Fibroblast growth factor-23 in canine chronic kidney disease

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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The Ohio State University
2017

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Abstract

Chronic kidney disease (CKD) is associated with hyperphosphatemia, reduced vitamin D metabolite concentrations, and hyperparathyroidism. This syndrome is known as CKD-mineral and bone disorder (CKD-MBD). Recently it has been shown that increased fibroblast growth factor-23 (FGF23) concentration is one of the earliest biomarkers of CKD in people and cats. It is an independent risk factor for both progression of kidney disease and survival time in humans and cats. FGF23 information in healthy and CKD dogs is lacking.

The primary objective of this study was to measure FGF23 concentration in dogs with different stages of CKD and to determine its association with factors involved in CKD-MBD, including phosphate and parathyroid hormone (PTH) concentrations. A secondary aim was to validate an ELISA for measurement of canine plasma FGF23. Thirty-two client-owned dogs with naturally occurring CKD and 10 healthy control dogs were enrolled in this prospective cross-sectional study. A human FGF23 ELISA was used to measure plasma FGF23 and its association with creatinine, phosphate, calcium, and PTH was determined. Plasma FGF23 concentration increased with severity of kidney disease and was significantly different between IRIS stages 1 and 2 versus stages 3 and 4 (P <0.0001).

Increased plasma FGF23 concentration occurred more frequently than hyperparathyroidism or hyperphosphatemia in this cohort. Serum creatinine and phosphate concentrations were the strongest independent predictors of FGF23 concentration. Plasma FGF23 concentration increased in CKD dogs as disease progressed. Plasma FGF23 concentration appears to be useful for further study of the pathophysiology of canine CKD-MBD.
Acknowledgments

I would like to acknowledge my mentor, Dr. Valerie Parker, for her continued support in completing my M.S. degree. Without her guidance this process would not have been possible.
Vita

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Publications


Fields of Study

Major Field: Comparative and Veterinary Medicine
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Chapter 1: Fibroblast growth factor-23 in chronic kidney disease-mineral and bone disorder

Chronic kidney disease (CKD) is a common disease in dogs, defined by progressive and irreversible renal injury of various causes. There are many causes of CKD including chronic glomerulonephritis, toxic insult, hypoxia, amyloidosis, and pyelonephritis, but underlying etiology in most patients is unknown. Chronic kidney disease has a reported prevalence of up to 25% in dogs presenting to a veterinary teaching hospital.

In humans, CKD is defined as a sustained (≥3 months) reduction in glomerular filtration rate (GFR [≤60 ml/min/1.73 m²]) or evidence of sustained (≥3 months) structural damage or proteinuria. A specific definition of CKD has not been determined in dogs. The International Renal Interest Society (IRIS) developed a staging system based on repeated fasting serum creatinine concentration to guide treatment and monitoring of dogs with CKD. This staging system consists of Stage 1: patients with nonazotemic renal abnormalities (creatinine < 1.4 mg/dL) such as inappropriate urine concentrating ability, proteinuria, chronic cylindruria, abnormal renal imaging (size, shape, echotexture) or renal biopsy results demonstrating chronic structural changes. Stages 2, 3, and 4 are based on the finding of serum creatinine concentrations that are consistently elevated at various levels (stage 2: 1.4-2.0 mg/dL; stage 3: 2.1-5.0 mg/dL;
stage 4: > 5.0 mg/dL). All IRIS stages are substaged by systolic blood pressure (minimal risk of target organ damage: <150 mmHg; mild to moderate risk: 150-170 mmHg; severe risk: ≥180 mmHg) and the presence of proteinuria (normal: urine protein-to-creatinine ratio [UPC] <0.2; borderline: UPC 0.2-0.5; overt: UPC >0.5). Clinical signs vary even within the same IRIS stage, though clinical signs associated with uremia such as hyporexia, nausea, and vomiting typically accompany stages 3 and 4.

The kidneys play a pivotal role in whole body homeostasis, contributing to fluid, electrolyte, acid-base, and blood pressure regulation, as well as participating in hormonal control of mineral balance and erythropoiesis. When GFR substantially decreases, there is a detrimental effect on multiple organ systems resulting from retention of compounds that should be excreted (eg, uremic toxins), loss of compounds due to excess filtration (eg, water and albumin), and loss of endocrine biosynthetic functions (eg, 1,25-dihydroxyvitamin D [1,25D] and erythropoietin).

In CKD, hyperphosphatemia largely occurs due to the reduced ability of damaged nephrons to excrete the dietary phosphate load. Normal serum phosphate concentration is initially maintained despite this decreased nephron mass in part by increased fibroblast growth factor-23 (FGF23) concentration. This hormone stimulates phosphaturia and decreases intestinal phosphate absorption by decreasing 1,25D synthesis. At the same time, minor decreases in serum ionized calcium (iCa) along with decreased circulating 1,25D concentration directly stimulate parathyroid hormone (PTH) synthesis from parathyroid glands. Over time the kidneys lose the ability to maintain normal serum phosphate concentration and persistent chronic increases in PTH occur, termed renal
secondary hyperparathyroidism (HPTH). The syndrome encompassing these disturbances in mineral metabolism is referred to as chronic kidney disease-mineral and bone disorder (CKD-MBD). This syndrome is characterized by the aforementioned biochemical abnormalities, bone disorders, and vascular calcification.\(^7\)

Renal secondary HPTH is a common complication of CKD-MBD in people, characterized by parathyroid hyperplasia and increased PTH concentration.\(^8\) The net effect of PTH is to increase serum calcium concentration and decrease serum phosphate concentration by acting directly on the kidney and bone and indirectly on the small intestine. Parathyroid hormone promotes renal calcium reabsorption while blocking phosphate reabsorption. It also increases serum calcium concentration by promoting osteoclastic bone resorption. The third function of PTH is to facilitate the conversion of 25-hydroxyvitamin D (25D) to its active metabolite, 1,25D by increasing 1-alpha-hydroxylase activity in the proximal renal tubules. Consequences of chronic excessive PTH secretion include high-turnover bone disease from excessive bone resorption, cardiovascular disease characterized by cardiac remodeling and vascular calcification, and increased mortality.\(^9,10\) This syndrome is termed renal secondary HPTH.

