SLED DOGS AS A MODEL FOR STUDYING DIETARY VITAMIN D

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Abstract

Vitamin D deficiency (VDD) has become a pandemic and has shown to be correlated with several poor health outcomes. Many factors that lead to VDD are environmental and lifestyle. Vitamin D has physiological implications involved in all areas of human health and is also important for animal health. Canines have shown adverse health outcomes similar to humans that correlate with vitamin D deficiency such as chronic kidney disease (CKD) and irritable bowel disease (IBD). Canine vitamin D requirements are largely unknown due to the lack of research and the wide ranges of supplementation throughout dog food manufacturers. Pre-active plasma vitamin D metabolites are used as the biomarker of vitamin D status in humans and dogs but may not be representative of overall vitamin D status. Therefore, other biomarkers representing vitamin D status are often used in conjunction to determine physiological relevance. To address this gap in knowledge, this study used parathyroid hormone concentrations as well as vitamin D binding protein concentrations to establish more of an overall status of vitamin D. In canines, clinical supplementation following VDD is usually administered orally with vitamin D olive oil tablets; however, supplementation is usually unsuccessful. Vitamin D and its metabolites are lipid soluble and stored in adipose tissue. Although few foods provide appreciable levels of vitamin D, wild salmon contain some of the highest dietary vitamin D levels. People living in Alaska are at an increased risk of VDD due to reduced zenith sun angles for much of the year. Consequentially sufficient vitamin D levels need to be acquired through diet or supplementation. Historically, Alaska Natives obtained sufficient amounts of vitamin D from traditional subsistence foods, but with the progressive shift away from these foods VDD has increased in Alaskan populations. The limited research available suggests that Alaskan sled dogs in particular are a group found to be generally VDD. Sled dogs are an important part of the traditional Alaska subsistence lifestyle and have evolved alongside humans in the circumpolar north. Sled dogs, therefore, provide a valuable model for studying health outcomes associated with VDD in both people and dogs in the far north. This study provides significant evidence showing wild Alaskan salmon as a dietary source of supplementation to raise 25(OH)Vitamin D serum in dogs after only 4 weeks. We also show significance in variation by confounding factors, age and sex.

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Chapter 1: Introduction

Vitamin D deficiency (VDD) has become a worldwide pandemic with over 1 billion people being affected; many of these deficiencies are linked to lifestyle and environmental factors. VDD has been associated with 18 different types of cancers and strong evidence supports the anti-tumorigenic effects of vitamin D. Many studies support the idea of cancer prevention through maintenance of sufficient vitamin D levels. Alaska Native populations, considered at risk of VDD, in the past have fulfilled their requirements of vitamin D through the use of their sustainable diets, which include high quantities of oily, fatty fish. Alaskan canines poor health outcomes are similarly correlated with VDD, underscoring the importance of vitamin D in our domesticated companion and working animals.

1.1 Vitamin D Metabolism & Physiology

Vitamin D, a steroid hormone, is crucial in most tissues of the body and plays important roles in many physiological processes. As humans, we are able to synthesize vitamin D from its precursor, 7-dehydrocholesterol, found in epidermal layers through exposure to UVB photons (290-320 nm). Once synthesized, the vitamin D precursor binds to circulating vitamin D binding protein (DBP) which delivers it to the liver where it is metabolized by vitamin D 25-hydroxylase (CYP2R1 and CYP27A1). When complete the vitamin D metabolite, now 25(OH)D, is able to bind again to the DBP for transport to the kidney. In the kidney proximal tubule, the vitamin D precursor is hydroxylated a second time by 1-alpha-hydroxylase (CYP27B1) to produce the most biologically active vitamin D metabolite, 1,25(OH)2D or calcitriol. The DBP is suggested to transport 95-99% of vitamin D metabolite, while the remaining vitamin D is transported by the minor transport proteins, albumin, and lipoproteins.[1] Biologically active calcitriol enters circulation, being picked up again by the DBP for delivery to target tissues. Cells of target tissues harbor vitamin D receptors (VDR) that are capable of binding active 1,25(OH)2 D to produce both genomic and non-genomic biological functions.[2] Tight regulation of vitamin D activation is accomplished by the 25(OH)D 24-hydroxlyase enzyme (CYP24A1), which produces inactive calcitroic acid to be excreted. This enzyme has shown major developmental importance, a CYP24A1 knockout mice model resulted in 50% perinatal death.[3] Along with this enzymatic negative feedback regulation, metabolism of vitamin D is also largely affected by the parathyroid hormone (PTH). When calcium levels are low, parathyroid cells are signaled and in turn increase the production of PTH. In circulation, the PTH is able to stimulate renal expression of CYP27B1 enzyme to increase active vitamin D production.[2]

