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ABSTRACT

The pathogenesis of increased serum phosphate concentration and proteinuria in dogs with spontaneous hyperadrenocorticism (HAC) is unclear. A potential link between proteinuria and calcium/phosphate metabolism has never been studied in dogs with HAC. The aims of the study were: (1) To evaluate calcium/phosphate metabolism in dogs with spontaneous HAC and compare to healthy dogs as well as to dogs with non-HAC illness; (2) to look for associations between markers of calcium/phosphate metabolism and biomarkers of kidney disease in dogs with HAC. Fifty-four dogs were included in the study, classified as HAC (n=27), non-HAC disease (n=17), and healthy (n=10). Serum calcium, phosphate, 25(OH)Vitamin D, 1,25$(OH)_2$Vitamin D, plasma intact parathyroid hormone concentration (iPTH), FGF23, and urinary fractional excretion of calcium and phosphate were evaluated in all dogs at diagnosis and compared between each group. The correlation between these variables and urine protein-to-creatinine ratio (UPC) and urinary N-acetylglucosaminidase-to-creatinine ratio (uNAG/C) was evaluated in the HAC group. Medians [range] of serum phosphate concentration, urinary fractional excretion of calcium (FE(Ca)), and iPTH were significantly higher in dogs with HAC than in dogs with non-HAC illness (P<0.01) and healthy dogs (P<0.01). Increased 1,25$(OH)_2$Vitamin D/25(OH)Vitamin D was also observed (P<0.001). In HAC group, UPC was significantly negatively correlated with 25(OH)Vitamin D (r(s): -0.54; P<0.01). Urinary NAG/C was significantly positively correlated with serum phosphate (r(s): 0.46; P=0.019). Increased serum phosphate, urinary excretion of calcium, and hyperparathyroidism were observed in dogs with HAC. Vitamin D metabolism may be shifted towards increased 1-alpha hydroxylation.

Introduction

Canine hyperadrenocorticism (HAC) is a common endocrine disease of older dogs and is accompanied by many metabolic disturbances. Among them, complications such as *calcinosis cutis,* internal organ mineralization, and calcium oxalate uroliths (Berry et al., 1994; Berry et al., 1994; Hess et al., 1998) suggest dysregulation in calcium and phosphate homeostasis. Indeed, adrenal secondary hyperparathyroidism has been previously reported in 60–95 % of cases (Ramsey et al., 2005; Corsini et al., 2021) along with increased calciuresis and decreased phosphaturesis (Fracassi et al., 2015a,2015b; Corsini et al., 2021). 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., 2015a,2015b). However, the pathogenesis of these dysregulations remains poorly understood. Hyperadrenocorticism is also associated with alterations in renal function (Smets., 2011; Smets et al., 2012a; Smets et al., 2012b, Marynissen et al., 2016). Impaired urinary concentrating ability, impaired electrolyte handling, and proteinuria of both high and low molecular weights are frequent complications (Mangos et al., 2003; Smets et al., 2010; Smets et al., 2012a) and provide evidence for both glomerular and tubular dysfunction in dogs with HAC.

Fibroblast Growth Factor-23 (FGF23) is a bone-derived phosphaturic hormone and one of the key regulators in phosphate metabolism.

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Increased serum phosphate concentration is an inducer of FGF23 secretion, which then promotes phosphaturia through the inhibition of phosphate reabsorption by the NaPiIIa transporter in the proximal tubule of the nephron. FGF23 also inhibits the 1α-hydroxylase activity, thereby leading to a decrease in calcitriol synthesis (Elliot and Geddes., 2022). In turn, calcitriol stimulates the synthesis and secretion of FGF23 by osteocytes. It is therefore key to assess FGF23 in diseases with calcium/phosphate metabolism disturbances. One recent study reported lower FGF23 concentration in dogs with HAC compared to healthy ones (Corsini et al., 2021). Furthermore, the severity of FGF23 increase correlated to the advancement of chronic kidney disease (CKD) due to decreasing renal phosphate excretion, and has been recognized as a potential prognostic biomarker in dogs with kidney disease (Rudinsky et al., 2018). In another study, aldosterone was suggested as a potential driver of FGF23 secretion in dogs, cats, and humans with CKD (Radloff et al., 2021). As plasma aldosterone concentration is reported to be decreased in dogs with HAC compared to healthy ones (Goy-Thollot et al., 2002), it may explain the lower FGF23 previously reported in dogs with HAC. One study reported increased FGF23 concentration to be independently associated with proteinuria in mice and humans (Vervloet et al., 2012). The kidney is also the primary site where 25(OH) Vitamin D is transformed, catalyzed by 1-alpha hydroxylase into its active form, 1,25$(OH)_2$ Vitamin D. Kidney disease has been linked to vitamin D metabolites disturbances (Miller et al. 2020). Given the role of the kidney in calcium and phosphate homeostasis, renal proteinuria and phosphocalcic metabolism regulators may be related in dogs with HAC. An evaluation of the key players in calcium and phosphate metabolism, along with biomarkers of kidney disease, is currently lacking and could provide a better understanding of the mechanisms leading to these disturbances in dogs with HAC.

The first objective of the study was to further characterize phosphate metabolism in dogs with naturally-occurring HAC, compared with healthy dogs and dogs with other diseases (dogs with non-HAC), by measuring calcium/phosphate metabolism biomarkers. The second objective was to investigate potential correlations between urine biomarkers of kidney dysfunction and lesions (using urine protein-to-creatinine ratio (UPC), urinary protein electrophoresis using a sodium dodecyl sulfate-polyacrylamide gel (SDS-AGE), and the tubular injury marker urinary N-acetyl-beta-glucosaminidase (uNAG)) and calcium/phosphate metabolism biomarkers. We hypothesized that FGF23 concentration would be increased despite increasing phosphatemia in dogs with hyperadrenocorticism and that the magnitude of proteinuria would be positively correlated to phosphatemia, and FGF23 concentration.

