeds.[153-155] Only small numbers of dogs were assessed in these studies and, due to differences in methodology, comparisons between studies are difficult. Thus, future studies should aim to assess GFR in conjunction with SDMA in greyhounds.

Compared with other breeds, greyhounds have several unique haematological, biochemical and drug metabolism characteristics,[152,154,158] and some of these factors could indicate differences in cellular production and metabolism. Indeed, increased production of SDMA due to an increased rate of cell turnover has been a proposed mechanism for higher SDMA concentrations in juvenile dogs[72] and could be a mechanism in greyhounds. Increases in SDMA concentration have shown an association with hypertension and endothelial dysfunction in people,[177,178] and a recent study found that the eicosanoid profile of greyhounds is shifted toward metabolites that promote vascular dysfunction, hypertension and proteinuria.[146] Whether there is a connection between elevated SDMA and vascular dysfunction in greyhounds remains to be elucidated.

Dogs in this study were not fed a standardised diet to provide a representative reference interval for the pet greyhound population. Previous studies suggest that the effect of diet on serum SDMA is negligible[179,180] unless diet is purposefully chosen to treat renal disease, in which case SDMA decreases.[44,181] Thus, diet is an unlikely explanation for the higher SDMA seen in greyhounds. Serum SDMA concentrations are not influenced by lean body mass,[44,182] and in the current study the greyhounds and non-

sighthounds were purposely chosen to be of similar weight and size to reduce any potential confounding effect of these factors on SDMA concentrations. SDMA is therefore unlikely to be higher and more variable because of differences in lean body weight between greyhounds and non-sighthounds.

In the non-sighthound group, four of the 24 dogs had high-normal SDMA concentrations of 14 µg/dL, which could suggest early renal disease. Serum SDMA occasionally reaches concentrations of 15µg/dL and rarely reaches up to 16 µg/dL, in dogs unaffected by renal disease, and these increases have been found to occur more commonly in young dogs.[72] Interestingly, three of the four dogs with SDMA concentrations of 14 µg/dL were 1–2 years of age. These dogs were included in the study because these small increases could indicate normal biological variation, and the goal of this study was not to establish accurate reference intervals for non-sighthounds but rather for them to be used as a comparison for greyhounds. The fact that the non-sighthound dogs studied here have, on average, significantly lower SDMA than greyhounds, despite the inclusion of dogs with mildly increased SDMA, strongly supports the existence of a breed-specific difference in SDMA concentration.

The reference interval for serum SDMA was established from 98 healthy greyhound dogs and was significantly higher than that of non-sighthound dogs of similar weight, age and sex. Thus, breed-specific reference intervals should be adopted when assessing SDMA in non-racing greyhounds.

# Chapter 6. Correlation between serum creatinine, urine creatinine, serum SDMA and muscle mass

## 6.1. Results

Greyhounds were found to have statistically significantly greater mean thigh circumference and height than non-sighthounds (t-test; both $P < 0.01$; see Table 6-1).

**Table 6-1. Body measurements for greyhounds and non-sighthounds**

| Category | Greyhound n = 98 | Non-sighthound n = 23 | P value |
|---|---|---|---|
| Weight (kg) mean ± SD | 31.6 ± 3.8 | 31.2 ± 5.2 | 0.73 |
| Thigh circumference (cm) mean ± SD | 45.4 ± 2.5 | 42.4 ± 2.0 | < 0.001 |
| Height (cm) mean ± SD | 66.3 ± 4.5 | 57.9 ± 6.1 | < 0.001 |
| Serum creatinine (µmol/L) mean ± SD | 125.6 ± 13.8 | 87.5 ±19.2 | <0.001 |
| UCr mean ± SD (µmol/L) | 27,562 ± 10,903 | 23,773 ± 8,237 | 0.07 |

Note: N/A, not applicable; SD, standard deviation.

Serum creatinine was weakly but statistically significantly correlated with both weight ($r = 0.24$, $P = 0.02$; see Appendix 6) and thigh circumference ($r = 0.25$, $P = 0.02$) and approached statistical significance with height ($r = 0.19$, $P = 0.06$), see Table 6.2.