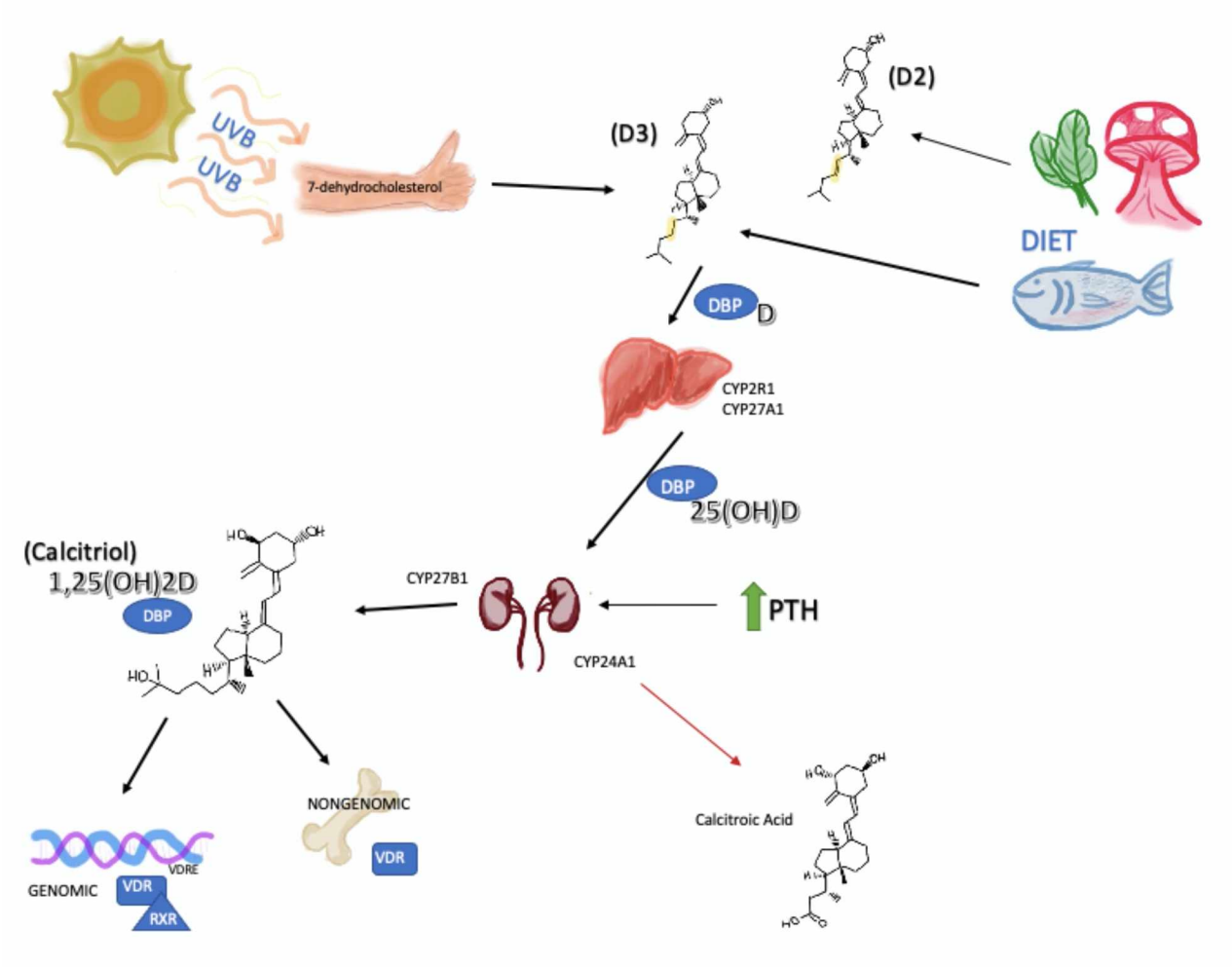

Figure 1.1. Metabolism of Vitamin D from different sources. Synthesis of the skin converts 7-dehydrocholesterol to vitamin D3 via UVB photons. Plants from the diet supply individuals with vitamin D2 through ingestion and oily fish or other animal sources provide individuals with vitamin D3. The DBP transfers metabolite from skin synthesis or diet to the liver where the first hydroxylation event occurs via the CYP2R1 or CYP27A1 enzymes. Hydroxylated vitamin D is then carried by the DBP to the kidneys where the second hydroxylation event occurs via CYP27B1 to create biologically active 1,25(OH)2D. The active metabolite may then use the VDR for local nongenomic effects or use the VDR-RXR complex to translocate into the nucleus and have genomic effects through DNA vitamin D response elements (VDRE). Negative feedback causes excretion of vitamin D in the form of calcitroic acid when the pre-cursor metabolite (25(OH)D) is transformed by the CYP24A1 enzyme in the kidneys. An increase in parathyroid hormone in circulation will result in an increase production of calcitriol via CYP27B1 enzyme in the kidney.

The DBP has 10- to 100- fold higher affinity for the 25(OH)D metabolite compared to the biologically active metabolite, 1,25(OH)2D, due to the binding cleft structure of the DBP and the "free hormone" hypothesis. This hypothesis states that the higher affinity, protein bound metabolite is used as

a circulatory reservoir for delivering local vitamin D to target tissues and cells.[1] Even though the DBP is crucial for vitamin D transportation, current studies fail to link vitamin D status with alterations in DBP concentrations. The DBP has a short half-life, suggesting high daily production. When enzymatically transformed to DBP-MAF (macrophage activating factor), the DBP may influence the function of macrophages and osteoclasts, similar to vitamin D's role in bone and immune health. In cancer, vitamin D is suggested to regulate tumorigenesis progression,[2] when the DBP-MAF is stimulated it acts by attacking growing malignancy.[1] The DBP is also largely involved in the clearance of actin filaments and the prevention of reformation in the event of severe injury.

