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ABSTRACT

Serum symmetric dimethylarginine (SDMA) and patterns of urinary protein separated by sodium dodecyl sulfate agarose gel electrophoresis (SDS-AGE) have not been investigated as biomarkers in dogs with ACTH-dependent hyperadrenocorticism (ADHAC). This exploratory prospective study aimed to evaluate SDMA, serum creatinine (sCR), and SDS-AGE in dogs with ADHAC with and without proteinuria (ADHAC-P and ADHAC-nP, respectively). Thirty-five pet dogs classified as ADHAC-P (n=16), ADHAC-nP (n=6) and healthy (n=13) were included. Renal biomarkers were evaluated in all dogs at diagnosis. Baseline concentration of SDMA was not significantly different between the three groups (P = 0.15) whereas sCr was significantly lower in dogs in ADHAC dogs compared to healthy dogs (88.0 µmol/L [70.4–132.6; 79.2–114.4]) whether they had proteinuria or not (P = 0.014 and 0.002, respectively). However, baseline concentrations of sCr and SDMA were not significantly different between dogs with ADHAC-P dogs (SDMA, 8 µg/dL [5–12; 7–9]; sCr, 57.2 µmol/L [35.2–212.2; 52.8–92.4]) and ADHAC-nP dogs (SDMA, 8.5 µg/dL [7–13; 8–10]; sCr, 70.4 µmol/L [61.6–79.2; 61.6–70.4]) (P = 0.35 and P = 0.41, respectively). Proteinuria in dogs with ADHAC-P was mainly of glomerular origin (SDS-AGE pattern: glomerular in 10/16 dogs; mixed glomerular/tubular in four dogs). In our study, SDMA was neither significantly different in dogs with ADHAC whether they were proteinuric or not, nor between ADHAC and healthy dogs. Urinary electrophoresis provides additional information to the UPC and further investigations are needed to determine whether it may help identify dogs with ADHAC-P requiring specific antiproteinuric treatment.

Introduction

The systemic effects of naturally occurring or iatrogenic hyperadrenocorticism (HAC) in dogs can lead to a variety of medical complications, such as proteinuria and systemic hypertension, which occur in 47–82% (Ortega et al., 1996; Waters et al., 1997; Hurley and Vanden, 1998; Mazzi et al., 2008; Lien et al., 2010; Smets et al., 2012a; Chen et al., 2016) and 31–84% (Ortega et al., 1996; Mazzi et al., 2008; Lien et al., 2010; Smets et al., 2012a; García San José et al., 2020) of dogs, respectively. Glomerulosclerosis and glomerulonephritis have been described in dogs with HAC but the causal relationship remains controversial and the occurrence of renal failure is very rare while tubular and glomerular dysfunction are frequent (Ortega et al., 1996; Mazzi et al., 2008; Lien et al., 2010; Smets et al., 2012a; Smets et al., 2012b).

Serum creatinine concentration (sCr) is the most commonly used indirect marker of glomerular filtration rate (GFR). However, it is an insensitive marker of early decrease in renal excretory function (Finco and Duncan, 1976) and non-renal factors (e.g., body mass) can affect its concentration (Hatton et al., 1989; Hall et al., 2015). Some data suggests

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that serum symmetric dimethylarginine (SDMA) is not affected by lean body mass, age, sex, or exercise (Moesgaard et al., 2007; Hall et al., 2015; Nabity et al., 2015) although it can be negatively associated with the percentage of body fat (Hillaert et al., 2021). Therefore, SDMA could provide additional information in dogs with ACTH-dependent HAC (ADHAC) which are known to have persistent proteinuria but no azotemia. Studies in dogs have also shown that urinary protein electrophoresis using a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) or SDS agarose-based gel (SDS-AGE) is an effective and noninvasive method to localize the origin of urine proteins, based on their molecular weights (Schultze and Jensen, 1989; Yalçin and Çetin, 2004; Zini et al., 2004; Schellenberg et al., 2008; Brown et al., 2010; Giori et al., 2011) as well as to detect glomerular and tubular dysfunction (Hokamp et al., 2018). Various types of glomerular lesions are described in dogs with HAC and dogs receiving glucocorticoid treatment (Smets et al., 2010). Use of urinary biomarkers has already been described in dogs with spontaneous HAC and suggested a proteinuria of mixed origin (Smets et al., 2012b). However, urinary markers there described are less available than urinary SDS-AGE. We therefore hypothesized that urinary SDS-AGE would also suggest the presence of tubular and glomerular dysfunction. Furthermore, GFR measurement using plasma endo-iohexol was higher in animals with Cushing's syndrome than in clinically healthy control dogs (Smets et al., 2012b; Smets et al., 2012c). Considering the inverse relationship between iohexol clearance and SDMA in non-azotemic dogs (McKenna et al., 2020), our hypothesis was that SDMA may be lower in ADHAC dogs compared to control dogs.

Serum SDMA and electrophoresis of urinary proteins might therefore be valuable in assessing the presence of renal dysfunction in dogs with spontaneous ADHAC.

The aims of the current exploratory study were (1) to describe SDMA concentrations in dogs with ADHAC with and without renal proteinuria at the time of diagnosis, using healthy dogs as a control group; (2) to evaluate patterns of proteinuria obtained with urinary protein SDS-AGE in dogs with ADHAC.

Materials and methods

Inclusion and group allocation

Dogs were prospectively enrolled at three French veterinary centres (Alfort Veterinary Teaching Hospital, Toulouse Veterinary Teaching Hospital, and Micen Vet Veterinary Clinic) from September 2017 to July 2019. The study protocol was approved by an institutional ethical committee (ComERC n°2017–02–04, approved on 13 April, 2017 [Ethics Committee of the Ecole Nationale Vétérinaire d'Alfort]) and informed owner consent was obtained for each dog.