The original hypothesis to explain development of increased PTH during CKD centered on a decreased serum ionized calcium (iCa) concentration\(^11\). With decreasing GFR the kidneys are transiently unable to excrete enough phosphate to match dietary phosphorus intake. Hyperphosphatemia directly stimulates PTH secretion and gene transcription.\(^12\) This relative increase in phosphate burden also causes a minor decrease in serum iCa concentration. This subtle change in serum iCa concentration is detected by
calcium-sensing receptors in the parathyroid glands, and PTH is released from chief cells in the parathyroid glands.

Parathyroid hormone directly increases serum iCa concentration by stimulating osteoclastic-mediated bone resorption of bone and increasing renal tubular reabsorption of calcium. Parathyroid hormone indirectly increases intestinal calcium reabsorption by increasing 1,25D concentration. Parathyroid hormone also decreases serum phosphate concentration by decreasing proximal tubular reabsorption of phosphorus. Serum phosphate remains within the reference range early in CKD due to this mechanism. Later in CKD, hyperphosphatemia develops as there are not enough functional nephrons to respond to PTH.

A second theory to explain development of renal secondary HPTH emphasizes the role of circulating 1,25D and its absolute or relative deficiency in CKD. Renal 1,25D synthesis is decreased by loss of proximal renal tubular mass but also by increased circulating phosphate, which decreases the activity of 1-alpha hydroxylase. An adequate concentration of circulating 1,25D is necessary to genomically inhibit PTH synthesis, thus the 1,25D deficiency seen in CKD removes inhibition of PTH synthesis. The resulting increased PTH concentration increases the activity of 1-alpha hydroxylase in an effort to stimulate 1,25D synthesis. Circulating 1,25D concentration is thus maintained in the normal range at the expense of increased PTH until advanced CKD develops. Serum phosphate concentration is also initially maintained in the normal range at the expense of increased PTH secretion. Excess PTH stimulation has adverse effects of soft tissue mineralization and bone resorption. Eventually, the kidneys are no longer able to
increase 1,25D synthesis and, despite persistent hyperphosphatemia and increased PTH concentration, serum 1,25D concentration remains low.\textsuperscript{13-15}

A third hypothesis for generation and maintenance of renal secondary HPTH centers on the role of increased FGF23 and decreased klotho. Fibroblast growth factor-23 inhibits PTH secretion in early stages of CKD, but in more advanced stages, the decreased 1,25D resulting from FGF23 indirectly promotes development of renal secondary HPTH because adequate amounts of 1,25D are needed to inhibit PTH gene transcription.\textsuperscript{16} The modifications in this theory help explain decreases in 1,25D that occur in early CKD and for PTH resistance in later CKD. There was an increased relative risk of death in people with CKD who have markedly increased PTH concentration.\textsuperscript{17,18}

In dogs, renal secondary HPTH has been shown to occur as early IRIS stage 1 CKD.\textsuperscript{19} Parathyroid hormone increased before overt hyperphosphatemia, with 36% of IRIS CKD stage 1 dogs having renal secondary HPTH despite only 18% having hyperphosphatemia. The incidence of renal secondary HPTH increased with progression of CKD, with 100% of IRIS CKD stage 4 dogs having renal secondary HPTH. Though renal secondary HPTH is associated with increased mortality in people with CKD, this has not been evaluated in canine patients. In an experimental model of induced kidney failure, parathyroidectomized dogs trended toward a higher survival rate but this did not reach statistical significance.\textsuperscript{20} Two studies that evaluated the calcium-phosphate product in dogs with naturally occurring CKD found that a total calcium-phosphate product (CPP) >70 mg^2/dL^2 was associated with a higher mortality rate compared to dogs with a
lower CPP.\textsuperscript{21} In a separate study of dogs with experimentally induced CKD, survival was associated with the iCa-phosphate product.\textsuperscript{20}

Fibroblast growth factor-23 is now considered a key regulator of phosphate homeostasis, forming a bone-kidney axis to control circulating phosphate and 1,25D concentrations. Plasma FGF23 concentration increases in order to decrease excess serum phosphate concentration by stimulating phosphaturia and inhibiting 1,25D synthesis.\textsuperscript{22} Fibroblast growth factor-23 has been studied in people with an emphasis on those with CKD and cardiovascular disease. New insights into the FGF23 pathway are discussed in this review, with an emphasis on its role in CKD-MBD in people and cats, as it is a strong predictor of morbidity and mortality in these species.\textsuperscript{23-29}

