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Calcium and phosphate homeostasis in dogs with newly diagnosed naturally occurring hypercortisolism

Andrea Corsini[1,2] | Francesco Dondi[1] | Daria G. Serio[1] | Silvia Zamagni[1] | Stefania Golinelli[1] | Mercedes Fernandez[1] | Federico Fracassi[1]

[1]Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Ozzano Emilia, Italy

[2]Department of Veterinary Medical Sciences, University of Parma, Parma, Italy

Correspondence
Federico Fracassi, Department of Veterinary Medical Sciences, Alma Mater Studiorum-University of Bologna, Via Tolara di Sopra 50, Ozzano dell'Emilia (BO), Italy.
Email: federico.fracassi@unibo.it

Abstract

Background: Hypercortisolism affects calcium and phosphate metabolism in dogs; however, the exact mechanisms are not completely understood.

Objectives: To evaluate circulating concentrations of whole parathormone (wPTH), 25-hydroxyvitamin D (25-(OH)D), calcitriol, and fibroblast growth factor-23 (FGF-23) in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs, and their association with calcium and phosphate homeostasis.

Animals: Twenty-three client-owned dogs with NOHC, and 12 client or staff-owned healthy dogs.

Methods: Prospective cross-sectional study. The circulating concentrations of total calcium, ionized calcium (iCa), phosphate, wPTH, 25-(OH)D, calcitriol and FGF-23, and the urinary fractional excretion of phosphate (FEP) and calcium (FECa) were compared between dogs with NOHC before treatment and healthy dogs.

Results: Dogs with NOHC had higher mean serum phosphate concentrations (4.81 mg/dL, SD ± 0.71 vs 3.86 mg/dL, SD ± 0.60; $P < .001$), median FECa (0.43%, range, 0.03-2.44 vs 0.15%, range, 0.06-0.35; $P = .005$), and median serum wPTH concentrations (54.6 pg/mL, range, 23.7-490 vs 24.6 pg/mL, range, 5.5-56.4; $P = .003$) as compared to the controls. Circulating concentrations of total calcium, iCa, and calcitriol and the FEP did not differ between groups, whereas the serum 25-(OH)D concentrations were lower in dogs with NOHC as compared to the controls (70.2 pg/mL, SD ± 42.3 vs 106.3 pg/mL, SD ± 35.3; $P = .02$). The dogs with NOHC had lower plasma FGF-23 concentrations than controls (316.6 pg/mL, range, 120.8-575.6 vs 448.7 pg/mL, range, 244.8-753; $P = .03$).

Conclusions and Clinical Importance: Urine loss of calcium and hyperphosphatemia could contribute to the adrenal secondary hyperparathyroidism.

Abbreviations: 25-(OH)D, 25-hydroxyvitamin D; ADH, adrenal-dependent hypercortisolism; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; FECa, urinary fractional excretion of calcium; FEP, urinary fractional excretion of phosphate; FGF-23, fibroblast growth factor-23; GGT, gamma-glutamyltransferase; iCa, ionized calcium; iPTH, intact parathyroid hormone; LDDST, low-dose dexamethasone suppression test; NOHC, naturally occurring hypercortisolism; PDH, pituitary-dependent hypercortisolism; PTH, parathyroid hormone; RI, reference interval; RIA, radioimmunoassay; uCr, urinary creatinine; UPC, urine proteins to creatinine ratio; USG, urine specific gravity; wPTH, whole parathyroid hormone.

KEYWORDS
calcitriol, FGF-23, hyperadrenocorticism, parathyroid hormone, urinary calcium excretion, vitamin D

1 | INTRODUCTION

Naturally occurring hypercortisolism (NOHC) is a common endocrine disease in dogs, characterized by a variety of clinical abnormalities resulting from chronic exposure to excessive concentrations of endogenous glucocorticoids. It is usually caused by an ACTH-secreting pituitary adenoma or a functional adrenocortical tumor, with the 2 forms defined as pituitary-dependent hypercortisolism (PDH) and adrenal-dependent hypercortisolism (ADH), respectively.[1] Calcium-containing urolithiasis is a possible, although uncommon, consequence of hypercortisolism, which suggest an influence of this condition on calcium balance.[2] Previous studies have not observed a difference in total and ionized calcium (iCa) concentrations between dogs with NOHC and healthy dogs.[3-6] Hyperphosphatemia occurs frequently in dogs with PDH, together with increased serum parathyroid hormone (PTH) concentrations, a condition previously called adrenal secondary hyperparathyroidism.[3-6] Furthermore, hyperphosphatemia in dogs with newly diagnosed PDH is an independent negative prognostic factor for survival, although the reason behind this finding remains unknown.[7] Dogs with PDH have lower urinary phosphate excretion and higher urinary calcium excretion compared to dogs without hypercortisolism.[5] Hypercortisolism in humans leads to an increase in both calcium and phosphate urinary excretion, occasionally resulting in hypocalcemia and hypophosphatemia.[8]