Dogs were categorized into three groups: dogs with ADHAC and no renal proteinuria (ADHAC-nP), dogs with ADHAC and renal proteinuria (ADHAC-P), and healthy control dogs.

Dogs with ADHAC

Suspicion of ADHAC was based on history, physical findings, and biochemical changes. The diagnosis of ADHAC complied with the American College of Veterinary Internal Medicine (ACVIM) consensus statement (Behrend et al., 2013). Dogs were included into this group if they had (1) three or more clinical signs/physical examination findings consistent with HAC (2) two or more clinicopathologic findings consistent with HAC and (3) if the results of a low-dose dexamethasone (Dexadreson, MSD Animal Health, Beaucouzé, France) suppression test (LDDST), or an ACTH (Synacthene, Alfasigma, Issy-les-moulineaux, France) stimulation test, or both, exceeded the upper value of the reference range (40 nmol/L for the cortisol concentration at 8 h for the LDDST, and 600 nmol/L for the post-ACTH cortisol concentration, respectively). Dogs with concurrent diseases such as diabetes mellitus, hypothyroidism, or diagnosed with any neoplastic disease were excluded as well as dogs previously known to have chronic kidney disease. A dog was diagnosed with ADHAC if at least two of the following criteria were present: a LDDST or high-dose dexamethasone suppression test result consistent with ADHAC (cortisol concentration at 4 h suppressed to < 40 nmol/L, or $\geq 50\%$ suppression of baseline cortisol concentration at 4 h or 8 h, respectively), plasma ACTH concentration $>$ 1.1 pmol/L (measured using Immulite ACTH, a 2-site solid-phase chemiluminescent immunometric assay) (Rodríguez Piñeiro et al., 2009), symmetric adrenal glands (on abdominal ultrasound or computed tomography [CT] scan), or presence of a pituitary mass identified by CT scan or magnetic resonance imaging (Benchekroun et al., 2010; Rodríguez Piñeiro et al., 2011).

Dogs were categorized as ADHAC–P or ADHAC-nP based on the presence or absence of persistent renal proteinuria. Renal proteinuria was defined as the presence of a urine protein to creatinine ratio (UPC) $>$ 0.5 on at least two samples collected 2 weeks or more apart, and no signs of hematuria, bacteriuria, or pyuria (evaluated by urinary dipstick, urine sediment examination, and urine culture). Dogs in the ADHAC-P group were further categorized into those with mild proteinuria (UPC $>$ 0.5 and < 2) and those with marked proteinuria (UPC ≥ 2) (Littman et al., 2013). Dogs in the ADHAC–nP group were defined as dogs with UPC $<$ 0.2. Descriptive and statistical analyses focusing on the UPC was conducted based on the results from the urine sample obtained at the time of inclusion, corresponding at the date of the diagnostic test performed.

Healthy control dogs

Dogs $>$ 6 years old were recruited among Alfort Veterinary School students and staff members, or their families. Health was defined by the owner's perception, the absence of relevant abnormalities detected by physical examination and hematologic, biochemical, and urine laboratory analyses, and a blood cortisol concentration ≤ 40 nmol/L at 8 h on LDDST.

Exclusion criteria

In all groups, dogs were excluded if they received within the last month before initial presentation any medication that could potentially affect renal function (e.g., trilostane, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers, or nonsteroidal anti-inflammatory drugs). Dogs with cardiac disease (other than that classified as stage A or B1 according to the ACVIM guidelines) were also excluded (Atkins et al., 2009). Dogs with borderline proteinuria (UPC between 0.2 and 0.5) and dogs with pyuria, hematuria, or bacteriuria were excluded. Dogs were also excluded if urine analysis at baseline could not be performed or if they received corticosteroids of any form within the last 3 months.

Initial exploration

All dogs underwent a thorough investigation including history, physical examination findings, systolic blood pressure (sBP) measurement, biochemical analyses, and urinalysis (urinary specific gravity [USG], urinary dipstick, sediment analysis, UPC, urine culture, and urinary protein SDS-AGE).

Systolic blood pressure measurement

Systolic blood pressure was measured in all dogs using Doppler method (Parks Medical Electronics Inc, Aloha, Oregon, United States of America) according to the ACVIM guidelines (Acierno et al., 2018). Systemic hypertension was defined as mild, moderate, or severe according to ACVIM guidelines (Acierno et al., 2018). Fundus examination was performed when sBP was ≥ 159 mmHg.

Blood analysis

Blood samples were collected in serum separator tubes, and centrifuged within 1 h of collection. The serum was aliquoted and stored at

−80°C until further analysis. The SDMA concentration was determined using a commercially available high-throughput immunoassay which was previously and extensively validated (IDEXX SDMA Test, IDEXX Laboratories, Inc.) (Relford et al., 2016). Creatinine, urea nitrogen, ALT, ALP, GGT, AST, sCr, total bilirubin, cholesterol, triglycerides, globulin, albumin, glucose, inorganic phosphate, Na, K, Cl, and tCa concentrations were measured by IDEXX Laboratories (Ludwigsburg, Baden-Württemberg, Germany, Beckman Coulter AU5800 Series Chemical Analyzer). Creatinine concentration was measured by Jaffe's reaction, a colorimetric method using picrate at alkaline pH. These samples were analysed in 6 successive batches throughout the study period. There was no change of instruments or methods over the whole study period.