Fibroblast growth factor-23 is a low molecular weight polypeptide hormone (32 kDa) produced by osteoblasts and osteocytes. It is characterized by an N-terminal that is the core domain for all fibroblast growth factors, and a C-terminal that is unique for FGF23. The N-terminal allows FGF23 to bind to FGF receptors while the C-terminal binds its co-receptor klotho. The amount of circulating FGF23 is precisely regulated by posttranslational processes. Intact FGF23 is responsible for biologic activity and without O-glycosylation the hormone is rendered inactive by intracellular cleavage.\textsuperscript{30} Canine FGF23 has 79\% homology with human FGF23.\textsuperscript{31}

The stimuli for FGF23 secretion are not completely clear. There is general agreement that FGF23 secretion is stimulated by 1,25D, PTH, phosphate and calcium concentrations.\textsuperscript{32-35} Fibroblast growth factor-23 binds fibroblast growth factor receptors (FGFRs) on multiple tissues throughout the body notably kidney, parathyroid gland,
heart, and vascular smooth muscle cells. Through its actions on kidney, bone, and parathyroid glands, FGF23 has an inhibitory effect on 1,25D and PTH synthesis, thus forming a negative feedback loop.

Effective FGF23 binding requires the presence of its cofactor, klotho. Klotho is a transmembrane or secreted glycoprotein expressed on renal distal tubular cells and to a lesser extent proximal tubular cells, as well as the parathyroid gland. It has been demonstrated that FGF23 requires klotho to influence phosphate homeostasis.

Fibroblast growth factor-23 induces activation of the mitogen-activated protein kinase (MAPK), extracellular signal-related kinase-1/2 (ERK-1/2), or early growth response-1 (EGR-1) pathways.

The kidney is FGF23’s primary target. Like PTH, FGF23 induces phosphaturia but it does this independently of both the 1,25D/vitamin D receptor system and PTH. The klotho/FGF23 complex binds to receptors on the apical membrane of the proximal tubular epithelial cells, downregulating type II Na+-dependent phosphate co-transporters NaPi-2a and NaPi-2c which normally reabsorb filtered phosphate. These cotransporters are principally located in the proximal tubule and to a lesser extent the distal tubule. The net effect is increased phosphaturia and decreased serum phosphate concentration.

Another way FGF23 acts to lower serum phosphate concentration is by decreasing circulating 1,25D. Fibroblast growth factor-23 decreases 1,25D synthesis via downregulation of 1-alpha-hydroxyalase activity as well as increased degradation of 1,25D to 24,25D via increased activity of 24,25-hydroxylase. Decreased circulating
1,25D results in decreased intestinal phosphate absorption and thus decreased serum phosphate concentration.

A second major target organ for FGF23 is the parathyroid gland. Fibroblast growth factor-23 directly downregulates PTH mRNA, thus suppressing PTH synthesis.\textsuperscript{39} Given the abundance of klotho in this organ, FGF23 is thought to suppress PTH synthesis in a klotho-dependent manner. However, in klotho-knockout mice FGF23 was still able to suppress PTH production through an alternate pathway.\textsuperscript{40}

Fibroblast growth factor-23 is also implicated in calcium and sodium regulation. FGF23 increases calcium reabsorption via the transient receptor potential vanilloid 5 calcium channel in the distal renal tubule.\textsuperscript{41} This calcium conserving function of FGF23 may help maintain serum iCa concentration despite decreased serum 1,25D concentration. Fibroblast growth factor-23 also stimulates sodium reabsorption via the sodium-chloride cotransporter in the distal renal tubules.\textsuperscript{42} Fibroblast growth factor-23 exerts these effects in a klotho-dependent manner.

Chronic kidney disease-mineral and bone disorder (CKD-MBD) is the syndrome resulting from increased FGF23, phosphate, and PTH concentrations combined with 1,25D and hypocalcemia. Clinically, this syndrome is characterized by bone abnormalities and vascular calcification, in addition to the aforementioned laboratory abnormalities.\textsuperscript{7} Increased FGF23 allows for the maintenance of serum phosphate concentration despite declining renal mass which comes at a cost. It is independently associated with risk of CKD progression, cardiovascular complications, and all-cause mortality in people with CKD-MBD.\textsuperscript{23,27,42-45}
Decreased GFR from loss of functional nephron mass results in a decreased filtered load of phosphate and reduced urinary phosphate excretion, and subsequent hyperphosphatemia. This is offset by increases in FGF23 and PTH secretion to increase phosphaturia. Decreased circulating klotho further increases phosphate retention in early CKD by creating renal resistance to FGF23. This system is efficient at maintaining normal phosphate concentration until advanced CKD develops. While FGF23 inhibits PTH synthesis, in advanced CKD, the parathyroid glands become resistant to FGF23, contributing to the development of renal secondary HPTH. This is brought about by a combination of klotho deficiency, 1,25D deficiency, and decreased klotho/FGF receptor expression in the parathyroid gland. In people, renal secondary HPTH is associated with increased risk of mortality and cardiovascular outcomes.\(^9,10,46\) The main complication of renal secondary HPTH is high-turnover bone disease known as renal osteodystrophy, which results from chronic osteoclastic bone resorption.\(^46\) This leads to bone pain, bone fragility, and increased risk of fracture.