Materials and methods

This study was conducted at the Alfort Veterinary Teaching Hospital from September 2019 to March 2022 as an observational cross-sectional study. The study was approved by the institutional ethics committee [COMERC 2020–05–21] on 15 October, 2020 and informed owner consent was obtained for each dog.

Study population and group inclusion criteria

Client-owned dogs were prospectively enrolled into the study and categorized into three groups: dogs with HAC (HAC group), dogs with a diagnosed condition other than HAC (non-HAC group), and healthy dogs (healthy group). The diagnosis of HAC complied with the American College of Veterinary Internal Medicine (ACVIM) consensus statement (Behrend et al., 2013). Dogs were included in HAC group if they had confirmed naturally-occurring HAC based on: 1) at least two major clinical signs consistent with HAC (e.g., polyuria-polydipsia, polyphagia, abdominal distension, dermatologic abnormalities, lethargy), 2) laboratory abnormalities consistent with the disease (e.g., elevated serum Alkaline Phosphatase [ALP] activity, hyperlipidemia, systemic arterial hypertension, decreased Urine Specific Gravity [USG], proteinuria, stress leukogram, elevated Urine Cortisol-to-Creatinine Ratio [UCCR]) and 3) confirmation with an endocrine test: dogs were diagnosed with spontaneous HAC if the results of a low-dose dexamethasone (Dexadreson, MSD Animal Health, Beaucouzé, France) suppression test (LDDST), or an adrenocorticotropic hormone (ACTH) (Synacthene, Alfasigma, Issy-les-Moulineaux, France) stimulation test, or both, were consistent with hypercortisolism, i.e., serum cortisol concentration $>$ 40 nmoL/L at 8 h, or post-stimulation serum cortisol concentration $>$ 600 nmoL/L, respectively. Diagnostic imaging (either abdominal ultrasound or computed tomography (CT) scan) had to be available to rule out major comorbidities and to help localize the disease as ACTH-dependent or ACTH-independent (Benchekroun et al. 2010; Rodríguez Piñeiro et al. 2011). To address the second objective of the study, dogs with HAC were subdivided into 3 groups according to their SDS-AGE pattern (physiologic vs glomerular vs tubular or mixed: HAC-Physio, HAC-Glom and HAC-Tub/Mixed respectively), and subdivided into proteinuric (HAC-P) and non-proteinuric (HAC-nP) (Lavoué et al., 2015).

Dogs were included in the non-HAC group if HAC was investigated but endocrine tests ruled out the disease and another condition was diagnosed with at least a one-year clinical follow-up of the initial presentation.

Healthy dogs were included as controls. These dogs were either owned by volunteer clients or Teaching Hospital staff members and were older than 6 years of age. Health status was defined by the owner's perception, the absence of any reported clinical signs, the absence of relevant abnormalities detected by physical examination and hematologic, biochemical, and urine laboratory analyses, and a serum cortisol concentration $\leq$ 30 nmoL/L at 8 h in an LDDST or a UCCR $< 10.10^{-6}$.

Dogs were not included in the study if they had been previously diagnosed with HAC, if they had received corticosteroids, ketoconazole, progestogens, diuretics, or medications affecting the renin-angiotensin-aldosterone system or calcium-channel blockers during the past month, if they suffered from primary hyperparathyroidism, primary hypoparathyroidism or comorbidities, other than IRIS stage 1 CKD, affecting calcium-phosphate metabolism. Moreover, for ethical reasons related to additional blood sampling, dogs were not included if they weighed less than 5 kg or were anemic on presentation.

Data collected

The day of inclusion corresponded to the day of the endocrine test. Systolic blood pressure was first measured, using an oscillometric method (HDO, S+B medVet GmbH, Germany). Blood was then collected by jugular venipuncture in the morning, after a minimum 12 h fast. In addition to samples for serum cortisol measurements, blood was collected at T0 for electrolyte measurement (including ionized calcium and magnesium), biochemistry, complete blood count, and serum aldosterone concentration. Additional EDTA samples were immediately centrifuged at 4°C and plasma was stored in aliquots at -80°C for intact PTH, FGF23, and ACTH levels. Serum was also stored at -80°C for 25 (OH)Vitamin D and 1,25$(OH)_2$Vitamin D levels (kept protected from light until completely processed). Finally, a spot urine sample was obtained by cystocentesis, and centrifuged and the supernatant was stored at -80°C for further UPC, SDS-AGE, fractional excretion of calcium (FE (Ca)) and phosphate (FE(P)) and uNAG measurements.

FGF23 concentration was measured in plasma using the previously validated ELISA method (Kainos Laboratories, Tokyo, Japan) (Harjes et al., 2017). Plasma PTH concentration was assessed with the intact PTH ELISA Immutopics© assay (San Clemente, United States of America). Serum cortisol and plasma endogenous ACTH concentration were assessed using the Immulite 2000 XPi (Siemens Healthcare Diagnostics, Tarrytown, United States of America). Serum 25(OH)Vitamin D concentration was measured with VIDAS® ELFA Biomérieux (France) and 1, 25$(OH)_2$ with Calcitriol ELISA Diasource assays (Belgium). Serum aldosterone concentration was assessed as an additional potential driver

of FGF23 concentration and was determined by radioimmunoassay (RIAZENco Aldosterone, ZenTech, Belgium).

