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Establishment of reference intervals for serum symmetric dimethylarginine in adult non-racing Greyhounds

Running header: Reference intervals for serum SDMA in Greyhounds

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ABSTRACT

Background: The reference intervals (RIs) for the renal biomarkers urea and creatinine in Greyhounds are higher than those for non-sighthound breeds. A recent study has demonstrated a higher concentration of another biomarker of renal function, symmetric dimethylarginine (SDMA), in Greyhounds compared to other dog breeds, and thus a breed-specific RI for serum SDMA may be appropriate for Greyhounds. Greyhounds appear to be predisposed to renal disease, and the establishment of an appropriate RI for SDMA may improve the ability to identify early renal dysfunction in this breed.

Objectives: The aim of this study was to establish an RI for serum SDMA in non-racing Greyhounds and to determine whether the RI for Greyhounds is different from that of non-sighthound breeds.

Methods: Blood samples were collected from 101 clinically healthy, non-racing Greyhounds for serum SDMA measurements. Results from Greyhounds were compared with serum SDMA concentrations measured in a group of non-sighthound dogs (n=24) of similar weight, age, and sex, and with a previously established canine serum SDMA RI.

Results: The serum SDMA RI for Greyhounds was 6.3-19.9 µg/dL (0.31-0.99µmol/L). Greyhounds had a significantly higher mean value (13.1 µg/dL) than that of the non-sighthound dogs (10.2 µg/dL) (P <0.001), and the RI of Greyhounds was different from previously established canine RIs for SDMA.

Conclusion: This study supports the use of a Greyhound-specific RI for SDMA. Using previously established canine RIs for this breed may result in the over-diagnosis of renal disease.

Key Words

Acute phase protein, C-reactive protein, dog,

immune-mediated hemolytic anemia

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INTRODUCTION

Symmetric dimethylarginine (SDMA) is released into cell cytoplasm following the intranuclear methylation of the amino acid arginine. Proteins carrying SDMA are involved in DNA repair, protein translocation, and signal transduction, and the degradation of these methylated proteins leads to free SDMA in the serum.[1] In both veterinary and human medicine, SDMA is used as a biomarker to assess glomerular filtration rate (GFR) since it appears to be eliminated exclusively by the kidneys.[2,3] Studies have demonstrated a strong correlation between SDMA and GFR, and suggest that measurement of SDMA may be more sensitive in the diagnosis of canine renal disease compared to serum creatinine (SCr).[4-7] An SDMA assay based on liquid chromatography-mass spectrometry (LC-MS) has been validated for use in dogs, and a reference interval (RI) of 6-13ug/dL has been established from 122 dogs.[5,8] IDEXX laboratories have since released a commercial immunoassay for SDMA that shows excellent correlation with LC-MS and has become widely adopted.[9] Currently, the IDEXX immunoassay adult canine SDMA RI is 0-14µg/dL.

Several veterinary studies have shown that unlike SCr, serum SDMA concentration does not depend on lean body mass, suggesting that the generic canine SDMA RI should be applicable for all breeds.[5,10] Indeed, a study comparing SDMA concentrations between 3 different breeds (Pointers, Cairn Terriers, and Cavalier King Charles Spaniels) found no significant differences.[11] In contrast, SDMA concentrations were significantly higher in Greyhounds (n=20) compared to other breeds (n=20), and 68% of Greyhounds had an SDMA concentration greater than the upper limit of the previously established canine RI.[12] Larger studies are required to confirm this latter finding, particularly since Greyhound pet ownership has become increasingly popular, and with this comes greater owner expectations for breed-specific veterinary knowledge.[13] In addition, Greyhounds appear predisposed to hypertension, proteinuria, and renal dysfunction, and thus there is a need for early detection of renal disease in this breed.[14,15]

The aim of this study was to establish an RI for serum SDMA concentration in non-racing Greyhounds using the commercially available immunoassay,[16] and compare this RI with serum SDMA concentrations measured in non-sighthound dogs of similar weight, age, and sex, and with previously established canine RIs.