Heart failure is the leading cause of death in people with CKD, with vascular calcification (VC) being a prognostic marker of cardiovascular mortality associated with left ventricular hypertrophy (LVH) and cardiovascular events.\(^47-50\) Vascular calcification is the result of abnormal calcium and phosphate deposition in blood vessels, valves, and heart. Hyperphosphatemia directly induces VC by its action on the type III sodium-dependent phosphate cotransporter PiT-1 on vascular smooth muscle cells.\(^51,52\) In vitro studies in human and mice have not demonstrated clear evidence as to whether FGF23 acts directly on vascular cells to promote or inhibit calcification.\(^53-57\) However, there is
clear evidence that klotho is protective against VC in CKD, and thus klotho deficiency is considered a major inducer of VC in this disease state.58,59

Cardiac remodeling characterized by left ventricular hypertrophy (LVH) is another risk factor for heart failure.60,61 The hypertrophic effect of FGF23 on cardiomyocytes has been demonstrated both in vitro and in vivo. This hypertrophic effect occurs independently of klotho, which is not expressed on cardiomyocytes, through a PLCy-calcineurin-NFAT pathway rather than the MAPK cascade through which FGF23 typically works.62 Left ventricular hypertrophy develops as FGF23 concentration increases and there is conflicting evidence that it is associated with increased PTH concentration as well.63,64 In people with heart failure of various etiologies, increased PTH and decreased 25D were independently associated with all cause and cardiovascular mortality.65 Experimental studies have demonstrated the protective effect the vitamin D receptor activator paricalcitol has on the prevention of cardiac hypertrophy.66

Increased plasma FGF23 concentration appears to be an early biomarker of mineral disturbances in people with CKD-MBD, but there is also experimental evidence in a rat CKD model that FGF23 administration causes LVH.62 In experimental rodent models of CKD-MBD, chronic administration of a neutralizing FGF23 antibody increased serum 1,25D, decreased PTH, and improved bone parameters but these subjects developed hyperphosphatemia, increased mortality, and VC.67 Thus, FGF23’s protective effect against hyperphosphatemia may be more important in decreasing all-cause mortality in patients with CKD-MBD.
Fibroblast growth factor-23 has been studied in cats with and without CKD, and results are consistent with what has been reported in human literature. Plasma FGF23 concentration increased with increasing stage of CKD and was an independent predictor of mortality in cats with CKD.\textsuperscript{29,68} Fibroblast growth factor-23 concentration in non-azotemic geriatric cats predicted future development of azotemic CKD in this population.\textsuperscript{69}

A human-specific FGF23 ELISA was validated for measurement of feline plasma FGF23. A reference interval from a population of healthy geriatric cats was 56-700 pg/mL.\textsuperscript{68} The FG23 concentrations from this population are higher than has been reported in people and may reflect different dietary phosphorus intake between the two species.\textsuperscript{70,71} The recommended daily allowance for phosphorus in humans >51 years old is 35 mg/100 kcal, whereas commercial adult maintenance feline diets have an average 250 mg/100 kcal (range, 135-600 mg/100 kcal) phosphorus. This chronic increase in dietary phosphorus intake may explain the higher reference range for feline plasma FGF23.

Plasma FGF23 concentration was an early predictor for the development of future CKD in cats. In one study, FGF23 concentration was higher in geriatric cats that went on to develop azotemic CKD over a 12-month period, as compared to geriatric cats that did not develop azotemia over the same time period.\textsuperscript{69} In cats with CKD, plasma FGF23 concentration increased in proportion to decreasing GFR, which partially paralleled serum creatinine concentration.\textsuperscript{68} Fibroblast growth factor-23 concentration was significantly increased with increasing severity of CKD as defined by IRIS stage.\textsuperscript{68}
Plasma FGF23 concentration was higher in hyperphosphatemic cats with CKD compared to normophosphatemic cats within the same IRIS stage. Fifty percent of normophosphatemic cats had FGF23 concentrations above the reference range, suggesting they were able to maintain a normal phosphate concentration at the expense of increased FGF23. As expected, there was also a positive linear association between increased FGF23 and PTH concentrations in cats with CKD.

Prior to the discovery of FGF23, serum phosphate was considered the most important predictor of survival in cats with CKD, with every 1 mg/dL increase in phosphate increasing risk of progression (defined by >25% increase in creatinine over baseline) by 41%. There was a clear concentration-dependent relationship between plasma FGF23 and survival time in cats with CKD, with FGF23 >10,000 pg/mL associated with an almost four-fold increased likelihood of death. This association between FGF23 concentration and all-cause mortality remained significant in multivariate analysis, whereas phosphate concentration was only significant in univariate analysis. This suggests plasma FGF23 may be a better predictor of disease progression and mortality in cats with CKD and illustrates the need for additional studies in domestic species, including dogs.

Clinical studies in cats with CKD have shown a clear association between feeding veterinary therapeutic renal diets and decreases in serum phosphate, PTH, and FGF23 concentrations. These diets contain 80-130 mg/100 kcal phosphorus. In one recent study, cats with stable CKD that were fed a phosphorus- and protein-restricted veterinary therapeutic renal diet over 4-8 weeks had significant decreases in serum phosphate, PTH,
and FGF23 concentrations as compared to cats eating maintenance diets. Even cats that were normophosphatemic at the start of the diet trial ended up having decreased phosphate concentration. In a different study, cats with stable CKD fed a phosphorus-restricted diet had significantly decreased plasma phosphate and PTH concentrations after several months. Further proof of this concept was demonstrated in a different study in which cats with CKD fed a veterinary therapeutic renal diet had increased survival, attributed in part to improvements in both serum phosphate and PTH concentrations. This would suggest veterinary renal diets are beneficial in cats with azotemic CKD irrespective of baseline phosphate concentration.