Calcium and phosphate homeostasis is tightly regulated by a complex interplay between different hormones, with calcitriol, PTH, and fibroblast growth factor-23 (FGF-23) being the most relevant ones.[9]

The influence of hypercortisolism on vitamin D metabolism is well-known in humans, mainly as a co-factor in the development of glucocorticoid-induced osteoporosis, the most common secondary cause of osteoporosis in humans.[10-12] Conversely, vitamin D metabolism has been poorly investigated in hypercortisolemic dogs, probably because osteoporosis is not a clinically relevant problem in these animals. In a previous study, 25-hydroxyvitamin D (25-(OH)D), calcitriol, and 24,25-(OH)D concentrations did not differ between dogs with PDH and healthy dogs.[13] No data exist regarding plasma FGF-23 concentrations in either hypercortisolemic dogs or humans. Moreover, the serum concentration, the urinary excretion of calcium and phosphate, and the serum PTH concentration have never been evaluated concurrently in hypercortisolemic dogs.

The aim of this study was to evaluate the regulators of calcium and phosphate homeostasis in dogs with hypercortisolism. The circulating concentrations of calcium, phosphate, PTH, 25-(OH)D, calcitriol, and FGF-23, together with urinary fractional excretion of calcium (FECa) and phosphate (FEP), were compared between dogs with NOHC at the time of diagnosis and healthy dogs. It was hypothesized that the circulating concentrations of PTH, 25-(OH)D, calcitriol, and FGF-23 differed between these samples.

2 | MATERIALS AND METHODS

2.1 | Study design

A comparative cross-sectional study was designed. Of the dogs presented at the Veterinary Teaching Hospital of the University of Bologna from December 2018 to January 2020, client-owned dogs with newly diagnosed NOHC were prospectively enrolled in the study. A diagnosis of NOHC was made based on clinical signs (eg, polyuria/polydipsia, polyphagia, dermatological abnormalities and abdominal distension) and laboratory findings, plus a low-dose dexamethasone suppression test (LDDST), an ACTH stimulation test, or both, which were consistent with NOHC. Differentiation between PDH and ADH was achieved based on the evaluation of the endogenous ACTH concentration, LDDST patterns, and diagnostic imaging (abdominal ultrasonography, computed tomography, or both). Age and weight-matched healthy dogs, both client- and hospital staff-owned, presented for routine health checks, were enrolled for comparison. The dogs were considered healthy on the basis of history, physical examination, and when the results of hematology, a serum chemistry profile, urinalysis, and urine protein to creatinine ratio were within the reference intervals (RIs).

Dogs diagnosed with a concurrent systemic disease (eg, diabetes mellitus, chronic kidney disease, protein-losing enteropathy, other malignant neoplasia, heart failure), dogs previously or currently treated for hypercortisolism, or treated with any formulation of corticosteroids in the month before presentation, dogs receiving diuretics, renin-angiotensin-aldosterone system inhibitors, levothyroxine, phenobarbital, or vitamin D supplementation, and dogs receiving therapeutic diets potentially affecting calcium and phosphate metabolism were excluded from the study. Approval for this study was given by the local Ethical Committee for Animal Testing (ID 1168).

2.2 | Clinical and clinicopathological data

At the time of inclusion, information regarding concurrent diseases, previous or ongoing medical treatments, and diets were recorded. Blood specimens were collected by standard venipuncture using a blood vacuum system. K_3EDTA samples for ACTH evaluation were immediately processed and analyzed within 30 minutes from collection.[14] The urine specimens were collected by cystocentesis or spontaneous voiding. Blood and urine samples were collected at the same

time after 12 hours of fasting. The plasma, serum, and urine samples were aliquoted and stored at −80°C for the measurement of PTH, 25-(OH)D, calcitriol, FGF-23, and the urinary excretion of electrolytes.

Venous blood gas analysis, including iCa concentrations, was carried out within 15 minutes from the collection on anaerobically handled heparinized whole blood samples, using a blood gas analyzer (ABL 800 Flex, Radiometer Medical ApS, Brønshøj, Denmark). A complete blood count (CBC) was carried out using an automated hematology system (ADVIA 2120, Siemens Healthcare Diagnostics, Tarrytown, New York). The chemistry profile included serum creatinine, urea, total proteins, albumin, glucose, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), cholesterol, triglyceride, haptoglobin, total calcium, and phosphorus. Serum chemistry was determined using an automated chemistry analyzer (AU 480, Olympus/Beckman Coulter, Brea, California). Hormone evaluations of cortisol and ACTH were carried out using an automated immunoassay analyzer (Immulite 2000 XPi, Siemens Healthcare Diagnostics, Tarrytown, New York). Urinalysis included urine specific gravity (USG), a dipstick examination, microscopic sediment evaluation, and urine chemistry, in particular the measurement of urinary creatinine (uCr), urine proteins, urine protein to uCr ratio (UPC), and urinary calcium and phosphate.