All urine samples were collected by ultrasound-guided cystocentesis. Urine analysis included in-house measurement of USG with a portable refractometer (FG-312, Index Instruments, Kissimmee, United States of America), urine dipstick (Multistix® 10 SG, Siemens Healthcare Diagnostics Inc, Tarrytown, United States of America) with visual reading and sediment microscopic examination. Bacterial culture was performed at the discretion of the attending clinician. The urine supernatant was then stored immediately at -80°C until shipment to external laboratories for further analysis. Urinary NAG, creatinine (uCr), urinary calcium (uCa), and phosphate (uP) were measured on urine supernatant. Fractional excretions of calcium and phosphate were calculated using the equation $FE(X) = \frac{uX \times sCr}{uCr \times sX}$, where sCr refers to concurrent serum creatinine concentration and sX to concurrent serum electrolyte concentration. Urinary NAG concentration was measured with N-acetyl-β-D-glucosaminidase Roche assay and was reported as uNAG-to-creatinine ratio (uNAG/C).

Urinary protein 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). Dogs with UPC $\geq$ 0.5 and no signs of inflammatory urinary tract disease were classified as proteinuric. After 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 (such a band is mainly associated with arginine esterase prostatic protein) (Schellenberg et al., 2008; Hokamp et al., 2018; Lavoué et al., 2015). 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.

Serum, EDTA plasma, and urine samples were sent to the external selected laboratories in two batches (March 2021 and March 2022). Storage time of the samples was therefore variable with a median of 197 days [6−383].

Statistical analyses

Continuous variables were presented as medians [range]. Medians of continuous clinicopathological variables were first compared between HAC, non-HAC, and healthy dogs 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_1 for the test comparing HAC and healthy groups, and P_2 for the test comparing HAC and non-HAC groups.

To assess the relationship between renal injury and calcium/phosphate metabolism in dogs with HAC, a Spearman correlation coefficient (r(s) and confidence interval (CI)) was calculated between kidney biomarkers (UPC, uNAG/C) and the following variables: serum phosphate concentration, FE(P), plasma FGF23 concentration, plasma iPTH concentration, serum 25(OH) and 1,25$(OH)_2$Vitamin D concentrations. Fibroblast Growth factor-23, iPTH, Vitamin D metabolites, FE(P), and serum phosphate concentration were only visually compared between HAC-Physio, HAC-Glom, and HAC-Tub/mixed dogs due to small sample sizes. Statistical analyses were performed using GraphPad Prism v9.5.

Results

Study population

Fifty-four dogs were included in the study, among which 27 were assigned to the HAC group, 17 were assigned to the non-HAC group, and 10 were apparently healthy. The signalment and final diagnosis of dogs enrolled in the study are presented in Table 1.

Table 1
Epidemiologic data and final diagnosis in dogs diagnosed with HAC, with a condition other than HAC, and healthy dogs (quantitative variables presented as median [range]).

		HAC (n=27)	Non-HAC (n=17)	Healthy (n=10)
Age (years)		12 [5.2–15.5]	10.7 [4–14]	7.5 [6.1–12.4]
Sex	Male (neutered)	13 (4)	6 (2)	6 (2)
	Female (neutered)	14 (12)	11 (9)	4 (2)
Weight (kg)		11.5 [5.0–29.0]	19 [5.0–40.0]	22.8 [5.9–44.2]
BCS (/9)		5 [3–7]	6 [4–7]	5 [4–6]
Breed		Beagle: 2 Border Terrier: 1 Cavalier King Charles Spaniel: 1 Crossbreed: 5 Dobermann: 1 English Cocker Spaniel: 1 Fox Terrier: 1 French Bulldog: 2 German Shepherd: 1 Jack Russell Terrier: 1 Jagd Terrier: 1 Lhasa Apso: 1 Maltese: 1 Miniature Pinscher: 1 Podengo: 1 Standard Poodle: 3 West Highland White Terrier: 1 Yorkshire Terrier: 2	Yorkshire Terrier: 2 Jack Russell Terrier: 2 Beagle: 1 Bernese Mountain Dog: 1 Polish Lowland Sheepdog: 1 Portuguese Sheepdog: 1 Bichon Frise: 1 English Bulldog: 1 Irish Setter: 1 Poodle: 1 Pont-Audemer Spaniel: 1 Husky: 1 Labrador Retriever: 1 Spitz: 1 West Highland White Terrier: 1	Australian Shepherd: 2 Beauceron: 1 Crossbreed: 2 Dalmatian: 1 Dachshund: 1 Golden Retriever: 1 Shiba Inu: 1 Yorkshire Terrier: 1
Final diagnosis		ACTH-dependant HAC: 22 ACTH-independant HAC: 3 Pituitary and Adrenal lesions: 2	Chronic kidney disease: 2 Diabetes mellitus: 4 Glomerulopathy: 1 Hypercoagulability: 1 Hyperlipidemia: 1 Idiopathic epilepsy: 1 Leishmaniosis: 1 Non-secreting adrenal nodule: 1 Ovarian tumor: 1 Pheochromocytoma: 1 Recurrent urinary stones: 1 Sudden Acquired Retinal Degeneration Syndrome: 1 Vacuolar hepatopathy: 1	

Median systolic blood pressure was 165 mmHg [133–220] in dogs with HAC, which was significantly higher than in healthy dogs (147 [122–163]; P_1<0.001) but not than in dogs with non-HAC (153 mmHg [120–200]; P_2=0.15).