Regression analysis was then performed to assess the effect of the body measurements on urine creatinine (corrected for urine concentration), as shown in Table 6.2. Significant weak associations for weight, height and thigh circumference were observed.

**Table 6-2. Regression coefficients for the effect of body measurements on laboratory results in greyhounds**

| | Serum creatinine | Urine creatinine | Corrected urine creatinine[109] | Serum SDMA |
|---|---|---|---|---|
| Weight | 0.24 (P = 0.02) | 0.21 (P = 0.04) | 0.23 (P = 0.03) | - 0.14 (P = 0.17) |
| Height | 0.19 (P = 0.06) | 0.18 (P = 0.08) | 0.20 (P = 0.05) | - 0.06 (P = 0.54) |
| Thigh circumference | 0.25 (P = 0.02) | 0.14 (P = 0.16) | 0.21 (P = 0.03) | - 0.086 (P = 0.40) |

The strongest predictor of serum creatinine was thigh circumference, while the strongest predictor of corrected urine creatinine was weight (see Appendix 7).

Serum SDMA did not show significant correlation with weight, height or thigh circumference (Table 6-2).

## 6.2. Discussion

Positive correlations for both serum creatinine and urine creatinine with weight, height and thigh circumference were identified. Previous studies suggest that morphometric measurements are often inaccurate as an assessment of muscle mass,[183-185] even with the use of complicated equations using several points of measurements.[186] This may explain why the correlations were weak. Despite the limitations of the morphometric measurements used in this study, the weak but statistically significant correlation suggests that the higher urine creatinine in greyhounds compared with non-sighthounds may be due to their higher muscle mass.[156,162-166] Stronger correlations may have been obtained with more sophisticated methods for muscle mass measurement. This study

concurs with previous studies[44,72,85,86], and shows that serum SDMA shows minimal correlation with body weight, and likely muscle mass in dogs.

Other methods were considered for this study. Body condition score was not useful because most greyhounds were of similar body condition. Muscle condition scoring has not been validated in dogs and uses a scale that grades degree of muscle loss rather than degree of muscling in healthy animals.[187] Bioimpedance is a safe, portable and non-invasive method of measuring body composition in dogs, but several studies have found it to be an unreliable indicator of body fat for some breeds of dogs, including greyhounds.[164,188] Other methods such as dual-energy X-ray absorptiometry scanning, computed tomography or deuterium oxide dilution require sedation and are expensive to perform; therefore, these were not used in this study.[186,189]

It is well established that greyhounds have higher muscle mass than other breeds.[145,169-173] Thigh circumference, height and body weight were used as simple estimators of muscle mass and a weak correlation between these measurements and urine creatinine was observed in greyhounds. Thus, greyhounds may have higher serum creatinine and urine creatinine due to their greater muscle mass. However, the correlation between creatinine and muscle mass was weak and muscle mass estimation based on morphometric measurements has limitations.[183-185] Therefore, further studies using more sophisticated methods of body composition measurement are needed to confirm these findings.

# Chapter 7. Limitations, conclusions and avenues for further investigation

## 7.1. Limitations

There were several limitations to this study. Attempts to exclude animals with disease were made based on history, physical examination and laboratory findings; GFR analysis, full haematology and biochemical testing and renal biopsies were not performed due to cost and welfare constraints. It is therefore possible that some dogs had subclinical disease, especially given that greyhounds are prone to renal disease and hypertension.[49,145] Efforts to minimise the effect of this limitation were made by utilising the American Society for Veterinary Clinical Pathology guidelines to calculate a *de novo* reference interval.

At least 120 subjects are recommended for the establishment of a reference interval; this was also the aim in this study and 149 greyhounds were initially screened.[150,182] While only 98 greyhounds were included in the final analysis, this number compares favourably to many other veterinary studies that have established reference intervals.[190-192] Financial and ethical constraints prohibited the inclusion of more dogs.