Urinalysis

All urine samples were collected by ultrasound-guided cystocentesis shortly after the dog had arrived at the hospital. The USG was measured with a portable refractometer (FG-312, Index Instruments, Kissimmee, United States of America, measuring range: 1.000–1.050) and urinary dipstick (Multistix10 SG, Siemens Healthcare Diagnostics Inc, Tarrytown, United States of America) was manually evaluated. A sample from the urine collected was submitted for bacterial culture in all dogs with ADHAC. The leftover was then centrifuged and the sediment was examined with a light microscope. The urine supernatant was stored immediately at −80°C until shipment to the laboratory for measurement of UPC and SDS-AGE of urinary proteins. These samples were analyzed in 2 batches.

Urinary proteins and creatinine concentrations were measured by pyrogallol red and kinetic Jaffé methods, respectively, using an automated analyzer (Indiko plus, ThermoFisher Scientific, Waltham, Massachusetts, United States of America) and UPC was calculated. A single urine sample was used for UPC determination in all dogs. Dogs with UPC ≥ 0.5 and no signs of inflammatory urinary tract disease were classified as proteinuric according to the International Renal Interest Society (IRIS) guidelines (IRIS Staging of CKD, 2023).

After the determination of UPC, urine samples were analyzed by SDS-AGE under non-reducing conditions with a semi-automated system (Hydrasys, Sebia Italia SRL, Italy), and interpreted as previously described (Lavoué et al., 2015). The urinary protein SDS-AGE pattern was considered physiological if there were no bands visible, if the only band visible was an albumin band of weak intensity (score of 1), or if, in samples from entire males, the only band present was a tubular band of approximately 25 kDa (compatible with arginine esterase prostatic protein) (Hokamp et al., 2018; Schellenberg et al., 2008; Théron et al., 2017). Proteinuria was considered to be of tubular origin if a low-molecular-weight protein band (LMWb) was detected (excepting a single tubular band of approximately 25 kDa in entire males), and of glomerular origin if there was an intense albumin band (score 2), or a high-molecular-weight protein band (HMWb). When both an LMWb and HMWb or LMWb and a score-2 albumin band were observed, proteinuria was considered to be of mixed origin.

Statistical analysis

Numerical data were reported as median [min-max; 1st quartile – 3rd quartile] and compared using nonparametric tests. Statistical analyses were performed using commercially available software (SAS University edition 9.04.01M6P11072018). Medians of continuous clinicopathological variables were first compared between ADHAC-P, ADHAC-nP, and healthy dog groups using the Kruskal-Wallis test. If significant differences between the three groups were observed (P of the Kruskall-Wallis test <0.05), pairwise comparisons were then conducted with the Mann-Whitney U test. Performing a Kruskal-Wallis test helps to circumvent the need for p-value correction for multiple tests, so the significance level was also set at $\alpha = 0.05$ (Bender and Lange, 2001). P-values of the Mann-Whitney test were further noted P_a for the test comparing ADHAC-nP and healthy groups, P_b for the test comparing ADHAC-P and healthy groups and P_c for the test comparing ADHAC-P and ADHAC-nP groups.

The correlation between SDMA and sCr was evaluated through the Spearman correlation test. Outliers were not excluded from the statistical analysis. The significance level was set at $\alpha = 0.05$. Due to the exploratory nature of the study and the lack of prior data on the variation of SDMA or SDS AGE pattern in dogs with ADHAC leading to an unknown effect size, a statistical power analysis was not performed.

Results

Study population

Sixty nine dogs were screened for study eligibility. Four dogs were excluded from the healthy group because cortisol concentration 8 h after an LDDST was $>$ 1.4 μg/dL, and eight were excluded because of abnormalities in their biochemical profile. Eight dogs were excluded because they could not be accurately classified as dogs with ADHAC. Nine dogs were excluded either because no urine could be collected or because bacteriuria was present, and five were excluded because of borderline proteinuria. A total of 35 dogs were enrolled, including 22 dogs with ADHAC (16 with ADHAC-P and six with ADHAC-nP) and 13 healthy control dogs. Hydration status was normal in all dogs. Signalment and endocrine test results are presented in Table 1.

Baseline clinical characteristics

The median duration of clinical signs was not significantly different between dogs with ADHAC-P and dogs with ADHAC-nP (6 months [2–24; 3–13] and 4 months [1–12; 1–9], $P = 0.22$).

Baseline clinical chemistry data are presented in Table 2. Serum creatinine concentration was significantly different between the three groups ($P = 0.007$) and was lower in ADHAC dogs compared to healthy dogs whether they had proteinuria or not ($P_b = 0.014$ and $P_a = 0.002$, respectively) but not between ADHAC-P and ADHAC-nP dogs ($P_c = 0.35$) (Fig. 1). Serum SDMA concentration was not significantly different between the three groups ($P = 0.15$). Serum phosphate was significantly different ($P < 0.001$) between the three groups and was significantly higher in ADHAC-P dogs than in healthy dogs ($P_a < 0.001$) but was not significantly different between ADHAC-P and ADHAC-nP dogs ($P_c = 0.09$) and nor between ADHAC-nP and healthy dogs ($P_b = 0.24$). Baseline sBP, routine urinalysis data, UPC, and urinary protein SDS-AGE pattern results are presented in Table 3 and Fig. 1. The sBP was significantly different ($P = 0.013$) between the three groups and was significantly higher in ADHAC-P dogs than in healthy dogs (P_b=0.002) but not between ADHAC-P dogs and ADHAC-nP dogs ($P_c = 0.48$) or between ADHAC-nP and healthy dogs ($P_a = 0.15$). sBP was $\geq$ 160 mmHg in 2/6 dogs with ADHAC-nP and in 8/14 dogs with ADHAC-P. None of these dogs had abnormal findings from the fundus examination.