Calcium / phosphate metabolism variables

Results of calcium and phosphate metabolism variables between groups are reported in Table 2 and Fig. 1. Some dogs could not have all tests performed due to more limited samples. Median [range] serum phosphate concentration was significantly higher in dogs with HAC (1.70 mmoL/L [0.80–3.20]) than in dogs with non-HAC (1.18 mmoL/L [0.69–1.50], P_2<0.01) and in healthy dogs (1.19 mmoL/L [0.94–1.46], P_1<0.01), with only one dog with HAC being hyperphosphatemic (based on the laboratory's reference interval). Dogs with HAC had significantly increased FE(Ca) (0.34 % [0.04–5.70]) compared with healthy dogs (0.08 %; [0.04–0.68]; P_1<0.01) but not with dogs with non-HAC (0.15 % [0.02–5.40]; P_2=0.06).

Median plasma iPTH was significantly increased in dogs with HAC (20.68 nmoL/L [3.50–261.2]) compared with healthy dogs (4.24 nmoL/L [1.91–13.47]; P_1<0.01) and dogs with non-HAC (8.38 nmoL/L [2.23–451.0]; P_2<0.01), and 13 dogs with HAC (50 %) had iPTH above the reference range. The median 1,25(OH)$_2$Vitamin D was significantly higher in dogs with HAC (179.7 pmoL/L [49–319.1] than in healthy dogs (115.7 pmoL/L [87.00–194.3]; P_1<0.01) but was not significantly different than in dogs with non-HAC (125.3 pmoL/L [61.10–320.0]; P_2=0.14). Similarly, median calcitriol/calcidiol ratio was significantly increased in dogs with HAC (1.70 [0.50–8.70]) compared with healthy ones (0.65 [0.47–1.18]; P_1<0.01) and dogs with non-HAC (0.97 [0.34–3.00]; P_2=0.03).

Markers of renal tubular and glomerular damage

Results for glomerular and tubular dysfunction are provided in Table 3. Median UPC was significantly different between groups, being increased in dogs with HAC (2.25 [0.16–9.33]) compared to healthy ones (0.06 [0.04–0.29]; P_1<0.01) and dogs with non-HAC (0.36 [0.04–4.70]; P_2<0.01). Urinary NAG/C was significantly increased in dogs with HAC (7.14 U/g [0.29–61.60] compared with healthy dogs (1.64 U/g [0.45–4.68]; P_1<0.01) and dogs with non-HAC (1.73 U/g [0.13–12.6]; P_2<0.01). Urinary protein SDS-AGE showed a physiological pattern in 7/26 dogs with HAC, in 9/9 healthy dogs, and in 9/14 dogs with non-HAC; a glomerular pattern in 16/26 dogs with HAC, in 0/9 healthy dogs and in 5/14 dogs with non-HAC and a mixed or tubular pattern in 3/26 dogs with HAC (including two mixed and one tubular), 0/9 healthy dogs, and 0/14 dog with non-HAC. Mixed and tubular patterns were combined in HAC dogs to pool cases with tubular lesions. In dogs with HAC, 23/26 were proteinuric (HAC-P) and 3/26 were non-proteinuric (HAC-nP). Further statistical analyses were not performed between these two subgroups due to small number of dogs with HAC-nP. Among dogs with HAC and a glomerular or mixed pattern (18/26), all, but one, had an intense albumin band and 15 had a HMWb.

Aldosterone concentration

Median serum aldosterone concentration was not significantly different between dogs with HAC, dogs with non-HAC and healthy ones (Table 2). In the 22 dogs with HAC of the study in which this parameter could be assessed, aldosterone concentration was significantly and positively correlated with FGF23 concentration (r(s)=0.47; P=0.03).

Assessment of association between calcium/phosphate variables and kidney dysfunction in dogs with HAC

UPC was significantly and negatively correlated with 25(OH) Vitamin D (r(s)= -0.54 (CI -0.77 to -0.17); P<0.01) (Table 4; Fig. 2). There was no significant correlation between UPC and the other calcium/phosphate variables (Table 4).

Table 2
Calcium and phosphate metabolism variables in dogs with HAC, non-HAC, and healthy dogs.

Variable	HAC (n=27)	Non-HAC (n=17)	Healthy (n=10)	Reference intervals	p-value
Total Calcium (mmoL/L)	2.80 [2.30–3.90] n=27	2.79 [2.49–3.18] n=17	2.75 [2.64–2.91] n=10	2.20–2.80	P=0.68
Ionized Calcium (mmoL/L)	1.23 [1.05–1.41] n=22	1.25 [1.03–1.47] n=14	1.21 [0.97–1.34] n=10	1.10–1.40	P=0.28
Ionized Magnesium (mmoL/L)	0.49 [0.32–1.10] n=20	0.43 [0.29–1.00] n=15	0.48 [0.36–0.59] n=10	0.40–0.60	P=0.20
Phosphate (mmoL/L)	1.70 [0.80–3.20] n=27	1.18 [0.69–1.5] n=17	1.19 [0.94–1.46] n=10	0.60–2.30	P<0.001 P_1<0.001 P_2<0.001
FE(Ca) (%)	0.34 [0.04–5.70] n=26	0.15 [0.02–5.40] n=15	0.08 [0.04–0.68] n=10	<0.33	P<0.01 P_1<0.001 P_2=0.06
FE(P) (%)	19.34 [1.62–60.00] n=26	13.42 [.62–45.78] n=15	20.34 [0.97–42.66] n=10	2.20–27.20	P=0.72
iPTH (nmoL/L)	20.68 [3.50–261.2] n=27	8.38 [2.23–451.0] n=17	4.24 [1.91–13.47] n=10	15.00–135.00	P<0.001 P_1<0.0001 P_2<0.01
25(OH)Vitamin D (Calcidiol) (nmoL/L)	136.2 [20.20–303.0] n=26	174.5 [20.22–309.8] n=17	172.0 [140.0–237.2] n=10	65.0–263.0	P=0.11
1,25(OH)$_2$ Vitamin D (Calcitriol) (pmoL/L)	179.7 [49–319.1] n=26	125.3 [61.10–320.0] n=17	115.7 [87.00–194.3] n=10	60.0–230.0	P=0.03 P_1<0.01 P_2=0.14
1,25(OH)$_2$Vitamin D/ 25(OH)Vitamin D ratio	1.70 [0.50–8.70] n=26	0.97 [0.34–3.00] n=17	0.65 [0.47–1.18] n=10		P<0.001 P_1<0.001 P_2=0.04
FGF23 (pg/mL)	335.30 [72.20–4301.6] n=27	279.80 [48.40–497.60] n=17	334.70 [87.54–464.70] n=10		P=0.81
Aldosterone (pmoL/L)	71.0 [22.0–446.0] n=24	90.0 [34.0–421.0] n=15	91.0 [43.0–229.0] n=9	15.0–350.0	P=0.67