Samples underwent prolonged storage or freeze–thaw cycles before analysis of SDMA, UPC and osmolality. The serum and urine samples in this study underwent one or two freeze–thaw cycles before SDMA and osmolality analysis, and were stored for up to 3 months at −80°C. The SDMA immunoassay has been validated for stability, with performance metrics within United States Food and Drug Administration guidelines.[193] Studies evaluating the effects of short-term storage and freezing on SDMA concentration

measurement using liquid chromatography–mass spectrometry showed no significant effect in samples stored for 14 days at 4°C or in samples subjected to three freeze–thaw cycles.[72,194] There are no published studies evaluating the long-term stability of SDMA in frozen serum samples, but anecdotal evidence suggests stability for at least 5 years when frozen at −80°C (IDEXX, Laboratories, Inc., Personal Communication).[195] In a recent small canine study, urine osmolality was 5% lower in samples stored for 90 days at −80°C compared with fresh samples.[103] There was no significant difference in samples that underwent one or two freeze-thaw events and therefore it is unlikely that the prolonged storage and freeze–thaw cycles had a clinically significant effect of urine osmolality measurement.

Urine and serum samples were refrigerated at 4°C for up to 72 hours before UPC and serum creatinine analysis, respectively. In one study, samples with inactive sediments showed no significant increase in urine protein or urine creatinine after 72 hours of storage at 4°C.[59] Serum creatinine is stable for up to 4 days at room temperature[57] and is likely to also be stable when stored at 4°C. It is unlikely that creatinine or protein measurements were significantly affected by prolonged refrigeration. It should be noted that in this study, the storage protocols and durations were the same for the greyhound and non-sighthound samples so that sample storage was not a confounding factor in any differences observed between groups.

Actively racing greyhounds were excluded from this study because exercise can affect GFR and urine protein excretion,[133-138] and muscle mass could be different in pet and racing greyhounds. Other sighthound breeds were excluded from this study. Sighthounds show some similarities and some significant differences in haematological

and biochemical results between breeds.[48,152,196] Therefore, the reference intervals established for greyhounds in this study should not be extrapolated to greyhounds in active race training or other sighthound breeds until further studies are performed to establish whether these reference intervals are applicable to these specific groups.

## 7.2. Conclusions and further avenues of investigation

Several measurements used in the assessment of renal function were investigated in a large group of non-racing greyhounds and a smaller group of non-sighthounds. The results show that for dogs with well-concentrated urine (USG ≥ 1.025), greyhounds and non-sighthounds had similar urine concentrations, as determined by both USG and urine osmolality. Additionally, there was no difference in urine protein concentrations between the two groups of dogs. However, some differences were observed. Greyhounds had significantly greater urine creatinine concentrations than non-sighthounds, likely due to higher muscle mass. It is possible that the higher urine creatinine in greyhounds could affect ratios other than UPC, such as electrolyte fractional excretion ratios and urine cortisol to creatinine ratios. Fractional excretion ratios have already been assessed in greyhounds, but the results were not compared to other breeds.[197] Thus, further studies investigating whether these ratios differ between greyhounds and non-sighthounds would be useful.

Greyhounds also had higher serum SDMA concentrations than non-sighthounds, and a greyhound-specific reference interval for SDMA was proposed. Further studies to investigate the causes of the higher SDMA and urine creatinine in greyhounds might include evaluating the rate of cellular SDMA production and further attempts to elucidate if GFR in greyhounds is different from that in other breeds.

UPC reference intervals were also established for greyhounds. The upper UPC reference limit was similar to that recommended by the IRIS committee, which is reassuring given that generic UPC reference intervals have historically been used in greyhounds, and this suggests that these reference intervals have been used accurately. Given the minimal difference in the UPC reference intervals for greyhounds and non-sighthounds, breed-specific UPC reference intervals for greyhounds are not recommended, but further studies would be helpful to define the UPC threshold for the classification of pathologic proteinuria in greyhounds. These studies would likely require 24-hour urine collection from greyhounds with renal disease, determination of urine protein excretion over this time and calculation of UPCs.

In conclusion, this study has contributed to breed-specific knowledge that can be used in the investigation of renal disease in greyhounds. A breed-specific reference interval should be used for SDMA in greyhounds, while the generic IRIS reference interval can be used for UPC. This information on clinicopathologic assessment of renal health in greyhounds should assist with screening greyhounds for renal disease.