A growing body of evidence shows that FGF23 plays a central role in the regulation of mineral metabolism in cats and people with CKD-MBD. Fibroblast growth factor-23 was a more sensitive indicator of disease progression and mortality than phosphate or PTH in cats with CKD-MBD. Increased FGF23 concentration is correlated with survival in humans and cats with CKD, so further research is warranted as to whether this is just a prognostic biomarker or could be targeted therapeutically as well. Little work has been done on FGF23 or other phosphatoninins in veterinary medicine, and there is a paucity of information on FGF23 in dogs.

We hypothesized that plasma FGF23 would be increased in dogs with CKD compared with healthy adult dogs, and that FGF23 concentration would increase with increasing severity of CKD as defined by IRIS stage. Additionally, we hypothesized that there would be a positive association between plasma FGF23 and serum creatinine, phosphate, and PTH concentrations.
Chapter 2: Fibroblast growth factor-23 in dogs with chronic kidney disease

Client-owned dogs diagnosed with CKD were prospectively recruited from the patient population referred to The Ohio State University Veterinary Medical Center (OSU-VMC) between January 2014 and July 2015. A diagnosis of CKD was made based on the presence of repeatedly minimally concentrated urine [urine specific gravity (USG) <1.030], ± renal proteinuria, ultrasonographic renal abnormalities, or azotemia in the absence of other diseases likely to cause polyuria or polydipsia. This definition of CKD is derived from the International Renal Interest Society (IRIS) guidelines, with azotemia defined as a serum creatinine ≥1.4 mg/dL. Ultrasonographic renal findings consistent with CKD included decreased corticomedullary distinction, and/or small, irregular kidneys.18 Controls consisted of OSU-VMC staff- and student-owned dogs deemed healthy on the basis of history, physical examination, and a normal complete blood count (CBC), serum biochemistry profile, and urinalysis with a USG > 1.030.

Dogs with CKD were classified into four stages as defined by IRIS guidelines based on serum creatinine concentration: Stage 1: <1.4 mg/dL, Stage 2: 1.4-2.0 mg/dL, Stage 3: 2.1-5.0 mg/dL, and stage 4: >5.0 mg/dL. They were substaged as defined by IRIS guidelines based on urine protein-to-creatinine ratio (UPC) and blood pressure. For UPC, dogs were considered non-proteinuric with a UPC <0.2, borderline proteinuric with a UPC of 0.2-0.5, and proteinuric with a UPC of >0.5. Dogs were considered hypertensive when systolic blood pressure was ≥150 mm Hg. Dogs were further divided
into normophosphatemic or hyperphosphatemic based on opinions from an expert panel suggesting that maintenance of serum phosphate within the following ranges is optimal management for dogs with CKD: 2.5-4.5 mg/dL for dogs with stages 1 and 2 CKD, 2.5-5.0 mg/dL for stage 3, and 2.5-6.0 mg/dL for stage 4.¹⁹

Dogs < 1 year of age, those diagnosed with concurrent diseases or receiving medications known to affect PTH concentration was excluded. Dogs diagnosed with acute kidney injury or suspected of acute exacerbation of CKD were excluded. Ethical approval for this study was obtained from the Institutional Animal Care and Use Committee and The Ohio State University’s Clinical Research Advisory Committee. All owners signed a consent form before dogs were enrolled in the study.

Each CKD dog had a complete physical examination performed and a systolic blood pressure measured via Doppler. Blood was anaerobically collected via jugular venipuncture, using a vacutainer, into red-top and EDTA-containing tubes. The CBC, serum biochemistry profile and serum iCa were performed using Advia 2120i Hematology Analyzer, Roche c501 Automatic Analyzer and Nova CCX electrolyte analyzer, respectively. Serum was harvested within 30 minutes of collection. Samples for iCa were handled anaerobically, and as such, pH correction equations were not used to correct for loss of CO₂ from the samples. Urine was collected via cystocentesis for urinalysis and UPC. Additional serum and plasma samples were aliquoted and stored at -80°C for PTH and FGF23 analysis, respectively. Information regarding medications, diets, and dietary supplements were recorded.
Serum whole PTH concentration was measured with a human-specific immunoradiometric assay. EDTA plasma FGF23 concentration was measured with a human-specific intact FGF23 ELISA. In order to achieve a reading on the standard curve, canine samples were diluted with the zero standard supplied with the assay. Precision and reproducibility were assessed by measuring intra- and interassay coefficients of variation (CV) in canine samples with low and high FGF23 concentrations.

Results are presented as median and range. Due to positive skewness and nonconstant variance, concentrations were log transformed prior to parametric modeling. FGF23 and PTH were compared by IRIS stage using a linear model with heterogeneous variance. The Tukey-Kramer adjustment was used to control for multiplicity. Pearson’s correlation coefficients were used to assess the relationships between variables. A multivariable model for FGF23 was created by utilizing a backwards variable selection method. All analyses were conducted in SAS v9.4 or Stata v13.1.