HAC, Hyperadrenocorticism; FE(Ca), urinary fractional excretion of calcium; FE(P), urinary fractional excretion of phosphate; iPTH, plasma intact parathyroid hormone concentration; FGF23, Fibroblast Growth Factor-23; P, p-value of the Kruskall-Wallis test comparing the three groups; P1, p-value of the Mann-Whitney test comparing HAC and Healthy groups; P2, p-value of the Mann-Whitney test comparing HAC and non-HAC groups.

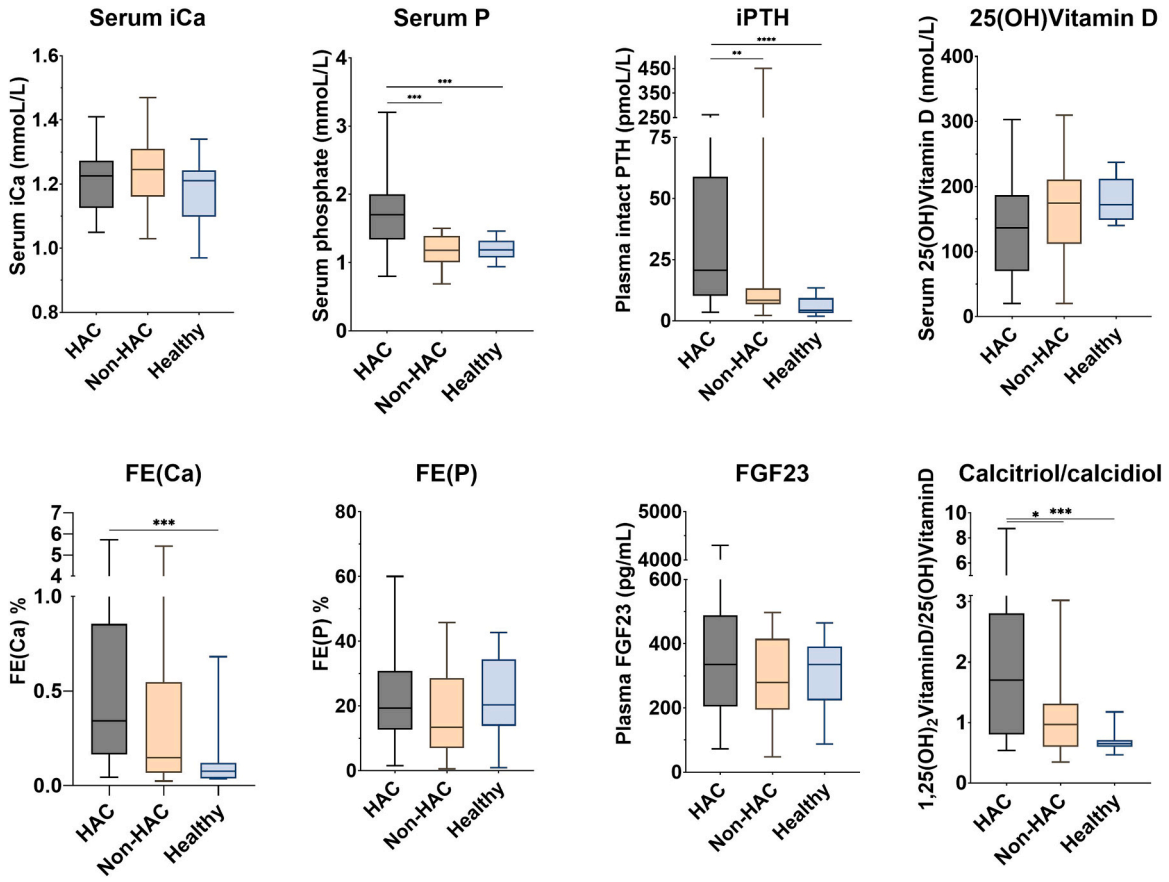

Fig. 1. Box and whiskers plots comparing ionized calcium concentrations, urinary fractional excretion of Ca (FECa) (%), serum phosphate concentration, urinary fractional excretion of P (FEP) (%), plasma intact parathyroid hormone concentration (iPTH), Fibroblast Growth Factor-23 (FGF23), 25(OH)Vitamin D and Calcitriol/Calcidiol ratio in dogs with HAC, dogs with non-HAC and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the minimum and maximum values. *, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

Table 3
Glomerular and tubular dysfunction markers in dogs with HAC, non-HAC, and healthy dogs.