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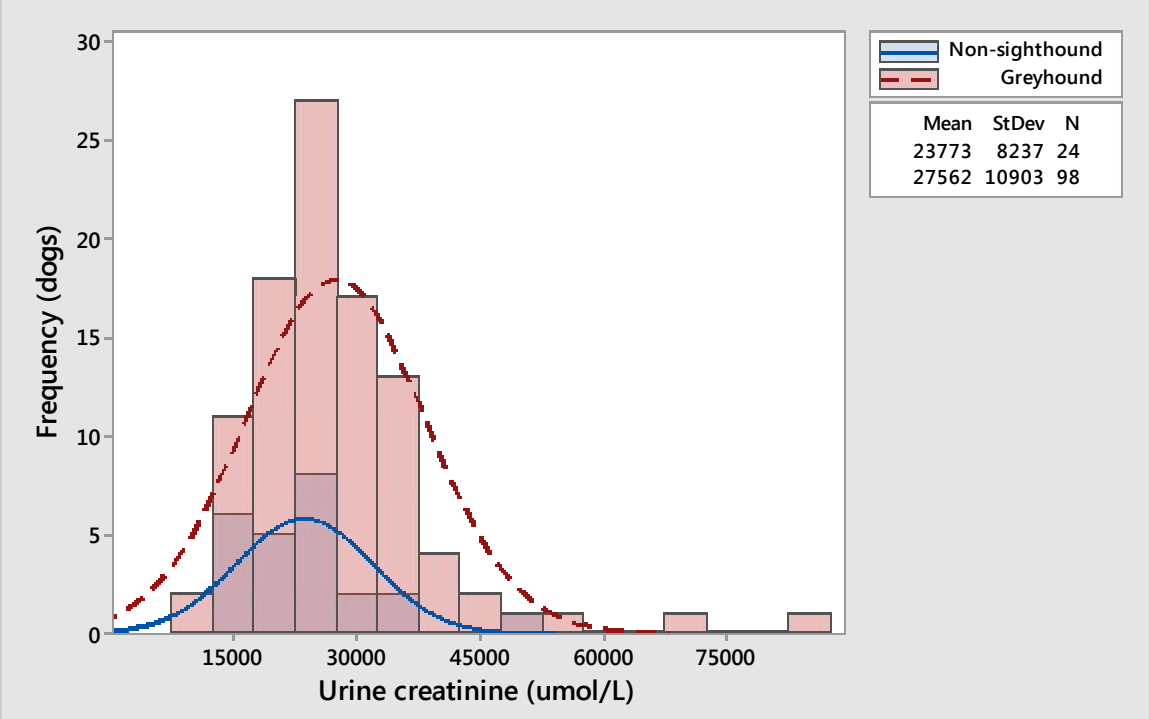
# Appendices

## Appendix 1. Osmolality results of one vs two freeze thaw cycles

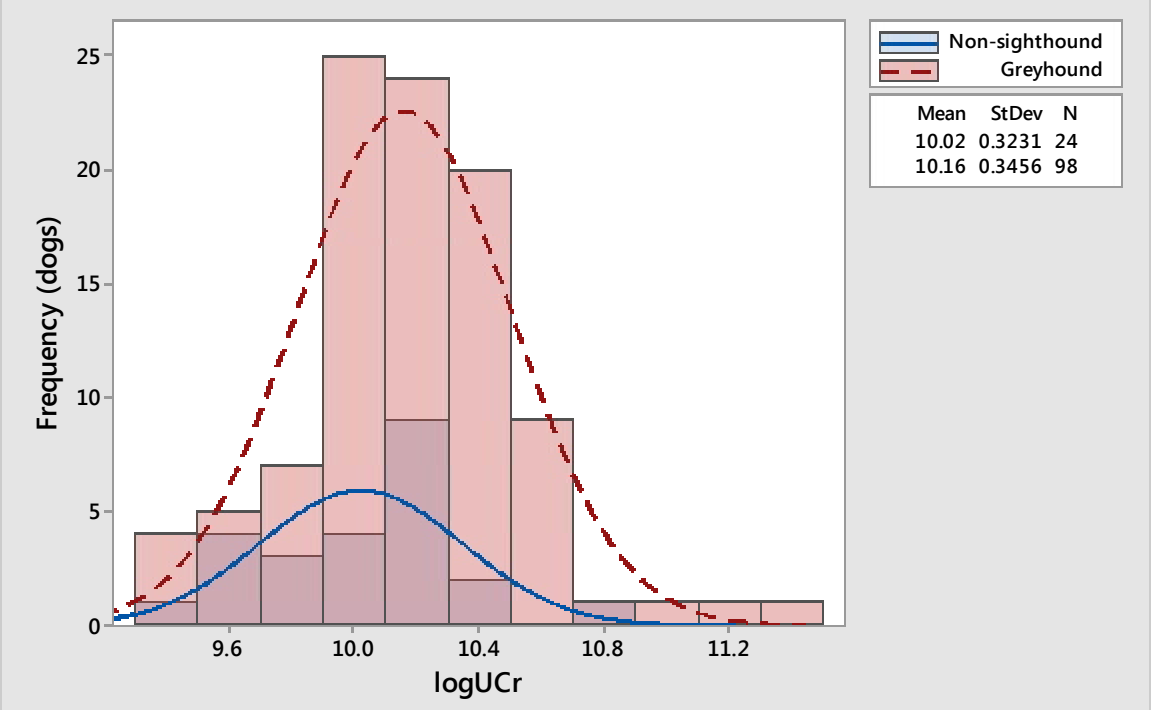
| | One freeze-thaw cycle | Two freeze-thaw cycles |
|---|---|---|
| Sample one (mOsm/kg) | 505 | 494 |
| Sample two (mOsm/kg) | 1,923 | 1,935 |
| Sample three (mOsm/kg) | 1,498 | 1,505 |
| Sample four (mOsm/kg) | 1,102 | 1,072 |
| Sample five (mOsm/kg) | 1,083 | 1,087 |