The FGF23 assay was performed using canine plasma, with all samples run in duplicate. The lowest and highest standards supplied with the ELISA were 0 and 800 pg/mL, respectively. Sample dilution (up to 1:128) was required to measure FGF23 concentrations in CKD dogs. Samples outside the standard curve were serially diluted before inclusion in the assay. The intra-assay coefficient of variation (CV) for samples measuring 223, 274, 3789, and 7242 (n=4) pg/mL FGF23 were 4.8%, 2.2%, 1.2%, and 7.2%, respectively. The interassay CVs for samples measuring 240, 289 (n=6), and 360 (n=4) pg/mL FGF23 were 6.7%, 3.1%, and 6.1%, respectively.
Thirty-two dogs met the inclusion criteria for enrollment in the CKD group. Median age was 10.5 years (range 3.1-15.7 years). The most common breeds were mixed breed (n=14) and Labrador retriever (n=2) and one each of the following breeds: Australian Cattle Dog, Boxer, Doberman Pinscher, Fox Terrier, German Shepherd Dog, Golden Retriever, Greyhound, Jack Russell Terrier, Miniature Schnauzer, Pekingese, Pomeranian, Shih Tzu, Viszla, Weimaraner, Welsh Terrier, and Whippet. Twelve castrated males, one intact male and 19 spayed female dogs were included. In accordance with the IRIS staging system, dogs were classified as stage 1 (n = 7), stage 2 (n=9), stage 3 (n=10), or stage 4 (n=6).

Ten healthy dogs were enrolled in the control group. The median age was 4.3 years (range 1.4-10.3 years) and represented breeds included mixed breed (n=4), Pitbull terrier (n=3), German Shepherd Dog (n=2) and Rottweiler (n=1). Six dogs were castrated males and four were spayed females.

Not all CKD dogs were enrolled at the time of diagnosis of CKD, and due to ethical considerations, treatment was not withheld prior to enrollment. These patients were receiving a variety of medications including enalapril (n=8), antibiotics (n=5), aluminum hydroxide (n=5), famotidine (n=5), tramadol (n=5), gabapentin (n=4), omeprazole (n=4), amlodipine (n=3), ondansetron (n=3), subcutaneous fluids (n=3), diphenhydramine (n=3), heartworm prevention (n=3), phenylpropanolamine (n=1), diethylstilbestrol (n=2), non-steroidal anti-inflammatories (n=2), ectoparasite prevention (n=2), mirtazapine (n=1), sevelamer (n=1), levothyroxine (n=1), trazodone (n=1), aspirin (n=1), and cyclosporine (n=1). The CKD dogs were fed a variety of diets, with 14 (44%)
eating a veterinary therapeutic renal diet, 2 (6%) a home cooked diet and 16 (50%) other commercial dog diets. All control dogs were fed commercial dog diets and 8/10 were receiving heartworm and ectoparasite preventatives.
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Table 1. Characteristics of dogs classified as having chronic kidney disease
Details regarding IRIS stage and substages of each enrolled dog are outlined in Table 1. Median serum creatinine for CKD dogs was 2.3 mg/dL (range, 0.6-12.9 mg/dL) (Table 2). Median serum phosphate concentration for CKD dogs was 4.5 mg/dL (1.6-14.4 mg/dL). According to previously defined optimal serum phosphate concentrations based on IRIS stage,19 41% (13/32) of dogs with CKD had hyperphosphatemia. Dogs with IRIS stage 3 CKD were subdivided into normophosphatemic (stage 3a; n=5) and hyperphosphatemic (stage 3b; n=5) groups. Median serum phosphate concentration in stage 3a was 3.4 (2.7-4.8) mg/dL and stage 3b dogs was 6.2 (5.6-6.6) mg/dL. Dogs in IRIS stages 2 and 4 were not subdivided because most dogs with stage 2 CKD were normophosphatemic (7/9) and most dogs with stage 4 CKD were hyperphosphatemic (5/6).
<table>
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<th>Laboratory Parameter and Reference Range</th>
<th>Control (n=10)</th>
<th>All CKD Dogs (n=32)</th>
<th>Stage 1 (n=7)</th>
<th>Stage 2 (n=9)</th>
<th>Stage 3 (n=10)</th>
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<td>FGF23 (pg/mL)</td>
<td>315 (211-449)</td>
<td>582 (142-41,265)</td>
<td>338 (221-684)</td>
<td>336 (142-704)</td>
<td>2,302 (455-24,409)</td>
<td>7,733 (2,520-41,265)</td>
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<td>PTH (0.5-5.8 pmol/L)</td>
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<td>1.7 (1.2-3.4)</td>
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Table 2. Comparison of creatinine, phosphate, FGF23, PTH, and calcium concentrations in control and CKD dogs. Results are presented as median and range.

Median (range) plasma FGF23 concentration in all CKD dogs was higher (582, 142-41,265 pg/mL) than in healthy dogs (315, 211-449 pg/mL) (Figure 1). Fibroblast growth factor-23 concentration was significantly lower in control dogs as compared to those with IRIS stages 3 and 4 (P <.0001). Using the upper FGF23 concentration from healthy dogs as the cutoff to define high FGF23 concentrations (449 pg/mL), 59% (19/32) of CKD dogs had increased plasma FGF23 concentration. Plasma FGF23 concentration increased with IRIS stage (stage 1: 338 [221-684]; stage 2: 336 [142-704];
stage 3: 2,302 [445-24,409]; stage 4: 7,733 [2,520-41,265]) pg/mL. Plasma FGF23 concentration was significantly increased in dogs with IRIS stages 3 and 4 as compared to stages 1 and 2 (P < .0001).