Vitamin D acts through its two receptors in order to have genomic and non-genomic actions; the nuclear vitamin D receptor is part of a superfamily of nuclear receptors and is found in the nucleus (nVDR), whereas the membrane receptors are found on the plasma membrane (mVDR).[4] VDR is found in almost every tissue of the body, but the best understood target tissues of active vitamin D include intestine, bone, and kidney, where vitamin D has a range of biological functions through genomic and nongenomic actions. The biologically active vitamin D metabolite (1,25(OH)2D) has 100- fold higher affinity than its precursor 25(OH)D for the VDR.[1] It's been suggested that the VDR has the ability to regulate up to 1,250 genes, up to 5% of the genome.[5] In the genomic pathway, vitamin D binds to the nVDR of target cell then through phosphorylation the receptor heterodimerizes with the retinoid-X receptor (RXR). In the nucleus the complex binds to DNA at the vitamin D response element (VDRE) and recruits transcriptional factors to regulate gene expression through activation or suppression of target genes. This process can have a range of functions through the body including the metabolism of both calcium and phosphorus.[2] In the nongenomic pathway, vitamin D still binds to the VDR of target cells, but instead of heterodimerization the VDR acts as a rapid response steroid-binding protein (1,25D-MARRS) to create changes in cellular signaling pathways. Nongenomic actions include the rapid generation of messenger molecules, and the rapid opening of calcium channels.[6] The VDR is found in most cancerous tissues and in animals, active vitamin D metabolites are found to inhibit cell proliferation, angiogenesis, invasion, and is able to promote cell differentiation and apoptosis.[7] Vitamin D affects almost every tissue in the body, as is evidenced by the observations of the VDR in immune, muscle, endothelium, myocardium, vascular smooth muscle, neurons, osteoblasts and adipose tissue cells.[6]