MATERIALS AND METHODS

Populations

This study was approved by The University of Melbourne Animal Experimentation Ethics Committee (ID: 1613906), and all owners signed a consent form prior to participation.

Non-racing Greyhound dogs (n=149) and non-sighthound dogs (n=35) were enrolled from September 2016 to July 2017. All dogs lived in Victoria, Australia. After exclusions were taken into account, the final analysis included 101 Greyhounds and 24 non-sighthound dogs. The Greyhound population was sourced from 35 different owners, which included a program that rehomes retired racing Greyhounds (n=40), 3 different racing trainers/breeders (n=27), an animal shelter (n=1), and private dog owners (n=33). Five dogs from the non-sighthound group were sourced from a shelter, and the remainder were owned by staff, students, or clients at the University of Melbourne Faculty of Veterinary and Agricultural Sciences. The non-sighthound group consisted of the following breeds; mixed breed (n=9), Labrador Retriever (n=8), Golden Retriever (n=1), Wirehaired Pointer (1), German Shepherd dog (n=1), Koolie (n=1), Belgian Shepherd (n=1), Kelpie (n=1), and a Gordon Setter (n=1).

Sample collection

Sampling took place where the animals were housed, at the University of Melbourne U-Vet hospital, or in public spaces during dog walking events. Free access to water was permitted unless it was withheld by veterinary staff due to a planned medical or surgical procedure (eg, prior to neutering surgery later that day). Each dog was leash-walked, allowed to urinate, and a midstream sample was collected into a clean container. Following urine collection, 3mL of blood were taken from the jugular vein (or cephalic if an IV catheter was placed) using a 21G needle (NIPRO Corporation, Osaka, Japan), and 3mL syringe (BD, Singapore), with 2.5mL placed into a serum separation tube ('Vacuette tube', Greiner Bio-One Frickenhausen, Germany), and 0.5mL into an EDTA microtube (MiniCollect, Greiner Bio-One Frickenhausen, Germany).

Inclusion and exclusion criteria

Inclusion criteria for Greyhounds comprised healthy dogs aged 1-12 years, of any gender or neutering status. Inclusion criteria for the non-sighthound population comprised healthy dogs aged 1-12 years, of any breed apart from sighthounds, in the weight range of 24-42kg, and of any gender or neutering status. Health status was assessed with histories, physical examinations, PCV and total solids (TS) measurements, SCr concentrations, and urinalyses. Owners provided information on the health of

each dog within the previous 14 days, including any medical conditions or surgical procedures, as well as racing status. Dogs were excluded if they had actively raced or trained within the last 7 days, had been administered medications that might interfere with GFR or SCr concentrations within the previous 14 days, had eaten in the 8 hours prior to sampling, or if a free catch urine sample could not be collected. Greyhounds were also excluded if they had values outside of Greyhound-specific RIs established at the University of Melbourne U-Vet hospital for the following: SCr>170µmol/L with a urine specific gravity (USG) < 1.030; PCV < 0.36 L/L; or TS < 48 g/L. Similarly, non-sighthounds were excluded if they had values outside the canine RI established at U-Vet: SCr >140µmol/L with USG <1.030; PCV < 0.37 L/L; or TS < 60 g/L. Based on published USG results in healthy Greyhounds,[17] dogs from either group were excluded if they had a USG < 1.025 regardless of SCr. Additionally, dogs were excluded if they had gross hematuria or evidence suggestive of urinary tract infection (≥5 WBC /high power field (HPF) or bacteriuria) on urine sediment examination.

Analytical methods

Serum tubes were centrifuged within 4 hours of sample collection, and approximately 0.5-1mL of serum was placed into an Eppendorf tube (Eppendorf AG, Hamburg, Germany) that was then immediately frozen for storage at -80°C for up to 3 months. Frozen serum samples were later thawed and sent to IDEXX laboratories for batch analysis of SDMA. The remaining serum was either immediately analyzed for SCr or refrigerated and analyzed within 36 hours of collection at the U-Vet hospital's clinical pathology laboratory.