SDMA and creatinine in dogs with ADHAC, with and without proteinuria

Serum creatinine concentration and SDMA were not significantly different between these two groups ($P_c = 0.35$ and $P_c = 0.41$, respectively). There were no significant differences between dogs with ADHAC-nP, dogs with ADHAC-P (mild), and dogs with ADHAC-P (severe) in the baseline concentrations of sCr ($P = 0.47$), urea nitrogen ($P = 0.78$), SDMA ($P = 0.56$), or phosphate ($P = 0.19$), USG ($P = 0.72$) or sBP ($P = 0.62$) (Table 3). SDMA and sCr were poorly correlated in dogs with ADHAC ($r = -0.07$, $P = 0.74$).

Proteinuria severity and pattern in dogs with ADHAC

In dogs with ADHAC-P, UPC was 2.95 [0.60–13.0; 1.6–6.2]. Seven dogs were classified as having mild proteinuria and nine had severe proteinuria.

Table 1
Signalment and endocrine test results for healthy control dogs and dogs with ADHAC. Data are median [minimum-maximum; 1st quartile-3rd quartile].

		Healthy control dogs	Dogs with ADHAC-nP	Dogs with ADHAC-P
		(n = 13)	(n = 6)	(n = 16)
Age, years		7.5 [6.0–9.5; 7.0–8.0]	11.4 [8–17; 10–14.0]	10.6 [5.6–12.0; 8.0–11.1]
Body weight (kg)		22.2 [4.2–32.0; 10.0–26.4]	11.4 [4.4–33.4; 6.7–14.0]	8.4 [5.6–34.0; 7.2–11.5]
Body condition score, scale 1–9		N/A	5.5 [3.0–7.0; 4.0–6.0]	5.0 [4.0–9.0; 5.0–7.0]
Breed (n)		Crossbreed (5), Australian Shepherd (3), Boxer, Labrador, Malinois Shepherd, Shetland, Dachshund (1 each)	Jack Russel Terrier, French Bulldog, Bichon Frisé, Cross shepherd dog, Whippet, Lhassa Apso (1 each)	Poodle (3), Cavalier King Charles (2), Crossbreed (2), Yorkshire Terrier, Jack Russel Terrier, Bichon Frisé, German Shepherd, Beagle, Spitz, Shi Tzu, Maltese dog, West Highland White Terrier (1 each)
Sex		9 female (6 N,3I), 4 male (3 N,1I)	4 female (1 N,3I), 2 male (2 N,0I)	9 female (5 N,4I), 7 male (2 N,5I)
Duration of clinical signs, months		N/A	4.0 [1.0–12.0; 1.0–9.0]	6.0 [2.0–24.0; 3.0–13.0]
LDDST	Number of dogs tested	13	6	11
	Basal cortisol, nmol/L (RI: 100–300)	63 [9–209; 34–121]	210 [146–281; 149–269]	160 [76–249; 86–215]
	Cortisol at 4 h, nmol/L (RI: < 40)	N/A	50 [22–258; 24–94]	74 [12–250; 44–225]
	Cortisol at 8 h, nmol/L (RI: < 40)	3 [2–25; 3–4]	162 [63–266; 97–177]	88 [44–343; 77–171]
ACTH-stimulation test	Number of dogs tested	0	0	5
	Basal cortisol, nmol/L (RI: 100–300)	N/A	N/A	191 [112–289; 172–225]
	Cortisol at 1 h, nmol/L (RI: < 600)	N/A	N/A	1047 [774–2623; 1 025–4 657]

ADHAC, ACTH-dependent hyperadrenocorticism; ADHAC-nP, ACTH-dependent hyperadrenocorticism without proteinuria; ADHAC-P, ACTH-dependent hyperadrenocorticism with proteinuria; I, intact; LDDST, low-dose dexamethasone suppression test; N, neutered; N/A, not available; RI: reference interval

Illustration of various SDS-AGE pattern are represented in Fig. 2. Urinary protein SDS-AGE showed a physiological pattern in two of 16 (12.5%) dogs with ADHAC-P. These two dogs were mildly proteinuric, (UPC of 0.6 and 1.1). A glomerular pattern was found in 10 of 16 (62.5%) dogs with ADHAC-P. A mixed glomerular/tubular pattern was found in four of 16 (25%) dogs with ADHAC-P. Among these dogs, all had an intense albumin band, 12 had a HMWb and four had a LMWb. No purely tubular patterns were identified. Among the 16 dogs with ADHAC-P, one had mild hypoalbuminemia (24 g/L [reference range, 28–43 g/L]); the UPC was 7.5. Among dogs with ADHAC-P and mild proteinuria, 4 (57%) had a glomerular, 1 (14%) had a mixed, and 2 had a physiologic (29%) SDS-AGE pattern. Among dogs with ADHAC-P and severe proteinuria, 6 (66%) had a glomerular and 3 (33%) had a mixed SDS-AGE pattern (Table 3).

Discussion

This is the first reported study of SDMA concentration and urinary protein SDS-AGE in dogs with ADHAC. The main findings were that SDMA concentration was not significantly different between healthy dogs and dogs with ADHAC, either proteinuric or not. sCr was lower in dogs with ADHAC compared to healthy control dogs. In ADHAC–P dogs, especially those with severe proteinuria (UPC >2), a glomerular SDS-AGE pattern was seen more frequently.