## Appendix 2. Urine creatinine concentration- untransformed



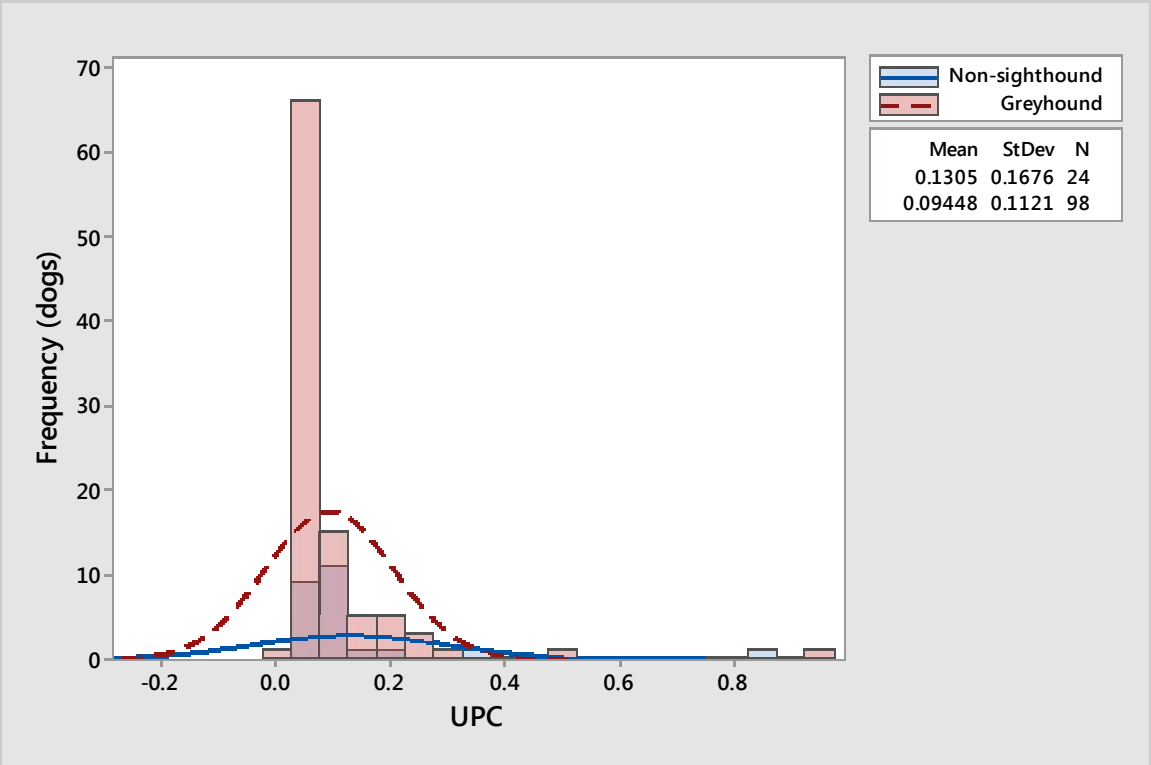

**Histogram of untransformed data of urine creatinine for greyhounds and non-sighthounds with a normal distribution overlaid**