Urine chemistry was determined using the same automated chemistry analyzer used for the serum chemistry (AU 480, Olympus/Beckman Coulter, Brea, California); the urinary proteins and uCr were measured using commercially available colorimetric methods (Urinary/CSF Protein OSR6170 and Creatinine OSR6178, Olympus/Beckman Coulter, O'Callaghan's Mills, Ireland). The USG was measured using a hand refractometer (American Optical, Buffalo, New York). Urine dipstick analysis was carried out using a commercially available method (Combur-Test 10 UX, Roche srl, Switzerland) and read by an automated reader (URISYS 1100, Roche srl, Switzerland), the results were additionally confirmed by visual inspection. Microscopic urine sediment examination was carried out at both a low (×100) and a high power field (×400).

The fractional excretion (FE) of calcium (FECa) and phosphate (FEP) were based on a spot urine sample and a contemporaneous serum sample. The FEs were calculated according to a previously reported equation,[15] that is, FEX: (uX × sCr)/(uCr × sX), where uX and sX were the concentrations of the specific analyte in urine and serum, respectively. The results of the FE were reported as percentages, and the reported RIs were the ones defined by the Clinical Pathology Laboratory of the Institution, which performed the analysis. The FECa was calculated using serum total calcium and urinary calcium concentrations. Serum samples on dry ice were sent to a commercial laboratory (Veterinary Diagnostic Laboratory, Michigan State University, Lansing, Michigan) to evaluate the serum whole parathyroid hormone (wPTH), 25-(OH)D, and calcitriol concentrations, using previously validated radioimmunoassays (RIAs).[16] The calcitriol to 25-(OH)D ratio was calculated. Plasma FGF-23 concentration was assessed using a human-specific FGF-23 ELISA kit (Kainos Laboratories, Tokyo, Japan) previously validated in dogs,[17] after the manufacturer's instructions.

2.3 | Statistical analysis

The data were analyzed using commercially available software (GraphPad Prism, version 8.0.2, GraphPad Software Inc, San Diego, California). Normality of the continuous data was assessed graphically using the Shapiro-Wilk test. Accordingly, the results were reported as mean and SD, or as median and range (minimum-maximum value).

TABLE 1 Clinicopathological findings regarding calcium and phosphate metabolism in the dogs included in the study

	NOHC dogs (n = 23)		Healthy dogs (n = 12)			
Variables	**n**	**Values**	**n**	**Values**	**RI**	***P* value**
Total calcium (mg/dL)	*23*	10.2 ± 0.7	*12*	10.3 ± 0.69	9.3 to 11	.69
Ionized calcium (mg/dL)	*23*	5.13 (4.65-6.1)	*12*	5.21 (4.85-5.61)	4.97 to 5.65	.26
Phosphate (mg/dL)	*23*	4.81 ± 0.71	*12*	3.86 ± 0.6	2.65 to 5.4	**<.001**
uCa/uCr	*23*	0.06 (0.00-0.29)	*12*	0.02 (0.00-0.03)	0.00 to 0.03	**<.001**
FECa (%)	*23*	0.43 (0.03-2.44)	*12*	0.15 (0.06-0.35)	0.00 to 0.33	**.005**
uP/uCr	*23*	0.97 (0.04-1.72)	*12*	0.61 (0.26-1.68)	0.00 to 0.97	.34
FEP (%)	*23*	12.8 ± 8.9	*12*	17.7 ± 12	2.2 to 27.2	.19
PTH (pg/mL)	*20*	54.6 (23.7-489.6)	*12*	24.6 (5.5-56.4)	4.5 to 52.8	**.003**
25-(OH)D (pg/mL)	*20*	70.2 ± 42.3	*12*	106 ± 35.3	43.7 to 169.5	**.02**
Calcitriol (ng/mL)	*20*	152 ± 44.6	*12*	164 ± 32.1	68.3 to 217.9	.43
Calcitriol to 25-(OH)D ratio	*20*	2.21 (0.83-13.87)	*12*	1.39 (1.03-3.59)	/	.08
FGF-23 (pg/mL)	*22*	316.6 (120.8-575.6)	*12*	448.7 (244.8-753)	/	**.03**

Note: Data are reported as mean ± SD or median and range (minimum-maximum value), based on their distribution. Significance set at *P* value <.05.
Abbreviations: 25-(OH)D, 25-hydroxyvitamin D; FECa, urinary fractional excretion of calcium; FEP, urinary fractional excretion of phosphate; FGF-23, fibroblast growth factor-23; NOHC, natural occurring hypercortisolism; PTH, parathyroid hormone; RI, reference interval; uCa/uCr, urinary calcium to creatinine ratio; uP/uCr, urinary phosphate to creatinine ratio.

Outliers were detected by the ROUT method ($Q = 5\%$) and removed if considered to be error outliers. Age, total calcium, iCa, phosphate, 25-(OH)D, calcitriol, and FEP were compared between the groups using an unpaired *t* test. Body weight, FECa, UPC, and the wPTH and FGF-23 concentrations were compared between the groups using the Mann-Whitney test. The categorical variables were compared between groups using the Fisher's exact test. Correlations between the variables in dogs with NOHC were assessed using the Spearman rank test. The Kruskal-Wallis test was carried out to compare dogs with NOHC with increased serum wPTH concentration (above 52.8 pg/mL) with dogs with NOHC with normal serum wPTH concentrations (below or equal to 52.8 pg/mL) and with healthy dogs. Significance was set at $P < .05$.