Lower sCr concentrations in dogs with ADHAC compared to healthy dogs were consistent with findings from previous studies (Pérez-Sánchez et al., 2023). It might be explained by a decreased muscle mass, a higher GFR, or both in dogs with ADHAC (Smets et al., 2012b). However, in our study, healthy dogs were younger and their body weight was higher than in dogs with ADHAC. It is therefore possible that the observed statistical difference for sCr in ADHAC compared to healthy dogs may be attributable to age or body weight rather than to muscle mass or GFR, as sCr is also affected by these factors (Braun et al., 2003; Craig et al., 2006). Ideally, the body condition score in healthy dogs should have been recorded, and they should have been age and weight-matched to dogs with ADHAC, and the muscle condition score assessed in all dogs.

The lack of significant difference in SDMA concentration at baseline between dogs with ADHAC and healthy dogs may be due to the small study population. However, it is possible that in dogs with ADHAC, the correlation between GFR and SDMA is lower than that of GFR and sCr, as suggested by recent observations in a study of healthy Beagles receiving immune-suppressive doses of steroids (Mantelli et al., 2022). Moreover, a previous study questioned the diagnostic value of SDMA in non-azotemic dogs with reduced GFR compared to sCr (McKenna et al., 2020). Interestingly, in our population of dogs with ADHAC, SDMA, and sCr were poorly correlated. Since glucocorticoids have various impacts on intracellular metabolism, increased endogenous production of SDMA due to glucocorticoid influence might be accountable for the contrast between SDMA and sCr in dogs with ADHAC. Such a phenomenon has not been investigated yet. Another possible explanation for the lower baseline sCr concentration associated with a non-significantly different serum SDMA concentration in dogs with ADHAC is that sCr changes are secondary to a smaller body mass rather than to a higher GFR (Smets et al., 2012a).

Most (16/22) dogs with ADHAC were proteinuric. In these dogs, UPC ranged from 0.6 to 13.9, which is in accordance with previous data (Smets et al., 2012b; Vidal et al., 2018). Urinary protein electrophoresis can be a valuable technique to investigate proteinuria in dogs. Indeed, a recent study that scored the intensity of electrophoretic bands showed SDS-PAGE to have a high sensitivity and specificity for both glomerular (97% and 100%, respectively) and tubular lesions (90% and 100%, respectively) as compared with a histopathological evaluation (Hokamp et al., 2018). Although SDS-AGE has been reported as highly sensitive but poorly specific to detect renal histological lesions in 2004 (Zini et al., 2004), no study has compared the diagnostic performance of renal histopathology with SDS-AGE being evaluated with a scoring system (such as the one adopted in the present study or the one previously described for SDS-PAGE [Hokamp et al., 2018]). In our study, 10/16 dogs with

Table 2
Baseline serum biochemistry results, systolic blood pressure, Urine specific gravity and urine protein creatinine ration in healthy control dogs and dogs with ADHAC. Data are median [minimum-maximum; 1st quartile-3rd quartile].

	Reference interval	Healthy control dogs	Dogs with ADHAC-nP	Dogs with ADHAC-P	P value
		($n = 13$)	($n = 6$)	($n = 16$)	
SDMA, µg/dL	0.0–14.0	9.0 [7–13; 8–12.0]	8.5 [7.0–13.0; 8–10]	8.0 [5.0–12.0; 7–9]	$P = 0.15$
Creatinine, µmol/L	44.0–133.0	88.0[a, b] [70.4–132.6; 79.2–114.4]	70.4[a] [61.6–79.2; 61.6–70.4]	57.2[b] [35.2–211.2; 52.8–92.4]	$P = 0.007$ $P_a = 0.002$ $P_b = 0.014$
Urea nitrogen, mmol/L	3.2–10.3	5.6 [3.3–8.7; 4.5–6.2]	6.2 [3.9–7.1; 5–7]	4.9 [3.5–19.5; 4.6–7.3]	$P = 0.76$
Inorganic phosphate, mmol/L	0.9–1.7	1.2[b] [0.6–1.7; 1.1–1.3]	1.3 [1.1–1.9; 1.2–1.8]	1.6[b] [1.2–3.6; 1.45–1.95]	$P < 0.001$ $P_a < 0.001$
Calcium, mmol/L	2.1–2.9	2.4 [2.2–2.7; 2.4–2.5)	2.6 [2.0–2.8; 2.5–2.8]	2.5 [1.7–2.9; 2.5–2.7]	$P = 0.12$
Potassium, mmol/L	3.9–5.8	4.6[a, b] [4.1–5.1; 4.2–4.7]	5.0[a] [4.7–6.7; 4.7–5.7]	4.9[b] [4.1–6.1; 4.6–5.2]	$P = 0.007$ $P_a = 0.007$ $P^b = 0.014$
Sodium, mmol/L	142.0–153.0	148 [143–153; 146–150)	152 [146–153; 149–153]	148 [143–152; 147–150]	$P = 0.1$
ALT, UI/L	0.0–122.0	49[a, b] [21–68; 33–59]	163[a] [50–485; 95–365]	145[b] [25–1396; 65–269]	$P = 0.003$ $P_a = 0.002$ $P_b < 0.001$
ALKP, UI/L	0.0–147.0	34[a, b] [19–79; 29–41]	504[a] [51–1082; 270–829]	368[b] [42–1012; 129–864]	$P < 0.001$ $P_a = 0.002$ $P_b = 0.005$
Total protein g/L	54.0–76.0	59 [56–70; 58–63]	66 [55–78; 62–70]	64 [55–71; 60–66]	$P = 0.29$
Albumin g/L	28.0–43.0	32 [29–41; 30–33]	34 [26–40; 30–38]	32 [24–35; 31–33]	$P = 0.73$
Glucose mmol/L	3.2–7.0	5.3 [4–6.5; 5.2–6.2]	5.6 [5.4–6.3; 5.5–6.1]	5.5 [3.4–6.6; 5.2–6]	$P = 0.74$
Systolic blood pressure, mmHg	110.0–140.0	120[b] [110–145; 120–140]	143 [110–170; 130–170]	160[b] [120–180; 135–170]	$P = 0.013$ $P_a = 0.002$
USG	1.025–1.050	1.050[a, b] [1.024–1.050; 1.042–1.050]	1.013[a] [1.005–1.050; 1.007–1.025]	1.014[b] [1.004–1.048; 1.011–1.020]	$P < 0.001$ $P^a = 0.01$ $P_b < 0.001$
UPC	< 0.2	0.04 [0.02–0.09; 0.03–0.05]	0.10 [0.04–0.19; 0.08–0.15]	2.95 [0.60–13.9; 1.6–6.2]	/