## Appendix 3. Urine creatinine concentration- log transformed




**Histogram of log transformed data of urine creatinine for greyhounds and non-sighthounds with a normal distribution overlaid**

## Appendix 4. Urine protein to creatinine ratios- untransformed data

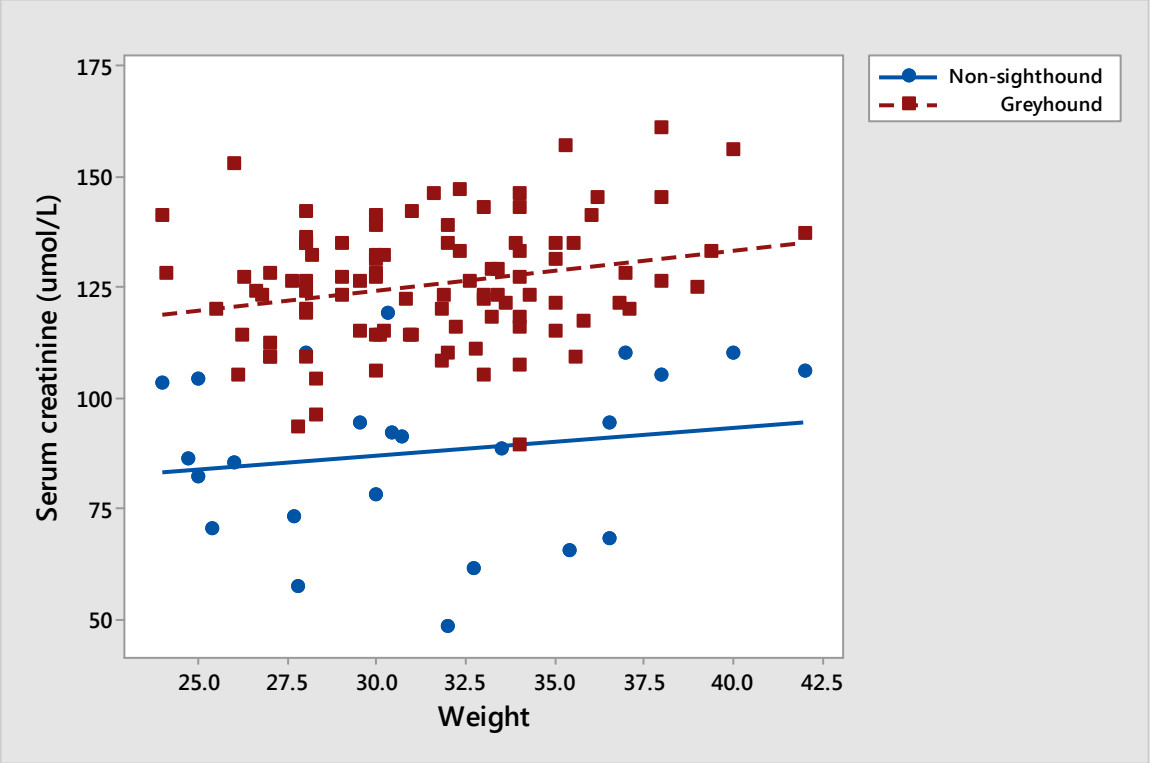



**Histogram of untransformed data of urine protein to creatinine ratio (UPC) for greyhounds and non-sighthounds with a normal distribution overlaid**