3 | RESULTS

3.1 | Study sample

Thirty-three dogs were presented with newly diagnosed NOHC during the study period. Ten dogs were excluded because of absence of blood or urine samples (n = 5), ongoing levothyroxine supplementation (n = 1), myxomatous mitral valve disease with ongoing diuretic treatment (n = 1), chronic kidney disease with severe proteinuria (n = 1), idiopathic epilepsy with ongoing phenobarbital treatment (n = 1), and ongoing topical steroid treatment (eye drops; n = 1). The remaining 23 dogs were enrolled in the NOHC group. Among these dogs, 19 (83%) were diagnosed with PDH and 4 (17%) with ADH. In the same period, 12 healthy dogs were enrolled as a comparison group.

The mean age was 11.1 years (SD ± 2.7) and 10 years (SD ± 1.8) in the hypercortisolemic and the healthy dogs, respectively. Median body weight was 12.7 (range, 3.4-71) and 14 (range, 5-38) kg in the hypercortisolemic and the healthy dogs, respectively. The NOHC group included 12 male (8 neutered) and 11 female (9 spayed) dogs while the healthy dog group included 6 male (1 neutered) and 6 female (2 spayed) dogs. Age, body weight, and sex did not differ between the groups. Breeds in the NOHC group included crossbreeds (n = 12), Maltese and Weimaraners (n = 2), and 1 each of American Bulldog, Beagle, Border Terrier, Cocker Spaniel, Dachshund, Irish Setter, and Newfoundland. The healthy dogs included crossbreeds (n = 4) and 1 each of Bolognese, Border Collie, Dachshund, German Shepard, Italian Shorthaired Segugio, Jack Russel Terrier, Labrador Retriever, and West Highland White Terrier.

3.2 | Calcium, phosphate, and UPC

The results for the circulating concentrations and the urinary fractional excretions of calcium and phosphate are reported in Table 1. Concentrations of total calcium, iCa, and phosphate, and FECa and FEP were available in all dogs. Ionized and total calcium concentrations did not differ between groups ($P = .69$ and $P = .83$, respectively). The FECa and the serum phosphate concentrations were higher in the dogs with NOHC as compared with the healthy dogs ($P = .005$ and $P < .001$, respectively). Of the dogs with NOHC, 5 (22%) of 23 had a serum phosphate concentration above the upper limit of the RI (4.5-5.4 mg/dL) as compared with none among the healthy dogs. The FEP did not differ between the hypercortisolemic and the healthy dogs ($P = .19$; Figure 1). The UPC was higher in hypercortisolemic dogs (0.70, range, 0.11-17) as compared with the healthy ones (0.16, range, 0.06-0.31; $P < .001$).

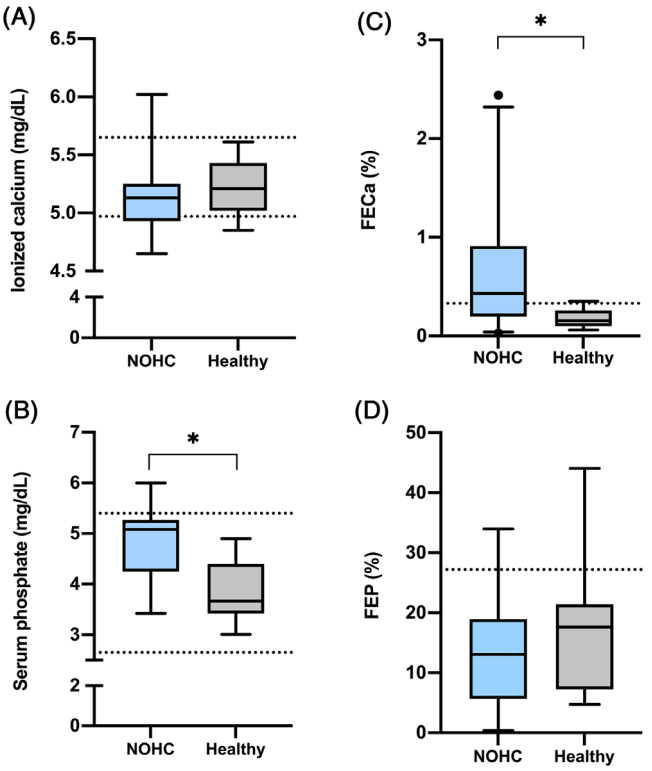

FIGURE 1 Box and whiskers plots comparing (A) ionized calcium concentrations, (B) serum phosphate concentrations, (C) urinary fractional excretion of calcium (FECa), and (D) urinary fractional excretion of phosphate (FEP) in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs. The 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 interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. * $P < .05$