Analyzers at both laboratories were calibrated as directed by the manufacturers. SCr was measured using the COBAS INTEGRA 400 plus (Roche Diagnostics Ltd, Rotkreuz, Switzerland) with a kinetic colorimetric assay based on the Jaffé method. PCVs were determined by centrifuging a plain microhematocrit tube (Frontline PTY Ltd., NSW, Australia) filled with EDTA anticoagulated whole blood at 14 800*g* for 5 minutes (Orbital 260 centrifuge; Clements NSW, Australia). TS was determined by refractometry using plasma from the centrifuged microhematocrit tubes.

Routine urinalysis was performed within 6 hours of collection; 5 mL of urine was centrifuged at 2100*g* for 3 minutes. The supernatant was used for USG, and dipstick analysis and the sediment were examined microscopically.

Statistical Analyses

Study data were collected and managed using Research Electronic Data Capture (REDCap) hosted at the University of Melbourne.[18] Statistical analyses was carried out using Minitab 17 Statistical Software (Minitab 17 Statistical Software, Minitab Inc., State College, PA, USA) and Microsoft Excel 2013 for Windows (Microsoft Corp., Redmond, WA, USA) with Reference Value Advisor v2.1 Add-In (freeware v2.1: http://www.biostat.envt.fr/reference-value-advisor/).[19] SDMA data were assessed

for compatibility with a normal distribution using frequency histograms and probability plots (or Q-Q plots), and the mean SDMA and SCr values for Greyhound and non-sighthound dogs were compared using an independent samples *t*-test. The association between SDMA and SCr was assessed using Pearson's correlation coefficient and a scatterplot. Age and weight of Greyhound and non-sighthound dogs were compared using an independent samples *t*-test. The chi-squared test was used to compare the proportions of gender and neutering status of Greyhounds and non-sighthound dogs. In accordance with American Society for Veterinary Clinical Pathology (ASVCP) guidelines,[20] reference limits and the 90% confidence intervals (CIs) were determined parametrically. The Tukey and Dixon method was used to detect outliers. The RI comprises the central 95% of the fitted distribution, with 90% CIs calculated around the lower (2.5%) and upper (97.5%) limits. Statistical significance was set at $P < 0.05$ for all analyses.

RESULTS

The final analysis included 101 Greyhounds and 24 non-sighthound dogs. A total of 48 Greyhounds and 11 non-sighthound dogs were excluded from the study; 41 due to USG <1.025, 10 due to pyuria, and 1 Greyhound due to an increased SCr with insufficiently concentrated urine. Of the remaining dogs, 5 were excluded due to insufficient sample quantity and 2 dogs were excluded as they were subsequently found to have been administered sedatives prior to blood collection.

No significant differences between the Greyhounds and the non-sighthound dogs for age (P=0.34), weight (P=0.65), gender proportions (P=0.63), or neutering status proportions (P=0.71 for male, P=0.97 for female) were found (Table 1). The SDMA data for both groups showed no significant deviation from normality (P=0.09 for Greyhounds, P=0.12 for non-sighthound dogs). No outliers were detected when using the Dixon or Tukey method. The serum SDMA RI for Greyhounds was 6.3-19.9µg/dL (0.31-0.99µmol/L). The upper end of this interval was higher than the upper limit of the published canine RI (6-13µg/dL)[8]. The mean SDMA concentration for the Greyhound group was significantly higher than the mean for the non-sighthound dog group, with a difference between the means of 2.85µg/dL (95% CI = 1.48, 4.23; P<0.001) (Figure 1). There were 37 Greyhounds and 4 non-sighthound dogs with SDMA concentrations higher than the published canine RI[8].