Figure 1. Box and whisker plot illustrating the plasma FGF23 concentration based on IRIS stage and in healthy control dogs. The boxes represent the 25th and 75th percentiles and the central lines in the boxes represent the median values. The whiskers represent the range of concentrations. Dots represent outliers. The scale for FGF23 is logarithmic. Asterisks represent statistically increased FGF23 concentration in dogs with IRIS stages 3 and 4 as compared to control dogs and those with stages 1 and 2 (P < .0001).
Plasma FGF23 concentration was positively correlated with creatinine (r = 0.87, P < .0001) (Figure 2) and phosphate (r = 0.68, P < .0001) concentrations (Figure 3). The estimated fold difference between FGF23 concentration in stage 3a versus 3b was 2.53 (95% confidence interval [CI]: 0.49, 13.06), but there was not a statistically significant difference between these two groups (P=.23) (Figure 4). There was no correlation between FGF23 and either total (P = .24) or iCa (P = .27).
Figure 3. Scatterplot illustrating plasma FGF23 concentration by serum phosphate concentration. The scales for both axes are logarithmic. Plasma FGF23 concentration was positively correlated with phosphate concentration (r=0.68, P <.0001).

Serum PTH concentration in all CKD dogs was 2.8 (0.9-229) pmol/L as compared to healthy dog PTH concentration of 1.1 (0.7-7.8) pmol/L (Figure 5). Plasma FGF23 concentration was positively correlated with serum PTH concentration in CKD dogs (r = .74, P <.0001) (Figure 6).
Figure 4. Scatterplot illustrating plasma FGF23 concentration by serum PTH concentration. The scale for FGF23 is logarithmic.

PTH concentration increased with IRIS stage (stage 1: 1.6 [0.9-5.2]; stage 2: 1.7 [1.2-3.4]; stage 3: 4.5 [1.6-14.2]; stage 4: 32.2 [5.1-229] pmol/L. Serum PTH concentration was significantly increased in stages 3 and 4 than all other stages and PTH concentration was significantly increased in stage 4 versus stage 3 dogs (P < .05). Eight CKD dogs had PTH concentrations above the upper limit of the laboratory’s reference range of 5.8 pmol/L. Of these dogs, three had stage 3 and five had stage 4 CKD.
Figure 5. Box and whisker plot illustrating serum PTH concentration based on IRIS stage (1-4) and healthy control dogs. The boxes represent the 25th and 75th percentiles and the central lines in the boxes represent the median values. The whiskers represent the range of concentrations. Dots represent outliers. The scale for PTH is logarithmic. The single asterisk represents a significantly increased PTH in dogs with IRIS stages 3 as compared to control dogs and those with IRIS stages 1 and 2 (P < .05) CKD. The double asterisk represents significantly increased PTH in dogs with IRIS stage 4 than all other groups (P < .05).

One control dog had a PTH concentration above the upper limit of the laboratory’s reference range (7.8 pmol/L). There was no additional serum available to recheck this value from the time of enrollment; however, 18 months later, the dog had a normal serum PTH concentration of 1.2 pmol/L. Removing this patient from the population did not significantly alter PTH results. Using this repeated value, median PTH concentration for control dogs would be 1.1 pmol/l (range, 0.7-1.8 pmol/l).

Comparing PTH concentrations of the CKD dogs to the upper range of the control dogs
(i.e., using a cut-off of 1.8 pmol/l), our data would suggest that 22/32 (69%) CKD dogs had hyperparathyroidism with a median PTH concentration of 5.0 pmol/l (range, 2.1-229 pmol/l). These included dogs with all stages of CKD: stage 1 (n=3); stage 2 (n=4), stage 3 (n=9), stage 4 (n=6).

Serum creatinine, phosphate, calcium and PTH were assessed in the multivariable model to predict FGF23 concentration. Of these, creatinine (P < .0001) and phosphate (P = .01) were retained in the final model (r² = 0.79). From this model, a doubling of creatinine was associated with a 150% (95% CI: 97%, 218%) increase in FGF23 and a doubling of phosphate with a 78% (95% CI: 15%, 176%) increase in FGF23 (Figure 8).

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<td>(mg/dL)</td>
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Table 3. Multivariable linear regression model to identify predictors of plasma FGF23. R² model = 0.79 (P<0.0001).

This study showed that plasma FGF23 concentration is increased in dogs with CKD and that FGF23 concentration increases with the degree of severity as assessed by
the IRIS staging system. The positive association between FGF23 concentration with serum creatinine, phosphate, and PTH concentrations supports a possible role of FGF23 in the pathogenesis of canine renal disease.

Under physiological conditions, FGF23 suppresses PTH and 1,25D secretion to reduce serum phosphate concentration. Fibroblast growth factor-23 activity requires the co-factor klotho. Klotho expression is decreased in CKD patients and results in an early reduction in serum klotho concentration. This early reduction in circulating klotho down-regulates the klotho-FGF receptor complexes in the parathyroid gland so that PTH synthesis is no longer adequately suppressed. Renal secondary HPTH occurs when PTH synthesis and secretion become excessive as a result of parathyroid gland hyperplasia and hypertrophy. Finding increased PTH concentration despite the elevated FGF23 concentration reported in this study suggest that FGF23 resistance is involved in the role of renal secondary HPTH in dogs. It is possible that PTH concentration would have been even higher without the documented increased FGF23 concentration. We attempted to measure serum, plasma, and urine klotho concentrations in this CKD cohort and healthy dogs; however, we were not able to detect measurable concentrations of klotho in any dog using a commercially available α-klotho ELISA kit.