[a,b] Significant differences between groups are shown with the same superscript symbols
ADHAC-nP, ACTH-dependent-hyperadrenocorticism without proteinuria; ADHAC-P, adrenocorticotropic hormone-dependent hyperadrenocorticism with proteinuria; SDMA, symmetric dimethylarginine.

ADHAC-P displayed a urinary protein SDS-AGE glomerular pattern (n=10/16) and only four displayed a mixed pattern. The presence of glomerular lesions in dogs with ADHAC-P is consistent with several studies in dogs with experimental or spontaneous HAC (Littman et al., 1988; Waters et al., 1997; Smets et al., 2012c). However, the low prevalence of a mixed pattern (4/16) and absence of a tubular pattern was partly unexpected as previous studies suggest that dogs with ADHAC have both tubular and glomerular dysfunction (Smets et al., 2012a; Smets et al., 2012b). Whether this finding is related to the chronicity or severity of HAC in our population is unknown.

SDS-AGE evaluation with a specific scoring system may therefore be complementary to the determination of UPC in dogs with ADHAC, as it may help to identify dogs with kidney lesions. Indeed, UPC measurement is unfortunately prone to high biological and analytical variations (Nabity et al., 2007; Rossi et al., 2016). Thus, dogs in our study with UPC close to 0.5 might may have had only transient or borderline proteinuria leading to possible misclassification of dogs with renal proteinuria (especially in proteinuric dogs with physiological SDS-AGE pattern). On the other end, a previous study including dogs with endocrine disease reported that glomerular proteinuria was the most frequent electrophoretic pattern (58.4%) but only 13.3% of dogs had UPC value of 2.0 or higher, a value considered to be the representative cut-off of glomerular dysfunction. This may highlight the utility of electrophoresis to identify the presence of glomerular dysfunction in initial or less severe stages, where, despite a UPC<2.0, damage is present at glomerular level (Pérez-Sánchez et al., 2023). In dogs with ADHAC-P, renal biopsy is rarely performed, and other renal biomarkers, such as urinary protein electrophoresis, are likely to offer diagnostic benefits in identifying dogs with more severe renal lesions that might require specific anti-proteinuric treatment.

Systemic arterial hypertension is a well-recognized complication of canine HAC, and the prevalence of systemic hypertension in dogs with ADHAC-P from our population was comparable to previous studies (García San José et al., 2020). Among our cohort, dogs with ADHAC-P presented significantly higher systolic blood pressure (sBP) compared to healthy dogs. There is an established association between systemic hypertension and kidney injury (García San José et al., 2020), making it challenging to ascertain whether glomerular damage and subsequent proteinuria are causative factors or consequences of systemic arterial hypertension in canine HAC. Interestingly, serum phosphate was significantly higher in ADHAC-P dogs than in healthy dogs. It's well known that serum phosphate concentration tends to be increased in dogs with HAC compared with healthy dogs and may be a negative prognostic factor (Fracassi et al., 2015). However, the higher magnitude of hyperphosphatemia in ADHAC-P dogs might suggest a potential link between increased phosphatemia and proteinuria. In one study, proteinuria was shown to increase serum phosphate concentration, independently of GFR, by altered tubular handling and decreased effect of FGF23 on NaPiIIa transporter (De Seigneux et al., 2015).

Limitations of this study include the small population size of dogs with ADHAC and of healthy control dogs. Applying strict exclusion criteria enabled us to ensure accurate group allocation, at the expense of reducing the population size. As mentioned earlier, this study is exploratory, and further studies based on statistical power calculation