Chronic inflammation can lead to disease and is correlated with poor health outcomes.[8] Data suggests that vitamin D has the ability to exert anti-inflammatory effects through at least four mechanisms including: inhibition of the prostaglandin pathway, suppression of p38 MAPK-mediated signaling pathway, inhibition of NFkB signaling pathway, and through the regulation of immune and cancer cells to suppress cytokine production.[2] With recent research supporting vitamin D as a cancer prevention & treatment option, some researchers have suggested that vitamin D itself is able to regulate the whole

process of tumorigenesis and the steps involved in cancer, including initiation, inflammation, antioxidant defense/DNA repair, promotion, proliferation/differentiation, and even apoptosis. [2] While there are limitations with vitamin-D based cancer therapies, those limitations do not negate the growing evidence supporting creation of therapeutic strategies.

It is unknown what the most effective source of vitamin D supplementation is; we do know that oily fish, and particularly wild Alaskan salmon, remains a "gold standard" for quantity of vitamin D per serving. Wild Alaskan salmon has up to 4 times the amount of vitamin D found in average farmed salmon.[9] The vitamin D from salmon and other animal sources is the same D3 (cholecalciferol) that can be produced in human skin from sunlight exposure, while the vitamin D from most plant sources is D2 (ergocalciferol). It's been suggested that vitamin D3 or cholecalciferol has a higher affinity for the DBP when compared with D2 or ergocalciferol, which could be due to the structure of the molecule or the metabolic pathway.[1] Research has found that vitamin D3 produced from the skin had a 100% binding rate with the DBP found circulating, while ingested vitamin D2 only has 60% respectively.[10] There are many factors that go into the synthesis of vitamin D from the skin, and not all animals are able to synthesize this steroid hormone from the skin; therefore, dietary vitamin D remains important for these populations.

1.2 VDD Risk Factors, Consequences, & Prevention

Most vitamin D deficiencies can be linked to lifestyle or environmental factors, making intervention relatively straightforward. Some researchers suggest that a main factor of deficiency is lack of awareness in at-risk groups.[11] In humans, the lack of skin exposure to sunlight, season, aging, obesity and medications may all be contributing factors of VDD. With skin exposure being a source of vitamin D synthesis, it is intuitive that the tone of skin along with the amount of skin being exposed are potential risk factors for VDD. The time of day and season can also affect our sun exposure due to the zenith angle of the sun; this is why more northern latitudes are found to have higher rates of deficiency. It's even been suggested that air pollution and elevation could play into these risks for developing VDD. Aging, obesity, and other medical dispositions can also be risk factors for VDD due to the internal synthesis of vitamin D. Though, it's been shown that diseases affecting the liver, intestines, or renal diseases all result in lower levels of transport proteins in circulation, resulting in VDD not necessarily from low vitamin D metabolites.[12]