While no significant differences in FGF23 concentration was found between hyperphosphatemic dogs with stage 3b versus normophosphatemic dogs with stage 3a, the trend for increased FGF23 in 3b dogs suggests that a larger number of dogs in these categories will provide further insight on the transition of CKD and how it affects FGF23 and the development of hyperparathyroidism. There was a tendency for FGF23
concentrations to be increased in stage 3b dogs as compared with stage 3a dogs, with a fairly large estimated fold difference between subgroups, but there were too few cases and too much variability to detect a significant difference. A larger cohort would be needed to determine whether there is in fact a significant increase in FGF23 concentration in hyperphosphatemic dogs within the same IRIS stage.

Depending upon which cut-off for increased PTH was used, the prevalence of renal secondary HPTH in this study was either 25% or 69%. There have been conflicting results about the prevalence of hyperparathyroidism in CKD dogs and cats. In a previous study, hyperparathyroidism was documented in 76% of dogs with CKD, including animals in IRIS stage 1. In cats, onset of renal secondary HPTH, defined by increased PTH, has been documented even prior to the onset of azotemia. It has also been reported that PTH concentration does not increase significantly in cats until end-stage renal disease is present. The discrepancies in these results may be the reflection of different laboratory reference ranges amongst studies or a reflection of patient variability we have yet to understand. Additionally, it has been shown that there is significant diurnal PTH variability in healthy dogs, with a distinct peak at 7:00 am. Longitudinal studies would be beneficial in determining the onset of hyperparathyroidism as most studies, including this one, only looked at a single time point. It should also be clearly documented at what time of day the samples are collected.

The multivariable regression analysis showed that creatinine and phosphate concentrations were independent predictors of plasma FGF23 concentration. These factors explained 79% of the variability in logFGF23 concentration. Interestingly, PTH
and calcium concentrations were not significant predictors in the presence of creatinine and phosphate. This model suggests we have identified the main variables influencing canine plasma FGF23 concentration.

The human intact FGF23 double antibody sandwich ELISA used in this study can measure canine FGF23 concentration over a wide range of values and could be valuable to assess disease status and progression in the clinical setting because of the fact that FGF23 concentration increased during progressive stages of CKD and concentrations were similar to those reported in cats with CKD, suggest that it is measuring canine FGF23. More rigorous standardization of this assay is warranted prior to clinical use, as CVs were from samples at all extremes of the standard curve instead of high on the standard curve (600-800 pg/mL).

There are a few limitations to note. Glomerular filtration rate (GFR) was not measured, making it is possible that some dogs were incorrectly classified as having CKD. Dogs were enrolled before the symmetric dimethylarginine (SDMA) test was routinely being performed on dogs. The majority of these patients had concurrent systemic hypertension, persistent renal proteinuria, or ultrasonographic changes consistent with CKD. Within IRIS stage 1, 2/7 dogs were enrolled solely based on repeated isosthenuria over at least 3 months. Within IRIS stage 2, 3/9 dogs did not have proteinuria nor was an abdominal ultrasound performed in these individuals for further evidence of CKD. In contrast to reports in humans and cats, Stage 1 dogs did not have significantly increased FGF23 concentrations than healthy control dogs. This could also be due to the incredibly high dietary phosphate intake in normal dogs increasing FGF to a higher baseline making
it harder to see an increases in IRIS stage 1. This may support an inappropriate diagnosis of CKD in the current study or may be a reflection of the small study population negatively impacting the ability to derive statistically significant differences between groups.

It would have been ideal to include dogs prior to initiating therapy, but due to ethical implications of withholding treatment, many CKD dogs were already receiving medications or veterinary therapeutic diets at the time of enrollment. Many of these therapies are known to affect phosphate homeostasis, including veterinary therapeutic renal diets (n=14) and intestinal phosphate binders (n=6). It has been shown in cats that eating reduced phosphorus diets can lower plasma FGF23. Since only one time-point was collected, it was impossible to determine if these treatments decreased FGF23 concentration in individual dogs. Additionally, venipuncture was not consistently performed on fasted patients, which may affect phosphate concentration. Despite this variability, there was no significant difference in FGF23 concentration based on diet or phosphorus binders within IRIS stage.

One control dog initially had an increased PTH concentration of 7.8 pmol/l. He was a young dog without documented hypercalcemia; thus, primary hyperparathyroidism was unlikely. He was eating a complete and balanced diet; thus, nutritional secondary hyperparathyroidism was unlikely. His serum PTH concentration was repeated approximately 18 months after initial enrollment due to lack of available serum from the first time-point. It was normal at 1.2 pmol/L. We hypothesize that since this dog’s blood was initially collected early in the morning that the PTH value was subsequently was influenced by diurnal variation. For reference, blood collected for recheck purposes was
collected at 11:00 am. It is unlikely that this diurnal variability influenced any of the CKD dogs as these dogs were typically presented to the hospital for late-morning or early afternoon appointments and their blood was collected midday. This finding highlights the variability in canine PTH concentration.

In conclusion, plasma FGF23 concentration increased as IRIS stage advanced in dogs with CKD. Our results indicate that increased FGF23 concentration occurs in early in canine CKD, and therefore can be used as a biomarker of disease progression as in other species. Whether increased FGF23 concentration in dogs with CKD merely reflects decreased GFR, with increases in total body phosphate burden, or contributes to progression of CKD and or mortality in dogs with CKD has yet to be established. It remains to be determined if FGF23 concentration affects survival and progression of canine CKD or if there may be a benefit to use FGF23 as a therapeutic target in this species.
References


34 Ito N, Fukumoto S, Takeuchi Y, et al. Effect of acute changes of serum phosphate on fibroblast growth factor (FGF)23 levels in humans. J Bone Miner Metab. 2007;25:419-