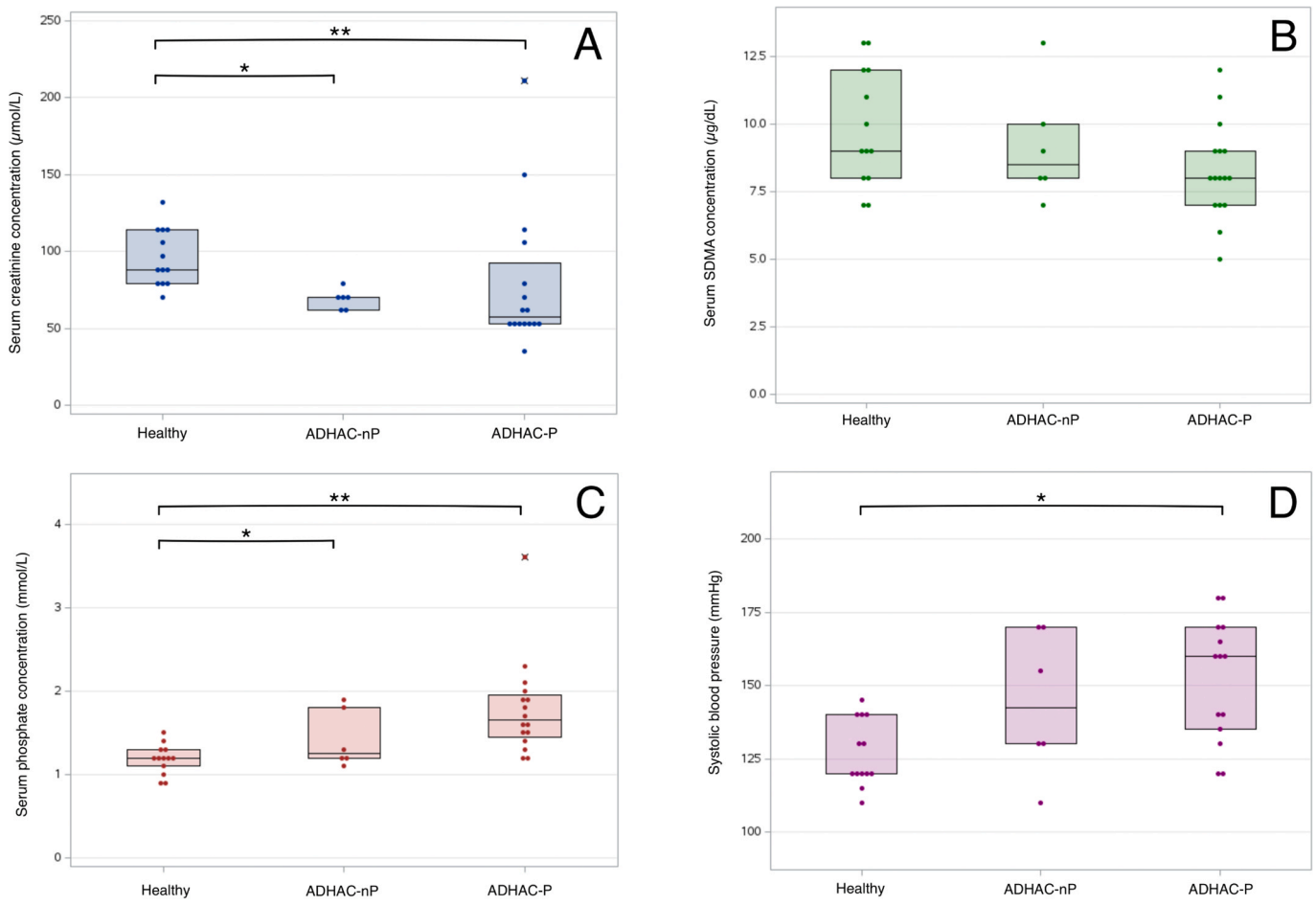

Fig. 1. Distribution of serum creatinine concentration (A), serum symmetric dimethylarginine (SDMA) concentration (B), serum phosphate concentration (C) and systolic blood pressure (D), in healthy dogs, dogs with ACTH-dependent hyperadrenocorticism (ADHAC) without proteinuria (ADHAC-nP) and dogs with ADHAC with proteinuria (ADHAC-P). The bottom and top of the box indicate the 25th and the 75th percentile, respectively, and the horizontal line inside the box shows the median of the distribution. Dots represent individual observations and crosses represent outliers. *: significantly different.

Table 3
Proteinuria magnitude and SDS-AGE pattern in healthy control dogs and dogs with ADHAC. Data are median [minimum-maximum; 1st quartile-3rd quartile].

Variable	Reference interval	Dogs with ADHAC-nP	Dogs with ADHAC-P mild	Dogs with ADHAC-P severe	P
		n = 6	*n* = 7	*n* = 9	
SDMA, µg/dL	0.0–14.0	8.5 [7.0–13.0; 8–10]	8.0 [7.0–10.0; 7.0–8.0]	9.0 [5.0–12.0; 7.0–9.0]	$P = 0.56$
Creatinine, µmol/L	44.0–133.0	70.4 [61.6–79.2; 61.6–70.4]	61.6 [52.8–211.2; 52.8–105.6]	52.8 [35.2–149.6; 52.8–70.4]	$P = 0.47$
Systolic blood pressure, mmHg	110.0–140.0	143 [110–170; 130–170]	148 [120–180; 130–165]	160 [120–180;140–170]	$P = 0.62$
UPC	< 0.2	0.10 [0.04–0.19; 0.08–0.15]	1.51 [0.6–1.94; 1–1.76]	6.01 [2.57–13.89; 3.89–7.48]	/
Pattern of urinary protein SDS-AGE					
Glomerular, *n*=		0	4	6	
Tubular, *n*=		0	0	0	
Mixed, *n*=		0	1	3	
Physiologic, *n*=		6	2	0	

ADHAC, ACTH-dependent hyperadrenocorticism; ADHAC-nP, ACTH-dependent hyperadrenocorticism without proteinuria; ADHAC-P, ACTH-dependent hyperadrenocorticism with proteinuria; n: number of dogs with the indicated result for a variable; n, number of dogs; UPC, urine protein to creatinine ratio; USG, urine specific gravity.