With so many physiological functions, there are consequences when vitamin D levels are deficient in the body, and many of these consequences directly impact the musculoskeletal system. In children VDD can lead to growth retardation and rickets. VDD in adults can lead to osteoporosis, increased risk of fractures, and creates muscle weakness.[13] Non-skeletal consequences of VDD include development of multiple types of cancers, and other physiological problems like diabetes, multiple

sclerosis, and hypertension.[13] Many studies have supported the use of vitamin D and its analogs from prevention and treatment of these chronic diseases and cancers. The administration of vitamin D has been shown to increase survival rates in chronic kidney disease patients, to reduce cardiovascular mortality, and to protect against the progression of age-related diseases like Alzheimer's and age-related macular degeneration of the eyes.[6] Recent studies show the importance of vitamin D in cardiovascular function, particularly important during developmental years. Several epidemiological studies have supported the correlation between VDD and various diseases.[12] The Framingham heart study reported a 60% increased risk of developing heart disease later in life when growing up with a VDD.[14] Epidemiological studies also suggest a correlation between chronically low levels of vitamin D and the development of Parkinson's disease. Vitamin D may even play a role in depression with the evidence of improved depressive symptoms after a year of supplementation with vitamin D.[14] Many major disorders occur due to an imbalance of chemical homeostasis in the body via the generation of reactive oxidative species; VDD leads to decreases in the regulation of these signaling networks, which may cascade into a wide variety of diseases and disorders.[15]

Assimilation of western diets has been a change to many populations and may have been one of the biggest changes to the Alaskan Native (AN) population. A study evaluating diet in women child-bearing age from AN populations between 1960-1990 showed a linear decline in traditional marine food intake that was associated with a significant decline in serum 25(OH)D concentrations between 1960-1970s.[16] It is suggested that the dramatic decline in the consumption of marine foods in the Alaska Native diet was a factor that contributed to the increase of rickets in children the 1990s. Traditionally Alaska Native populations were not reported to have problems with VDD, perhaps because their diet included up to 90% fish, yet this population has become at increasing risk in recent years.[17]

It's been shown that vitamin D improves postural stability and reaction times in older people who fall.[18] This outcome may be associated with vitamin D's role in muscles and bone fitness overall. Furthermore, vitamin D supplementation is known to increase the size and amount of type 2 muscle fibers, associated with fast twitch muscles and fall prevention.[19] The link between vitamin D and fitness has recently been explored in more detail. One of the first studies examining this link found a 56% increase in fitness children who were taught in classrooms with irradiated UVB lights, compared to their average classroom counterparts. Furthermore, when children in the control group were exposed to only 1 dose of 250,000 IU of vitamin D their fitness performance significantly improved.[20] It has also been previously reported that higher vitamin D levels are associated with higher VO2max in athletes.[19] It is likely that athletes of many kinds may be at risk for developing VDD compared to sedentary counterparts. A study done comparing student-athletes and their sedentary counterparts at the same time during the winter living on the same campus revealed that athletes had significantly lower vitamin D concentration

while ingesting significantly more vitamin D.[21] The same study also showed that males had significantly lower concentrations of 25(OH)D when compared with their female counterparts. Similar results have been shown in dogs, where racing sled dogs were generally found to be insufficient or deficient in vitamin D.[22] Endurance athletes in particular may be at risk for developing VDD. Willis suggests that intensive training put athletes at risk for illness and overtraining syndrome; he found a 42% prevalence of VDD in runners.[23] Higher serum 25(OH)D levels in human athletes is associated with reduced injury rates and better sports performances;[11] it is likely that this relationship would follow in other animals.