## Appendix 5. UPC values depending on USG exclusions

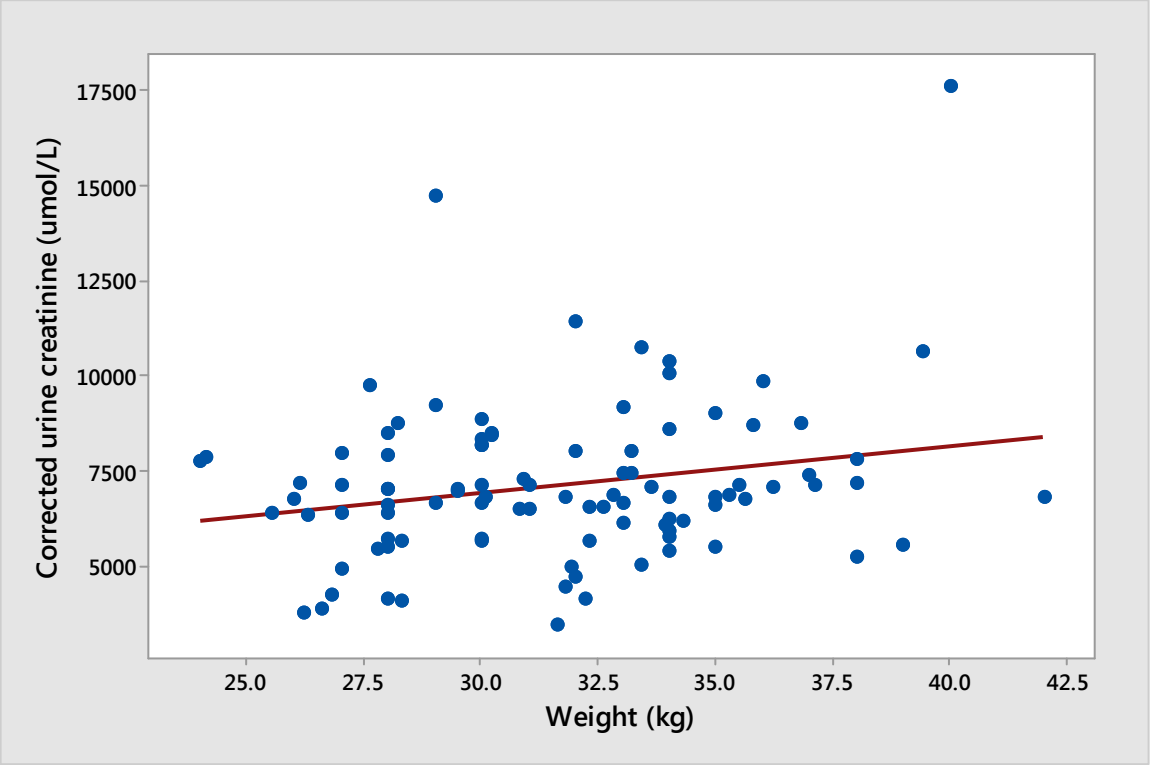
| Category | Greyhound with USG ≥ 1.025 (n= 98) | Greyhounds with USG ≥ 1.015 (n= 120) | Non-sighthound with USG ≥ 1.025 (n= 24) | Non-sighthound with USG ≥ 1.015 (n= 26) |
|---|---|---|---|---|
| UPC median | 0.061 | 0.061 | 0.081 | 0.081 |
| Range | 0.02–0.96 | 0.023–0.98 | 0.04–0.85 | 0.038–0.85 |
| Estimated lower and upper limits | 0.037–0.42 | 0.036–0.50 | N/A | N/A |
| 90% CI for lower limit | 0.023–0.038 | 0.023–0.038 | N/A | N/A |
| 90% CI for upper limit | 0.23–0.96 | 0.24–0.98 | N/A | N/A |

## Appendix 6. Serum creatinine vs weight for both groups




**Scatterplot with regression lines showing the association between serum creatinine and body weight for greyhounds and non-sighthounds**

## Appendix 7. Corrected urine creatinine vs body weight in greyhounds




**Scatterplot with regression line showing correlation between corrected urine creatinine and body weight in greyhounds.**

## Appendix 8. Owner consent form

| | |
|---|---|
| VETERINARY HOSPITAL<br>University of Melbourne<br>Consent Form | **Owner Details:** |

## Determination of urinary creatinine and protein levels in the Greyhound

**Clinical Investigator**:
Dr Rebekah Liffman BVSc
Email: rebekah.liffman@unimelb.edu.au
Phone: 03 8001 2563 (business number) or 03 9731 2398 (clinical pathology lab)
If you require veterinary assistance outside of business hours, please call U-Vet on 03 9731 2000

**Purpose of Study**: The purpose of this study is to determine the concentration of urinary creatinine and protein in greyhounds, and compare it to the concentration of these metabolites in the urine of healthy dogs of other breeds. Blood will also be collected to compare the creatinine levels in blood with urine. The blood results will also allow us to exclude unhealthy animals from the study. The collected blood and urine samples will also be saved for future research use.