3.3 | Whole PTH, 25-(OH)D, calcitriol and FGF-23

The results for the calcium and phosphate regulators are reported in Table 1. The concentrations of wPTH, 25-(OH)D, and calcitriol were measured in 20 (86%) of the 23 dogs with NOHC and in all the healthy dogs. Plasma FGF-23 concentrations were measured in all the dogs included in the study. Serum wPTH concentrations were higher in the dogs with NOHC as compared to the healthy dogs

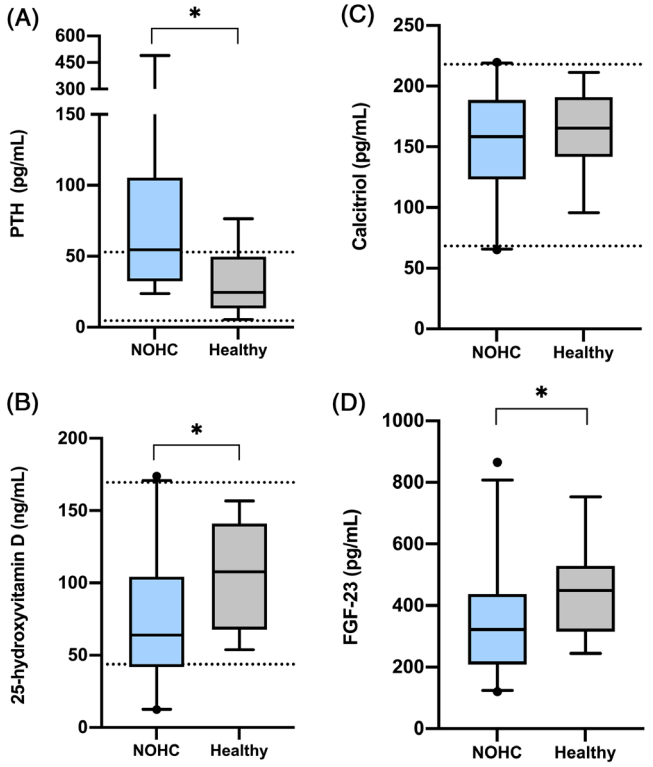

FIGURE 2 Box and whiskers plots comparing serum concentrations of (A) whole parathyroid hormone (wPTH), (B) 25-hydroxyvitamin D, (C) calcitriol, and (D) plasma fibroblast growth factor-23 (FGF-23) concentrations in dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs. The boxes represent the interquartile range from the 25th to the 75th percentile. The horizontal bar in each box represents the median value. The whiskers represent the interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. * $P < .05$

($P = .003$). Twelve (60%) out of 20 dogs with NOHC and 2 (17%) out of the 12 healthy dogs had serum wPTH concentrations above the upper limit of the RI (4.5-52.8 pg/mL). Serum 25-(OH)D concentrations were lower in the dogs with NOHC as compared to the healthy dogs ($P = .02$). Five (25%) out of the 20 NOHC dogs and none of the healthy dogs had serum 25-(OH)D concentrations below the lower limit of the RI (43.7-169.5 pg/mL). Serum calcitriol concentrations did not differ between the hypercortisolemic and the healthy dogs ($P = .43$); only 1 hypercortisolemic dog out of all the dogs included in the study had a value below the lower limit of the RI (68.3-209.5 ng/mL). Plasma FGF-23 concentrations were lower in the dogs with NOHC as compared to the healthy dogs ($P = .03$; Figure 2). Calcitriol to 25-(OH)D ratio did not differ between the dogs with NOHC and the healthy dogs ($P = .08$). When the dogs with NOHC were stratified based on serum wPTH concentrations, those with increased serum wPTH concentrations had a higher median calcitriol to 25-(OH)D ratio (2.92, range, 1.19-13.97) as compared to both the hypercortisolemic dogs with normal wPTH concentrations (1.46, range, 0.83-8.29; $P = .02$) and the healthy dogs (1.39, range, 1.03-3.59; $P = .007$). The calcitriol to 25-(OH)D ratio did not differ between the

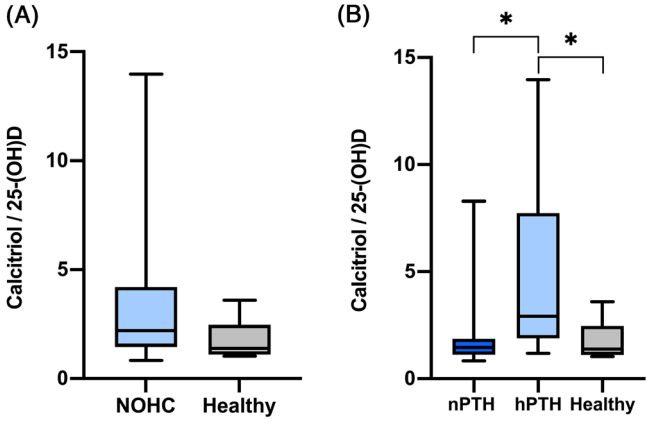

FIGURE 3 Box and whiskers plots comparing the calcitriol to 25-hydroxyvitamin D (25-(OH)D) ratio between (A) dogs with naturally occurring hypercortisolism (NOHC) and healthy dogs and between (B) NOHC dogs with increased serum wPTH concentrations (hPTH), NOHC dogs with normal serum wPTH concentrations (nPTH), 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 interquartile range from the 2.5th to the 97.5th percentile, with the outliers represented as dots. The dotted lines represent the limits of the reference interval. *$P < .05$