In 2013 Wacker suggested a 3-part strategy to prevent global VDD: increase food fortification programs with vitamin D, obtain sensible sun exposure, and encourage ingestion of vitamin D supplements.[10] An adequate amount of vitamin D is only found in few foods naturally. After the turn of the 20th century, when almost 85% of children in northern latitudes had rickets, food started being fortified with vitamin D. Milk was one of the first fortified foods made available to the public. While many people still believe that milk builds strong bones, the Vitamin D fortification is actually the cause. We currently have a variety of multivitamins available on the market with anywhere from 400-50,000 IU of vitamin D in both D3 and D2 forms. Prevention of VDD seems within reach of all populations through different measures. Healthy vitamin D levels, as of 2018, range between 75-125 nmol/L; sufficient is 50-70 nmol/L and deficient is any level less than 50 nmol/L.[24] When patients are found to be VDD, clinical supplementations suggest increasing vitamin D doses. Furthermore, it's been suggested that 90% of vitamin D in humans is obtained from the sunlight;[24] in the absence of sun exposure, like Alaska in the winter, it's recommended to take 800-1000 IU of vitamin D daily to maintain sufficient levels.[10] Due to age and skin type being a risk factors, individuals should change their supplementation to accommodate their own risk categories.

1.3 Vitamin D in Canines

Many animals, like humans, are able to synthesize vitamin D from the skin to get their requirements. However, dogs lack the ability to do so and even lack the precursor (7-dehydrocholoesterol) found in skin; thus, all vitamin D requirements come from their diet. It has been suggested that canines in the past were able to maintain vitamin D levels by eating the fat stores of prey animals because vitamin D is stored in adipose tissues.[25] Industrialization resulted in a transition to commercially produced foods as more canines became our domesticated companion animals. One result of this transition is the wide range serum 25(OH)D values in dogs. Research on topic is insufficient: there is little research on the efficacy of which form of dietary vitamin D is most bioavailable to dogs, dogs' general requirements for vitamin D, or the relationship between vitamin D intake and overall health in dogs.[26] The health consequences of VDD overlap with humans and dogs suggests that canines may be a good animal model to study vitamin

D. For example, currently serum 25(OH)D in dogs is being used clinically as a marker for various diseases including chronic kidney disease, primary hyperparathyroidism, irritable bowel disease, induced endotoxemia, and a variety of cancers.[26] Similar to humans, there are confounding factors that could affect the bioavailability and status of vitamin D in dogs. Factors that are known to influence blood 25(OH)D serum in humans and dogs include sex, sex hormone concentration, age, body condition score, muscle condition score, dietary intake, blood calcium levels, as well as blood parathyroid hormone levels.[27]

The lack of research about vitamin D and dogs has resulted in a wide range of vitamin D requirements suggested for dog food companies. The current supplementation range reported by the Association of American Feed Control Officials (AAFCO) from 2007 is 500-5,000 IU/kg/day and the suggestion reported by the Nutrient Requirements for Adult Dogs (NRC) from 2006 134 IU/day with an upper limit range of 800 IU/day. These suggested requirements are based on past literature; however, very few studies have been published as references, and those available lack the information necessary to scientifically correlate canine health based on the vitamin D. A study from 1933 showed that puppies who were receiving low phosphorus and calcium and vitamin D developed rickets, while puppies with "normal" levels of calcium and phosphorus but low vitamin D were protected from developing rickets.[28] Similar results came from other studies done around the same time. However, recent studies showing poor development due to lack of vitamin D have not been shown in dogs. It is unknown how important vitamin D is in dogs, but we do know that VDD is associated with multiple chronic diseases and cancers.

Research regarding vitamin D toxicity in dogs is similarly inadequate to determine optimal canine diet. One study giving puppies 17 times the amount of NRC suggested dose of vitamin D daily found no vitamin D toxicity;[29] however, in 2019 the FDA recalled several dog food products due to potential vitamin D toxicity.[30] Pet owners who's dogs consumed these products witnessed toxic effects 12-36 hours after consumption. Symptoms included vomiting, loss of appetite, increased thirst, increased urination, and weight loss. Given the wide range of suggested vitamin D requirements, dog food companies could unintentionally produce foods that prove harmful to dogs due to improper vitamin D levels. Companies may not know the endogenous levels of vitamin D in their ingredient list, and likely do understand vitamin D metabolism or dietary intake needs. More research in this field is clearly needed.