This study has been granted animal ethics approval (ID: 1613906) and is a registered study at the U-Vet Werribee Animal Hospital (project number: 000010SA6)

**Eligibility**: Any adult dog (1-12 years) that weighs 24-42kg that is deemed clinically healthy is eligible to participate in this study.

**Procedures**: Your dog will be leash-walked, and if he/she voluntarily urinates, a container will be used to catch a urine sample. If a sample is obtained, your dog will proceed to have blood collected. If we are not able to obtain a urine sample, your dog is returned to you and cannot be used in our study.

During blood collection, your dog will be gently restrained to allow blood to be taken from the jugular (neck) vein. A single blood sample of 3ml will be collected. Your dog is then placed back into your care and is free to resume normal activities. If you dog is at all distressed by the procedure, we will abort the procedure and your dog will be immediately returned to your care.

If your dog is undergoing surgery, urine may be collected via gentle expression of the bladder whilst under general anaesthesia. Blood will be collected from the intravenous catheter placed for surgery.

**Associated Risks**: The risks of blood collection include potential minimal bruising at the collection site and slight swelling at the site of blood collection. Introduction of skin contaminants into the blood are possible but very unlikely, and even if it does happen, it is rare that it would cause any problems.

**Compensation**: There is no monetary compensation for participation in this study. The owner will be responsible for all other appropriate medical fees if an abnormality is found on bloodwork and further tests are recommended.

**Incentives**: Participation in this study includes complimentary measurement of several analytes from both blood and urine that will help to determine kidney function. This information will be forwarded to you via email or post. Please note it may be several weeks before the full set of results are made available.

**Confidentiality**: Owner and patient confidentiality will be maintained at all times. Identification of study participants shall never be made when reporting or publishing the data arising from this study.

**Questions** about this project may be directed to Rebekah Liffman on 03 8001 2563 or via email: rebekah.liffman@unimelb.edu.au

I understand that my dog(s) participation in this study is entirely voluntary. I am free to withdraw my dog(s) from this study at any time without compromising the quality of care provided to my animal. I understand that my voluntary removal is final.

I also understand that my dog(s) may be required to withdraw from the study for violation of eligibility requirements, or noncompliance with restrictions and/or procedures during the study. This also constitutes disqualification. I may also be required to withdraw from the study to protect my dog's health (such as with the occurrence of significant injury, adverse reactions, or illness whether or not a consequence of the study), or if the study is terminated prematurely.
I have not withheld information regarding my dog's medical history.

*I acknowledge that I have read and understand this consent form and all my questions have been answered to my satisfaction. I have been assured that all personal identifying information will be kept confidential.*

*I am aware that this research has been reviewed and approved by the Office of Research Ethics and Integrity at the University of Melbourne.*

*As a volunteer, I give my informed consent to the Board of Trustees of the University of Melbourne and the Veterinary Teaching Hospital to enroll my animal/s in this study, according to the explanations and conditions presented in this document. I agree to hold harmless the Board of Trustees of the University of Melbourne, the Veterinary Teaching Hospital, and its officers, employees, agents and assigns from any and all liability, claims and actions that may arise from participation in this study.*

☐ I have received a copy of the study information form.

_____

Printed Name: Owner

_____ _____

Signature Owner Date

_____

Printed Name: Witness

_____ _____

Witness Signature Date

## Appendix 9. Data collection sheet

Owner information

Name: _____

_____

Please provide a postal address or email address to send results to: _____

_____

Pet information

Name: _____ Breed: _____

Gender: _____ Age: _____

Desexing status: _____ Weight: _____

For researchers to fill out

Patient ID _____

Time and date of sample collection: _____ Body Condition Score /5

Height: _____ Thigh circumference: _____