Figure legends

are warranted. Moreover, the dogs included in the healthy group were younger and larger than ADHAC dogs and a control population with characteristics similar to the ADHAC dogs would have been more relevant. Another limitation of our study is the use of a single measure of UPC rather than an average obtained from urine collected over 3 consecutive days (LeVine et al., 2010) which may have led to wrong group allocation, but is a better reflection of routine clinical practice. We have not measured GFR in our dogs, as the gold standard renal clearance test (Watson et al., 2002) is not always available and may be difficult to perform in clinical settings. The relationship between SDMA

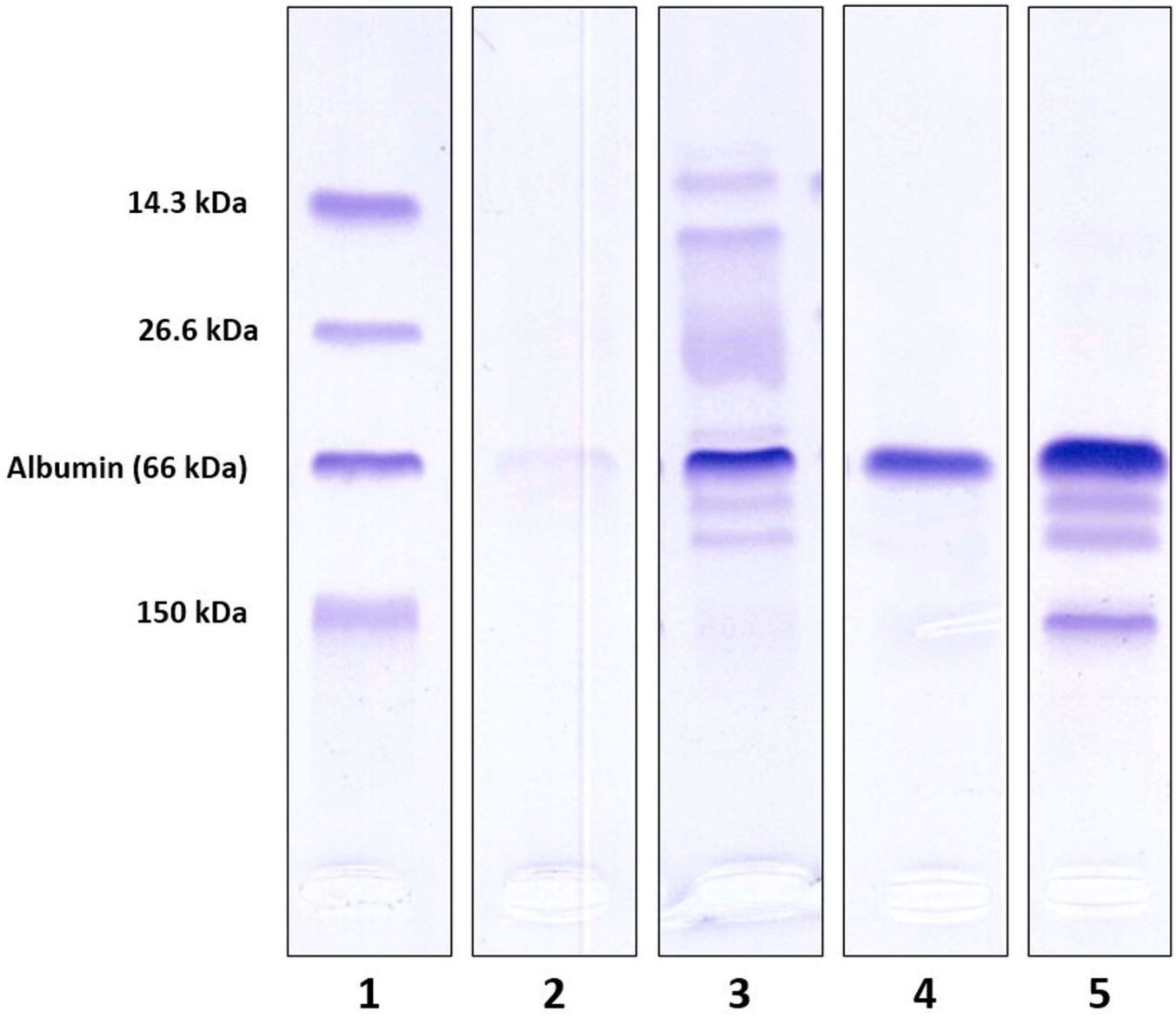

Fig. 2. Illustration of various SDS-AGE patterns obtained in ADHAC dogs. Lane 1 represents the migration of molecular markers, lane 2 a normal pattern in an ADHAC-nP dog, lane 3 an abnormal mixed pattern in an ADHAC-P dog and lane 4 and 5 an abnormal glomerular pattern from an ADHAC-P dog, one with an intense albumin band but no high molecular weight band (lane 4) and one with an intense albumin band and a high molecular weight band (line 5). The numbers on the left side correspond to molecular weight of molecular markers in kDa.

concentration and GFR in dogs with ADHAC remains to be investigated. Renal histopathology may have provided information about the nature of renal lesions in dogs with ADHAC. However, the risk of complications and cost precluded kidney biopsy. Renal biopsy could have been performed post-mortem, but the results would have been hard to interpret due to interference of the disease duration, effect of treatment with trilostane, and aging. We have therefore evaluated urinary protein electrophoresis as a surrogate indicator for the identification of renal injury in our population. Urinary protein electrophoresis, however, may indicate renal dysfunction that is not necessarily always associated with the presence of histological renal lesions (Hokamp et al., 2018).

Conclusions

Our results show that SDMA is not significantly different in dogs with ADHAC whether they are proteinuric or not and between dogs with HAC and healthy dogs. Urinary electrophoresis in ADHAC dogs with proteinuria indicates mainly signs of glomerular dysfunction and whether it may help to identify dogs with ADHAC-P that need specific anti-proteinuric treatment requires further studies.

Declaration of Competing Interest

Michael Coyne and Rachel Murphy are employees of IDEXX Laboratories, Inc. None of the authors had any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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