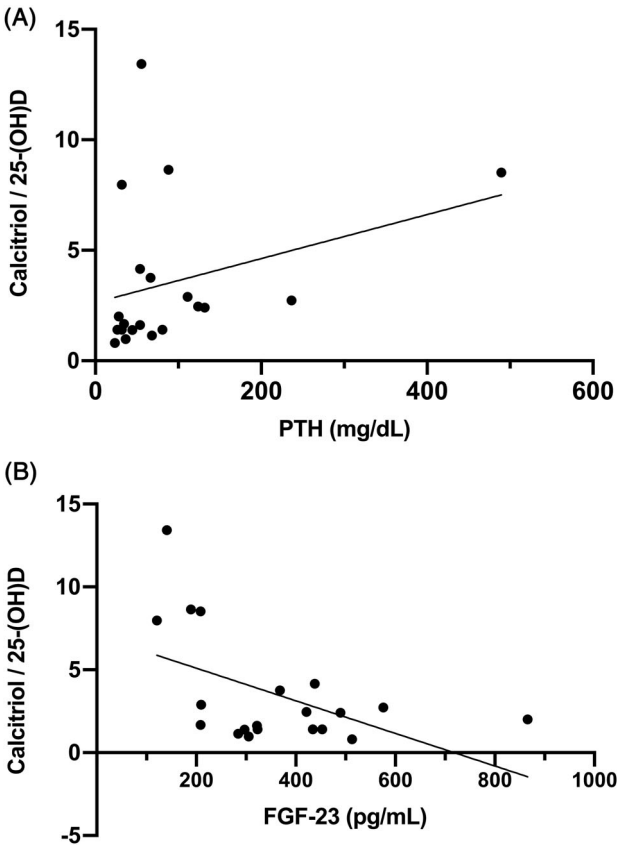

FIGURE 4 Spearman's correlation between (A) the calcitriol to 25-hydroxyvitamin D (25-(OH)D) ratio and serum whole parathyroid hormone (wPTH) concentrations and (B) the calcitriol to 25-(OH)D ratio and plasma fibroblast growth factor 23 (FGF-23) concentrations, in dogs with naturally occurring hypercortisolism

hypercortisolemic dogs with normal wPTH concentrations and the healthy dogs ($P = .98$; Figure 3).

3.4 | Correlation analysis in the dogs with NOHC

Serum phosphate concentration did not correlate with the FEP nor with the serum concentrations of wPTH, 25(OH)D, calcitriol and plasma FGF-23. The FECa, the FEP, and the iCa, serum wPTH, serum 25-(OH)D, and serum calcitriol concentrations did not correlate with any other parameter. No correlation was found between serum 25-(OH)D concentrations and UPC ($r = .36$, 95% CI = −0.70 - 0.12; $P = .12$). The calcitriol to 25-(OH)D ratio showed a moderate positive correlation with the serum wPTH concentration ($r = .47$, 95% CI = 0.02-0.76; $P = .04$) and a moderate negative correlation with the plasma FGF-23 concentration ($r = .51$, 95% CI = −0.78 to −0.08; $P = .02$; Figure 4). No other correlations were detected.

4 | DISCUSSION

This study described the circulating concentrations of the main regulators of calcium and phosphate homeostasis in hypercortisolemic dogs. Dogs with NOHC had higher serum phosphate concentrations, FECa, and serum wPTH concentrations compared to the controls. Whole PTH concentrations were commonly above the upper limit of the RI in the dogs with NOCH. Serum 25-(OH)D concentrations were lower in the dogs with NOHC than in the controls, while calcitriol concentrations did not differ. Also, the dogs with NOHC had lower plasma FGF-23 concentrations.

Hypercortisolism affects calcium homeostasis in different ways.[18,19] In the present study, neither total calcium nor iCa concentrations differed between the groups while dogs with NOHC had increased urinary excretion of calcium. Hypercalciuria is common in hypercortisolemic humans and is described in dogs with PDH.[5,20-22] It is a major risk factor for the development of calcium-containing uroliths, possibly explaining why dogs with NOHC are more likely to develop calcium-containing uroliths as compared to dogs without NOHC,[2] although, in humans with glucocorticoid-dependent nephrolithiasis, multiple factors are involved in kidney urolith formation.[21] Nonetheless, hypocalcemia is rarely reported in humans, and the circulating calcium concentration is largely unaffected in the numerous studies evaluating it in dogs with hypercortisolism.[3-7,13] The vast majority of studies reports only the total calcium concentrations, and only 2 of them report the iCa concentration.[3,6] Circulating iCa and total calcium concentrations remain within the RI in most of the cases likely because of effective compensation. This compensation could be explained by increased bone resorption, but the present study did not specifically assess the effects of hypercortisolism on bone metabolism. Despite decreased bone mineral density is described in dogs with NOHC compared to healthy dogs, there is no evidence of bone metabolism alterations in dogs with hypercortisolism based on the evaluation of some markers of bone formation and resorption.[6,23] Alternately, increased intestinal absorption could compensate for increased urinary calcium excretion, but it seems less likely considering that hypercortisolism decreases intestinal calcium absorption in humans.[18] The influence of hypercortisolism on intestinal calcium absorption in dogs was evaluated neither in the present nor in other studies.