In critically ill patients, VDD has been used as an independent predictor Similarly, in ICU dogs, VDD was shown to also be a predictor of survival and correlated with illness severity.[31] The dogs from this study that made it out of the ICU had a significant increase in vitamin D levels compared with the dogs that passed away in the ICU. There was a nonlinear relationship found between serum 25(OH)D levels and the vitamin D binding protein (DBP) which could have a potential influence of severity. Research in bioavailability will strengthen our understanding of vitamin D status and overall health in

both dogs and humans. Finally, further research is needed in the relationships between biomarkers and overall vitamin D status. The PTH remains a solid marker in humans and dogs of vitamin D status and deficiency. In dogs this relationship is established as a declined PTH shown to plateau when 25(OH)D levels were 100ng/ml (insufficient).[29] The most physiologically relevant definition for the ideal dose of supplementation and serum concentration of vitamin D for dogs must include a PTH plateau.[25] This plateau is used to define sufficient levels of vitamin D concentration ranges in dogs. Ranges for optimal health of 25(OH)D serum is 100-120 ng/mL and insufficiency at 30-100 ng/mL[32].

Chapter 2: Increases in serum 25(OH)D in sled dogs due to wild Alaskan salmon supplementation[1]

2.1 Abstract

Background: Vitamin D deficiency has become a pandemic; both canines and athletes are high-risk groups. Further research regarding optimal intake and supplementation is needed to establish the importance of Vitamin D status in canines and create a physiological relevant definition for vitamin D health.

Hypothesis/Objectives: (1) Compare the levels of 25(OH)D in plasma of Alaskan sled dogs before and after receiving a diet supplemented with wild Alaskan salmon and (2) correlate biomarkers associated with vitamin D metabolism to 25(OH)D plasma levels in dogs.

Animals: Alaskan sled dogs (n=14) from a kennel in Salcha, Alaska; all working dogs between ages of 10 months and 7 years old with similar body condition scores.

Methods: Plasma samples were collected from sled dogs before and after a 4-week supplementation with approximately 0.45 kg of salmon/dog/day. Samples were analyzed using enzyme-linked immunosorbent assays (ELISA) for parathyroid hormone and vitamin D binding protein; 25(OH)D levels were examined by Michigan State University Veterinary Diagnostic Laboratory using commercial radioimmunoassay (RIA) kits.

Results: Serum 25(OH)D values in sled dogs increased significantly after a 4-week treatment with salmon (P=0.0011), along with increases in DBP (P=0.0367), using 95% confidence intervals. Furthermore, there was a significant difference when separated by sex (P=0.0404). Separation by age also yielded significance among various groups.

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[1]Striker K, Høe-Raitto M, Jerome S , Dunlap KL. Formatted for submission to the Journal of Veterinary Medicine

Conclusions and clinical importance: Wild Alaskan salmon remains an efficient dietary supplement to raise canine serum 25(OH)D concentrations. Confounding factors such as age and sex affect serum 25(OH)D levels in the same population.

2.2 Introduction

Currently over 1 billion people worldwide have a vitamin D deficiency (VDD), with most being attributed to lifestyle or environmental factors.[1] Low vitamin D status in canines has previously been linked to adverse health outcomes, similar to humans; furthermore, sled dogs maintain insufficient levels of circulating 25(OH)D in plasma.[2] Limitations to current knowledge of vitamin D in canines include the unestablished relationship between vitamin D intake and resulting health status, the efficacy of supplementation methods, and overall dietary requirements.[3] The focus of this research is assess the impact of a natural dietary source of vitamin D, wild salmon harvested sustainably in Alaska, as a vitamin D supplement in sled dogs.