The Greyhounds had a significantly (P<0.001) higher SCr concentration compared with the non-sighthound dogs (Table 2). There was a significant but weak correlation between SDMA and SCr concentration (r= 0.22, P =0.03) in the Greyhound group but not in the non-sighthound dog group (r= 0.36, P = 0.08) (Figure 2).

DISCUSSION

The RI for the serum SDMA concentration in Greyhounds was 6.3-19.9 µg/dL (0.31-0.99µmol/L) and the mean was significantly higher compared with that of a group of non-sighthound dogs of similar weight, age, and sex. The upper end of the Greyhound RI is higher than the reported canine RI of 6-13 µg/dL (0.30-0.64 µmol/L),[8] indicating that Greyhounds require a wider serum SDMA RI than dogs of other breeds.

The cause for higher SDMA concentrations in Greyhounds is uncertain. Increases in serum SDMA and SCr concentrations have been shown to predict a lower GFR.[4,5] It is possible that the GFR of Greyhounds is physiologically lower than that of other breeds, and this contributes to higher SDMA and SCr concentrations. In the current study, GFR was not assessed. Other published studies have reported contradictory results, with Greyhounds having higher, comparable, or potentially lower GFRs than other dog breeds.[21-23] 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 possess a number of unique hematologic, biochemical, and drug metabolism characteristics[12,23,24] and some of these factors could indicate differences in cellular production and metabolism. Indeed, increased SDMA production due to an increased cell turnover rate has been a proposed mechanism for higher SDMA concentrations in juvenile dogs,[5] and could be a potential mechanism in Greyhounds. Interestingly, increases in SDMA concentration have shown an association with hypertension and endothelial dysfunction in people,[25,26] and a recent study demonstrated that the eicosanoid profile of Greyhounds is shifted toward metabolites that promote vascular dysfunction, hypertension, and proteinuria.[27] Whether there is a connection between elevations in SDMA and vascular dysfunction in Greyhounds remains to be elucidated.

Dogs in this study were fed a varied diet to reflect a representative RI for the Greyhound population. Previous studies suggest that the effect of diet on serum SDMA is negligible,[28,29] unless the diet is purposefully chosen to treat renal disease, in which case SDMA decreases.[10,30] Thus, diet is an unlikely explanation for higher SDMA values seen in Greyhounds. Serum SDMA concentrations are not influenced by lean body mass[10,31] and, in the current study, the non-sighthound and Greyhound groups were purposely chosen to be of similar weight and size to reduce any potential 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 the Greyhounds and non-sighthound dogs.

In the non-sighthound dog group, 4 of the 24 dogs had mildly elevated SDMA concentrations of 14µg/dL, which could support early renal disease. However, serum SDMA intermittently reaches concentrations of 14-15µg/dL, and rarely up 16µg/dL, in dogs unaffected by renal disease, and these increases were found to occur more commonly in young dogs.[5] Interestingly, 3 of the 4 dogs with SDMA concentrations of 14µg/dL were 1-2 years of age. We decided to include these dogs in the study since mild increases could indicate normal biological variation, and the goal of this study was not to establish accurate RIs for non-sighthound dogs but rather to use these RIs as a comparison for Greyhound RIs. The fact that non-sighthound dogs studied here have, on average, had significantly lower SDMA than Greyhounds, despite the inclusion of dogs with mildly increased SDMA, strongly supports a breed-specific difference in SDMA concentrations.

There were limitations to this study. International recommendations state the preferred method for establishing RIs is with the use of nonparametric determinations from at least 120 reference individuals.[32,33] In this study, 149 Greyhounds were enrolled; however, 48 Greyhounds were excluded, mostly due to insufficient urine concentrations. This was an unexpected finding and lead to the inclusion of only 101 Greyhounds in the final analysis. We decided to forgo sampling more Greyhounds, as the data demonstrated an approximate Gaussian distribution, and the upper and lower CIs fell within the recommended guidelines.[33,34]

Although we attempted to exclude animals with renal disease on the basis of history, physical examination, and laboratory findings, we did not measure GFR or blood pressure, and the health screen used to determine inclusion eligibility did not include a CBC. Therefore, it is possible that some of the dogs included in the study had subclinical disease; especially given that Greyhounds are prone to renal disease and hypertension.[14,15] By following ASVCP guidelines for establishing RIs as closely as practically possible, the presence of some animals with subclinical disease should have minimal impact on the tolerable level of uncertainty.