Variable		HAC	Non-HAC	Healthy	p-value
UPC		2.25 [0.16–9.33] *n*=26	0.36 [0.04–4.70] *n*=14	0.06 [0.04–0.29] *n*=9	*P*<0.0001 P_1<0.0001 P_2<0.01
SDS-AGE Pattern	Physiologic Glomerular Tubular/Mixed	7 16 3	9 5 0	9 0 0	
uNAG (U/L)		2.15 [0.20–16.90] *n*=26	1.30 [0.10–10.40] *n*=15	3.75 [0.50–8.50] *n*=10	*P*=0.15
uNAG/C (U/g)		7.14 [0.29–61.60] *n*=26	1.73 [0.13–12.60] *n*=14	1.64 [0.45–4.68] *n*=9	*P*<0.001 P_1<0.01 P_2<0.01
Urea (mmoL/L)		7.16 [1.83–32.13] *n=27*	5.66 [2.50–10.50] *n=15*	5.74 [4.50–8.99] *n=10*	*P*=0.0489 *P1*=0.043 *P2*=0.052
Creatinine (μmoL/L)		53.04 [17.68–168.0] *n=27*	53.04 [26.52–114.9] *n=15*	66.30 [44.20–97.24] *n=10*	*P*=0.21
USG		1.018 [1.002–1.044] *n=26*	1.028 [1.002–1.050] *n=16*	1.044 [1.009–1.050] *n=8*	*P*<0.01 *P1*<0.01 *P2*=0.023

HAC, Hyperadrenocorticism; UPC, Urinary protein-to-creatinine ratio; USG, Urine Specific gravity; SDS-AGE, Sodium dodecylsulfate-agarose gel electrophoresis; uNAG, urinary N-acetylglucosaminidase; uNAG/C, urinary N-acetylglucosaminidase-to-creatinine ratio; P1, p-value of the Mann-Whitney test comparing HAC and Healthy groups; P2, p-value of the Mann-Whitney test comparing HAC and non-HAC groups.

A significant and positive correlation was observed between uNAG/C and serum phosphate (r(s)=+0.46 (CI 0.072–0.732); *P*=0.02; Table 4, Fig. 2). There was no significant correlation between uNAG/C and the other calcium/phosphate variables (Table 4).

Median of serum phosphate, ionized calcium, FE(Ca), FE(P), iPTH, FGF23, 25(OH)Vitamin D, 1,25(OH)2VitaminD was overall similar

Table 4
Correlation between UPC or uNAG/C and calcium and phosphate variables in dogs of the study with HAC.

Calcium/phosphate variable	Correlation with UPC			Correlation with uNAG/C		
	r(s)	p-value	*n*	r(s)	p-value	*n*
Serum Phosphate (mg/dL)	0.12	0.56	26	.46	0.02	26
Serum ionized Calcium (mg/dL)	-0.10	0.68	21	-0.23	0.31	22
Serum Magnesium (mg/dL)	0.08	0.75	19	-0.04	0.86	19
FE(P)%	<0.01	0.98	26	0.19	0.35	26
FE(Ca)%	0.19	0.35	26	0.26	0.20	26
FGF23	0.08	0.69	26	-0.14	0.48	26
iPTH	-0.32	0.11	26	-0.01	0.95	26
25(OH)Vitamin D	-0.54	<0.01	25	-0.19	0.36	25
$1,25(OH)_2$Vitamin D	-0.28	0.18	25	-0.28	0.17	25
$1,25(OH)_2$Vitamin D /25(OH) Vitamin D ratio	0.29	0.16	25	0.02	0.93	25

iPTH, plasma intact parathyroid hormone concentration; UPC, Urinary protein-to-creatinine ratio; FE(Ca), urinary fractional excretion of calcium; FE(P), urinary fractional excretion of phosphate; FGF23, Fibroblast Growth Factor-23; r(s), Spearman's correlation coefficient; uNAG/C, urinary N-acetylglucosaminidase-to-creatinine ratio;

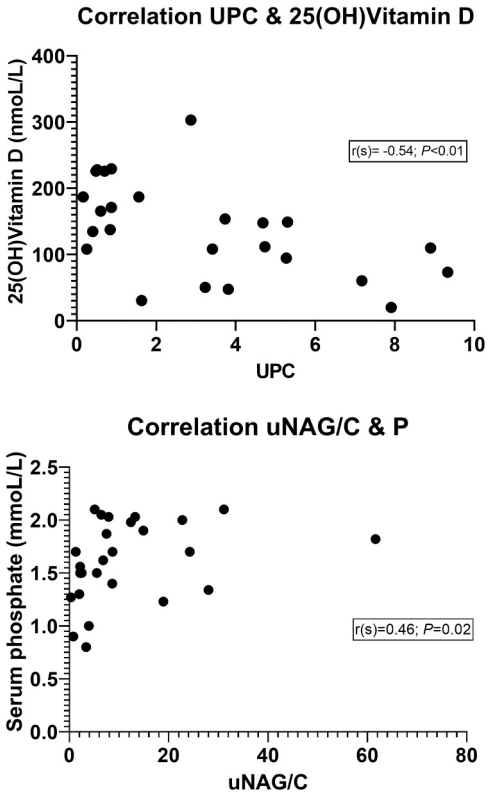

Fig. 2. Spearman's correlation between Urinary Protein-to-Creatinine ratio (UPC) and serum 25(OH) Vitamin D concentration, between urinary N-acetylglucosaminidase-to-creatinine ratio (uNAG/C) and serum phosphate, and between uNAG/C ratio and $1,25(OH)_2$Vitamin D.

between dogs with HAC with physiologic, glomerular or tubular/mixed types of proteinuria.