Hyperphosphatemia was described in hypercortisolemic dogs in the present study, as had been reported in previous studies.[3,4,6,7,13] The reasons behind this finding are not clear. The urinary excretion of phosphate is decreased in 167 dogs with PDH as compared with healthy and sick control dogs.[5] At least in part, hypophosphaturia could be involved in the development of the hyperphosphatemia described in these dogs. The urinary fractional excretion of phosphate did not differ when dogs with NOHC were compared to healthy dogs in the present study. However, considering that the present results were similar to those reported in the study cited, we believe that the most likely explanation for the findings in the present study is a lack of statistical power because of the markedly lower number of dogs.

In the present study, wPTH concentrations were increased in dogs with NOHC (median value 54.6 pg/mL) as compared to an age and weight-matched sample of healthy dogs. Increased circulating PTH concentrations are reported in dogs with NOHC.[3,6] Mean intact parathyroid hormone (iPTH) concentration, measured using RIA, is approximately 105 pg/mL (exact data not reported, extrapolated from graphs) in a cohort of hypercortisolemic dogs, with 92% among them showing iPTH concentrations above the upper limit of the RI.[3] Whole PTH assays are usually regarded as the most precise indicator of actual PTH concentrations since, unlike intact PTH (iPTH) assays, they do not cross-react with large 7-84 PTH fragments. However, the superiority of wPTH assays in routine clinical decision-making remains controversial.[24] Both iPTH and wPTH concentrations, measured using RIAs, are increased in dogs with NOHC as compared to control dogs. In both these groups, the iPTH is approximately 50% higher than wPTH (median values in dogs with NOHC are 87.2 and 43.74 pg/mL, respectively), with a strong correlation between them. Thus, the measurement of either provides similar information.[6] Overall, the occurrence of adrenal secondary hyperparathyroidism in hypercortisolemic dogs could be supported by all these studies. Adrenal secondary hyperparathyroidism had been investigated as a cause or as a consequence of calcium and phosphate abnormalities in hypercortisolemic dogs. However, the major function of PTH is to stimulate calcium retention together with phosphate excretion, markedly differing from the calcium and phosphate alterations described in hypercortisolemic dogs. Hypercalciuria occurred in the hypercortisolemic dogs of the present study, supporting the role of abnormal calcium balance in the development of adrenal secondary hyperparathyroidism. Sustained hypercalciuria could stimulate PTH secretion by inducing a mild negative calcium balance despite that the circulating calcium concentrations remained largely unaffected, as reported in this and in previous studies.[5,6,25] Moreover, hyperphosphatemia stimulates the parathyroid glands to produce PTH independently of changes in serum calcium concentrations.[25] Nonetheless, a correlation between circulating

concentrations of calcium and phosphate and PTH was not identified either in the present study or in the previous studies, supporting a multifactorial origin for adrenal secondary hyperparathyroidism.[25] Cortisol seems to be capable of influencing human and rat parathyroid glands activity both directly, stimulating PTH secretion, and indirectly, decreasing the sensitivity of parathyroid cells to the inhibitory action of calcitriol, thus allowing for an increase in PTH secretion.[26,27] However, hypercalcemia and hypophosphatemia would be expected if these mechanisms contributed primarily to hyperparathyroidism. Adrenal secondary hyperparathyroidism seems more likely to be a consequence of calcium and phosphate abnormalities.