A further limitation is that serum samples in this study underwent both a freeze-thaw cycle before analysis and were stored for up to 3 months at -80°C. The SDMA™ immunoassay has been validated for stability, with performance metrics within FDA guidelines.[16] Studies evaluating the effects of short-term storage and freezing on SDMA using LC-MS showed no significant effect of storage time on SDMA concentrations in samples stored for 14 days at 4°C.[5,35] Additionally, no significant differences were found when samples were subjected to 3 freeze-thaw cycles when compared with unfrozen samples.[5] There are currently no published studies evaluating the long-term stability of SDMA in frozen serum samples; however, anecdotal evidence supports stability for at least 5 years

when frozen at -80°C (IDEXX, Laboratories, Inc., Personal Communication).[36] Furthermore, in this study, the storage protocol and timespan were similar for the Greyhound and non-sighthound dog samples (data not shown), suggesting sample storage was not a factor in the differences between groups.

Of all the sighthound breeds, only Greyhounds were included in this study. Among the sighthounds, hematologic and biochemical RIs show some similarities but also significant differences;[24,37,38] and therefore, the RIs established in this study should not be extrapolated to other sighthounds. In addition, actively racing Greyhounds were excluded from this study. Further work is required to determine these group-specific RIs.

In conclusion, the RI for serum SDMA was established from 101 healthy Greyhound dogs and was significantly higher than that of non-sighthound dogs of a similar weight, age, and sex. We, therefore, propose that, when assessing SDMA, this breed-specific RI should be adopted for non-racing Greyhounds.

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Table 1 . Summary of population characteristics for Greyhounds and non-sighthound dogs

Category		*Non-sighthound* n = 24	*Greyhound* n = 101
Age in years:	mean (SD)	4.5 (2.4)	4.0 (2.2)
Sex			
	Male intact, n (%)	2 (8.3)	12 (11.9)
	Male neutered, n (%)	10 (41.7)	44 (43.6)
	Female intact, n (%)	5 (20.8)	19 (18.9)
	Female neutered, n (%)	7 (29.2)	26 (25.7)

Weight (kg)	mean (SD)	31.2 (5.2)	31.7 (3.8)