Discussion

Regarding calcium and phosphate metabolism, dogs with HAC were found to have increased serum phosphate concentration, increased plasma iPTH, and serum $1,25(OH)_2$VitaminD concentrations as well as higher FE(Ca) than healthy dogs. Dogs with HAC were frequently proteinuric, with glomerular profiles being predominant. In this group of dogs, a significant moderate negative correlation was found between UPC and 25(OH)Vitamin D, whereas no correlation was found with serum phosphate or plasma FGF23 concentration.

In this study, plasma FGF23 concentration was not significantly different between HAC, non-HAC, and healthy dogs. This is in contrast to a previous study reporting lower FGF23 concentration in dogs with naturally-occurring hyperadrenocorticism (Corsini et al., 2021). This difference might be due to variations in dog population characteristics (regarding dogs with HAC but also healthy dogs and dogs with non-HAC to which they are compared), differences in clinical severity in dogs with HAC, and the magnitude of kidney dysfunction. Indeed, the median FGF23 concentration in our healthy dogs was lower than previously described (334.70 pg/mL [87.54–464.70] versus 448.7 pg/mL [244.8–753]) (Corsini et al., 2021). However, the other findings regarding plasma iPTH, serum phosphate, and FE(Ca) are similar to previous studies (Ramsey et al., 2005; Fracassi et al., 2015a,2015b; Corsini et al., 2021). Another explanation could then be analytical or pre-analytical variations leading to differences in FGF23 results. However, sample collection, storage conditions, and measurement conditions were the same for all groups although storage time differed between dogs. However, it seems unlikely that storage time would have affected significantly the results as FGF23 plasma concentration was shown to be relatively stable during long-term storage (El-Maouche et al. 2016; Tang et al. 2021). Other possibility includes lack of statistical power due to a low number of cases, increasing the risk of type 2 error. However, a true lack of difference regarding FGF23 concentration in dogs with HAC due to compensatory mechanisms, is also possible. Finally, there were slight epidemiological differences between groups, with healthy dogs being generally larger and younger than dogs with HAC and non-HAC. Although a correlation between body weight and FGF23 concentration has not been demonstrated in dogs, this has been occasionally reported in humans (Marsell et al., 2009) and cannot be excluded. When considering age, the decision to use a lower threshold of 6 years for healthy dogs was made to match with the minimal age of dogs with HAC. While older dogs might have been more suitable, it was challenging to enroll perfectly healthy older dogs in clinical practice. As FGF23's action in the nephron is mediated by its cofactor alpha-Klotho, this could have been a relevant variable to assess in order to further evaluate FGF23 activity.

Vitamin D status has not been clearly established in dogs with HAC or following glucocorticoid therapy. Previous reports describe variable results whereas in humans, patients with Cushing's disease usually have lower 25(OH) Vitamin D concentration. In the present study, 25(OH) Vitamin D was slightly lower in HAC dogs compared to non-HAC dogs and healthy dogs, but this was not statistically significant, which is in contrast with a previous study (Corsini et al., 2021) and with what is described in human patients. A lack of statistical power, or differences in populations between studies might explain this difference. Beyond the evaluation of 25(OH) Vitamin D and 1,25(OH) Vitamin D separately, we have evaluated the $1,25(OH)_2$Vitamin D/25(OH)Vitamin D ratio which is believed to better assess the 1-alpha hydroxylase activity and hydroxylation efficiency (Pasquali et al., 2015). Indeed, the 1-aplha hydroxylase being the target of FGF23, and being also affected by kidney disease, its hydroxylation efficiency was a relevant parameter to assess. The increased $1,25(OH)_2$Vitamin D/25(OH)Vitamin D ratio we observed in HAC dogs compared to non-HAC dogs and healthy dogs might reflect increased 1-aplha hydroxylase activity due to increased PTH stimulation.

Adrenal-secondary hyperparathyroidism has been previously described in dogs with hyperadrenocorticism and in our study, half of dogs with HAC had plasma iPTH above the reference range, which is similar to previous reports (Ramsey et al., 2005; Corsini et al. 2021). This finding has been reported both using intact and whole PTH assays. Hyperparathyroidism is suspected to be multifactorial in dogs with HAC,

with negative calcium balance (as shown by increased FE(Ca)), increased serum phosphate, and possible direct action of corticosteroids on parathyroid glands being the most retained hypotheses.

Serum phosphorus and plasma PTH concentration have been shown to differ significantly in a postprandial state (2 h after feeding) compared with fasted state in dogs fed high-phosphate diet, particularly of inorganic source (Dobenecker, Kienzle, and Siedler, 2021; Dobenecker, Reese, and Herbst, 2021)). In our study, all dogs were fasted (for 12 h) and although diet was not controlled nor similar between dogs, the postprandial state is unlikely to have played a major role in our results.

Our second goal was to evaluate possible correlations between glomerular and tubular dysfunction and calcium/phosphate metabolism variables in dogs with HAC. There was no visual difference when looking at variables between proteinuric (HAC-P) and non-proteinuric (HAC-nP) dogs with HAC. Statistical tests were not conducted, as only 3 dogs with HAC were found to be nonproteinuric. Indeed, as the number of HAC-nP was very small, it may be difficult to conclude. We did not detect any significant association when assessing the quantitative correlation between UPC and the values of serum calcium, phosphate, $1,25(OH)_2$Vitamin D, plasma iPTH, and FGF23 concentrations, as well as FE(Ca) and FE(P). In humans, FGF23 is positively correlated to Urinary Albumin-to-Creatinine Ratio (Silva et al., 2016) in patients with diabetic nephropathy. Moreover, elevated FGF23 is an independent predicting factor of negative renal outcome in patients with macroalbuminuric diabetic nephropathy (Titan et al., 2011; van Der Vaart et al., 2021). More generally, there is compelling evidence in human medicine for a correlation between proteinuria and increased phosphatemia (Vervloet et al., 2012; de Seigneux et al., 2015). In one of these studies, proteinuria was shown to increase serum phosphate concentration, independently of GFR, by altered tubular handling and decreased effect of FGF23 on NaPiIIa transporter, which simultaneously lead to compensatory increase in FGF23 concentration (de Seigneux et al., 2019). A causal relationship between proteinuria and serum phosphate was also suspected in a study where patients with high phosphate levels were more likely to have more severe proteinuria, increased FGF23, and decreased cofactor alpha-Klotho concentration (Jung et al., 2020), this last finding probably explaining the decreased biological effect of FGF23, (state of FGF23-resistance leading to hyperphosphatemia). Such mechanisms could be extrapolated to dogs with HAC, given the spontaneous model of proteinuric kidney disease with relative hyperphosphatemia despite normal to increased GFR. Our study's lack of significant results could be explained by a lack of statistical power, possible heterogeneity in disease severity in dogs with HAC, or by additional compensatory mechanisms not investigated.