Vitamin D metabolism has been only scarcely investigated in hypercortisolemic dogs. In the present study, the dogs with NOHC had lower serum 25-(OH)D concentrations, whereas the serum calcitriol concentrations did not differ when compared to the healthy dogs. Vitamin D metabolism seems unaffected in a small cohort of 12 dogs with PDH, in which 25-(OH)D, calcitriol, and 24,25-(OH)D do not differ as compared to healthy dogs.[13] Long-term administration of prednisone at 1.5 mg/kg/day slightly decreases serum calcitriol concentrations, but not 25-(OH)D concentrations, in healthy mixed-breed dogs.[28] These findings are similar to what happens in humans undergoing long-term steroid treatment or affected by naturally occurring Cushing's Syndrome, in which circulating concentrations of 25-(OH)D and calcitriol are decreased or unchanged depending on the study.[8,10,29-31] The controversial results reported in the human literature might be because of differences in the nature of the underlying diseases, the dose and duration of the glucocorticoid excess, and the degree of mineral bone disorders.[32] These hypotheses could also apply to the present results. The mechanism of action by which glucocorticoid excess influences vitamin D metabolism in humans is not completely understood; however, it has been proposed that cortisol directly upregulates 24-hydroxylase expression, leading to the increased inactivation of 25-(OH)D and calcitriol.[33,34] Circulating concentrations of 25-(OH)D and calcitriol are decreased in proteinuric non-azotemic dogs in which hypercortisolism was excluded.[35] Urine loss of vitamin D-binding proteins-complexed and albumin-complexed vitamin D metabolites has been suggested as part of a multifactorial explanation, and it could play a role also in hypercortisolemic dogs. However, in the present study no correlation was found between UPC and serum concentrations of 25-(OH)D and calcitriol in dogs with NOHC, despite UPC being higher in dogs with NOHC compared to healthy ones. Despite our study could have failed to demonstrate the impact of proteinuria on circulating 25(OH) concentrations because of inadequate statistical power, it is reasonable to suppose that multiple mechanisms affect circulating 25(OH) concentrations in hypercortisolemic dogs. To additionally assess vitamin D metabolism in the dogs with NOHC, the ratio between calcitriol and 25-(OH)D was calculated. This variable has been proposed to be representative of vitamin D hydroxylation efficiency, possibly improving the understanding of the vitamin D status in the course of the disease.[36] Based on the present results, vitamin D hydroxylation efficiency seemed to be increased in dogs with NOHC with elevated serum wPTH concentrations, as is suggested in the human literature.[8,31] This could partially explain why serum calcitriol concentrations did not differ between the groups in the present study, despite 25-(OH)D being significantly lower in the hypercortisolemic dogs as compared to the healthy dogs. Elevated circulating concentrations of PTH are probably the major factor responsible for the increased 1-α-hydroxylase activity. Nonetheless, the interpretation of the calcitriol to 25-(OH)D ratio remains speculative.

The circulating concentration of FGF-23 had never been evaluated before either in dogs or in humans with hypercortisolism. The major determinant of FGF-23 secretion is the circulating concentration of phosphate, with hyperphosphatemia leading to an increase in plasma FGF-23 concentration.[9,37] In dogs with chronic kidney disease, there is a positive correlation between serum phosphate and plasma FGF-23 concentrations, with FGF-23 markedly increased in more advanced stages of the disease. Serum phosphate concentration is an independent predictor of plasma FGF-23 concentration.[17] Furthermore, phosphate-enriched diets induce a mild increase in circulating FGF-23 concentrations in humans, while feeding a phosphate-restricted diet is associated with a decrease in circulating FGF-23 concentrations in cats with stable CKD.[38-40] Based on these assumptions, an increased plasma FGF-23 concentration would be expected in hypercortisolemic dogs. In the present study, however, hypercortisolemic dogs showed lower plasma FGF-23 concentrations compared to healthy dogs. No correlation between serum phosphate concentrations and plasma FGF-23 concentrations was found. Notably, even if a difference between hypercortisolemic and healthy dogs exists, its relevance from a clinical standpoint remains unknown. In humans, the clinical consequences of FGF-23-dependant disorders are more commonly reported when an excess of FGF-23 develops, usually because of hereditary genetic diseases or acquired disorders, such as McCune-Albright syndrome or tumor-induced osteomalacia. The clinical phenotype associated with a FGF-23 deficiency had been described only in familial tumoral calcinosis or in experimental FGF-23 knockout mice, and it is characterized by hyperphosphatemia, hypophosphaturia, increased calcitriol concentrations, and ectopic tissue calcification.[41,42] It could be speculated that, in hypercortisolemic dogs, a decreased plasma FGF-23 concentration plays a role in the development of hyperphosphatemia and hypophosphaturia as described in the previous study.[5] However, it is unknown whether the magnitude of the decrease in the plasma FGF-23 concentrations described in the present study could relevantly impact phosphate metabolism. Moreover, the biological activity of FGF-23 depends not only on its circulating concentrations but also on the tissue expression of the Klotho : FGF receptor complex,[9] which was not assessed in this study. Overall, the interpretation of circulating FGF-23 concentrations in the hypercortisolemic dogs remains unclear.

The present study had some limitations. First, this was an observational comparative cross-sectional study, and the hypothesis proposed remains theoretical. Second, a relatively small number of dogs were included in the study, possibly affecting the statistical power. Thus, the number of dogs included could have been inappropriate for detecting significant differences in some of the variables evaluated, resulting in a type II error. This limitation should be considered when interpreting the lack of difference between groups for some variables

described in the present study. For example, a difference in FEP between the groups would have been expected, based on previously published results.[5] Third, the dietary intake of phosphorus, calcium, and 25-(OH)D was not assessed in any of the dogs included in this study, although dogs assuming therapeutic diets were excluded from the study. Thus, even if unlikely, the influence of diet on these results could not be completely ruled out.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Scientific Ethical Committee for Animal Testing of the University of Bologna (ID 1168).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

ORCID

Andrea Corsini https://orcid.org/0000-0003-1673-8715
Francesco Dondi https://orcid.org/0000-0001-8259-6458
Federico Fracassi https://orcid.org/0000-0003-3121-2199

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