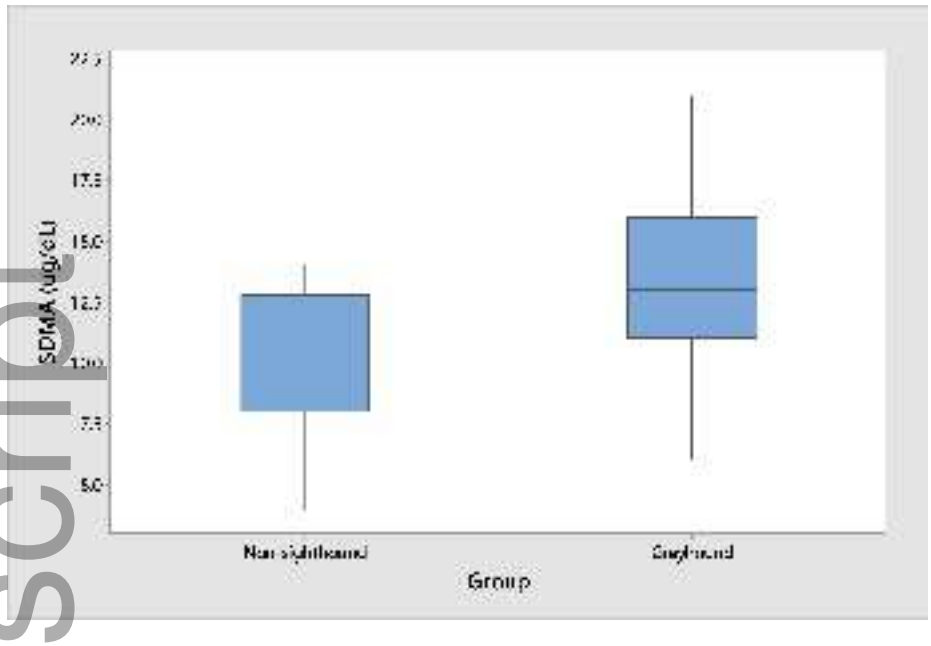
n= number SD= standard deviation

Table 2. Summary of laboratory results for Greyhounds and non-sighthound dogs

Category	Non-sighthound n= 24	Greyhound n=101	Adult canine RIs[8] n= 122
SDMA (µg/dL) mean (SD)	10.2 (2.9)	13.1 (3.4)	N/R
Estimated lower (2.5%) and upper (97.5%) limits (µg/dL)	N/A*	6.3-19.9	6-13
90% CI for lower limit (µg/dL)	N/A*	5.5-7.2	N/R
90% CI for upper limit (µg/dL)	N/A*	18.9-20.8	N/R
Range (µg/dL)	4.0-14.0	6.0-21.0	5-17
Serum creatinine (µmol/L) mean (SD)	87.5 (19.2)	126.0 (14.0)	N/R
Range (µmol/L)	48.0-119.0	89.0-161.0	44.2-141.4
PCV (L/L) median	44.0	52.0	N/R
Range	37-55	37-67	
TS (g/L) median	6.7	6.0	N/R
Range	60-81	52-81	

CI = confidence interval; N/A= not assessed; N/R= not reported.

**The non-sighthound dog group was too small to accurately calculate the reference interval.*



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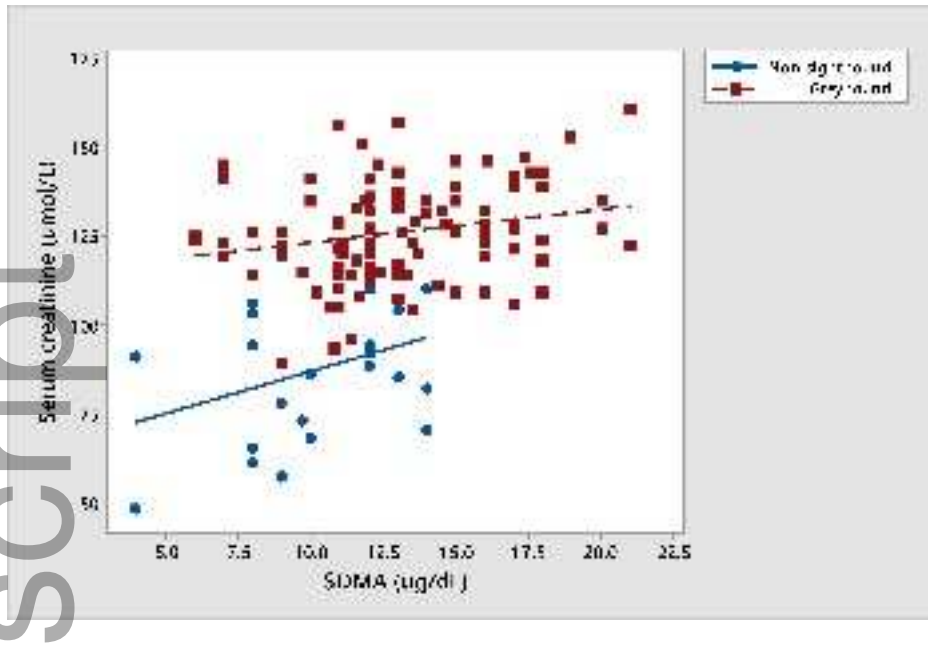

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