For instance, a positive significant correlation between aldosterone and FGF23 has been reported in dogs and cats (Radloff et al., 2021), and results from studies in mice suggested aldosterone to be a direct driver of FGF23 secretion (Radloff et al., 2021). In diabetic humans, plasma aldosterone level is described as either normal or increased (Hollenberg et al., 2004) whereas in dogs with HAC, serum aldosterone level is reported to be decreased (Goy-Thollot et al., 2002), which may influence the final plasma FGF23 concentration in opposite ways. Further in that concept, dietary sodium and potassium have been show to influence phosphatemia and FGF23 concentration (potassium supplementation leading to an increase in phosphatemia and a decrease in FGF23, sodium supplementation the opposite way) (Humalda et al. 2020). Increased dietary potassium load might affect aldosterone concentration by decreasing it, which could thereafter explain the decline in FGF23. One could therefore suspect an interrelationship between dietary mineral intake, aldosterone, FGF23 and calcium/phosphate concentrations. Blood pressure might also influence aldosterone concentration, and further insights into blood pressure and FGF23 metabolism would be beneficial, even though no significant correlation was found between blood pressure and FGF23 or aldosterone in our data.

To better understand pathophysiological mechanisms underlying the association between proteinuria and phosphatemia, future studies with a larger number of dogs and extended laboratory panels including plasma alpha-Klotho concentration would be warranted.

The only significant correlation found between proteinuria and calcium/phosphate metabolism was a negative one between UPC and serum 25(OH)Vitamin D. Such findings have been previously reported in dogs with non-azotemic protein-losing nephropathy (Miller et al., 2020), where dogs had significantly lower vitamin D metabolites. There might be an interrelation between proteinuria and vitamin D metabolism in dogs with HAC. Further studies are needed to investigate these hypotheses.

There was a positive correlation between uNAG/C and serum phosphate, which could suggest a link between tubular lesions and increased phosphatemia, through altered tubular phosphate handling. One explanation could be altered local alpha-Klotho expression and therefore decreased FGF23 activity, leading to an increased proximal tubular NaPi-IIa expression (De Seigneux et al., 2015)). Further studies would be required to specifically assess this feature in dogs with HAC with a more thorough renal evaluation.

This study has some limitations. There was a relatively small number of cases, particularly in the HAC-nP subgroup, impacting statistical comparisons. We did not calculate statistical power before enrolling subjects because of the paucity of data regarding FGF23 in dogs with HAC (the study was initiated before data were published from Corsini et al., 2021), impacting the ability to predict the expected difference in FGF23 concentration between groups. Blood samples were analyzed in two batches at 2 different timepoints; this might have introduced technical variability in our results. Moreover, the non-HAC group was heterogeneous in final diagnoses, which can also make interpretation difficult. However, this group was chosen specifically because it comprised dogs that were clinically suspicious of HAC, and therefore they constitute a positive control group with which to compare dogs with HAC, to evaluate the potential association of hypercortisolism in itself with selected variables and not the impact of the presence of any disease process. Although dogs with CKD were included in non-HAC group, they were classified in IRIS stage 1 and therefore a significant impact on FGF23 concentration was unlikely. Third, dietary intake of calcium and phosphorus was not assessed in this study. Finally, the evaluation with UPC and SDS-Age might not have been sufficient to fully characterize proteinuria in dogs with HAC: the evaluation of urinary albumin might have been of additional value. Moreover, a single urine sample was used to classify proteinuria, therefore persistence and variability of proteinuria were not assessed and some dogs might have been misclassified.

Conclusions

This study further reports increased serum phosphate concentration, increased plasma PTH concentration, and increased calciuresis in dogs with spontaneous HAC compared with healthy dogs and dogs with non-HAC diseases. No significant difference in plasma FGF23 concentration was found. In dogs with HAC, proteinuria was frequent and mostly glomerular, with no significant correlation found between FGF23 or serum phosphate concentrations and proteinuria. Vitamin D metabolites were negatively correlated with markers of renal dysfunction (25(OH) Vitamin D and UPC, $1,25(OH)_2$VitaminD and uNAG/C), which warrant further investigation. The positive correlation between uNAG/C and serum phosphate could still suggest that altered tubular phosphate handling participates in increasing serum phosphate concentration in dogs with HAC. The mechanisms underlying the relative increase in serum phosphate and decreased phosphaturesis in dogs with HAC remain to be elucidated. Further prospective studies with a larger number of dogs and broader metabolic evaluation (including the coreceptor alpha-Klotho) are warranted, as well as longitudinal evaluation of all these parameters after treatment of hyperadrenocorticism.

Conflict of interest

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