Commercial dog foods following the Association of American Feed Control Officials (AAFCO) guidelines must provide 500-5000 IU/kg of daily vitamin D. However, many companies fail to account for the endogenous vitamin D in the ingredients being used. Given this problem and the large recommendation range, extremes on both ends of the vitamin D supplement spectrum have been observed: over-supplementation leading to toxicity and under-supplementation leading to developmental abnormalities (i.e.rickets).[4] A recent study using a dosage of five times the daily recommended allowance (within safe upper limit) only yielded significant results after 9-10 weeks of supplementation, and still found apparently healthy dogs with insufficient plasma vitamin D levels.[5]

Canines rely on diet for their vitamin D requirements, and few foods contain appreciable amounts of endogenous vitamin D. Fatty fish is among the best food sources for vitamin D, and wild Alaskan salmon is considered one of the best sources: vitamin D amounts in wild Alaskan salmon are more than double that of farm-raised salmon.[6] Some research has been published exploring vitamin D and dogs, but few studies are successful in raising serum 25(OH)D. This lack of efficacy may be related to the bioavailability of vitamin D in the diet.[7] One study using fish oil, salmon oil, and a fortified dog biscuit

only saw an increase in serum vitamin D with the salmon oil supplement.[8] Vitamin D circulation is dependent on the vitamin D binding protein (DBP), which has been shown to have higher affinity for D3 compared to D2.[9] Fish liver has an abundance of vitamin D stores, making fatty fish an excellent source of vitamin D3. Using wild Alaskan salmon caught by gill net on the Yukon River, we observed changes in plasma 25(OH)D levels after 4 weeks of supplementation. To explore the relationship between vitamin D intake and status, biomarkers of vitamin D metabolism were used. Parathyroid hormone (PTH) is responsible for the regulation of vitamin D metabolites, and vitamin D binding protein (DBP) is responsible for the transportation of metabolites, among other physiological functions. While previous canine research has established the inverse relationship between vitamin D metabolites and serum PTH any DBP link has yet to be established in canines. The DBP has been acknowledged as an important factor in human health to regulate immune function and inflammation, as well as being used as a marker for chronic disease in canines. [10] The inverse relationship of the PTH has been suggested to represent a physiologically relevant point where vitamin D deficiency becomes sufficient, the point at which the inverse PTH decreasing hits a plateau. Clinical determination of vitamin D status should be based on this range with the PTH plateau. [11]

2.3 Materials/Methods

Animals

Sled dogs (n=14) from a private mushing kennel in Salcha, Alaska (64°N,146°W) were used for this study. This study was approved by the University of Alaska's Institutional Animal Care and Use Committee (IACUC, approval #1343101-5). All dogs underwent a similar training routine and were from a similar genetic lineage. The dog owner/kennel manager is a competitive musher, competing at the national and world levels for more than 3 decades. Maintaining an ideal body condition score is an important factor in performance. The kennel owner fed the dogs to maintain an ideal body condition scores between 4-5 based on previous literature.[12] Body condition scores were checked before and after supplementation; dogs included were all at an ideal body condition score and no dogs were excluded throughout the study. All dogs acted as their own control throughout the study; dogs were chosen by

kennel manager prior to study, and the dogs' identities were not blinded or randomly chosen. Dogs were selected to provide diversity in both age and sex; there were females (n=8) and males (n=6) with ages ranging between 10 months old and 7 years old.

Grouping of dogs were based on treatment and confounding factors. Age and sex were suggested to be the main differences in this pool of dogs, because environment and diet remained consistent. Age was separated into 3 categories, to view variations based on developmental states. Puppies were all 10 months old (n=4), adults were between ages 2-4 years old (n=7), and oldies were 7 years or older (n=3).

Supplementation

Prior to treatment, sled dogs were fed with a high protein, high fat (32% and 20%, respectively) commercial diet for performance canines with supplementation of corn oil and egg powder. This commercial diet is reported to have 2,221.12 IU/kg of Vitamin D in dry matter. An average sized sled dog from this kennel (~25kgs) eats approximately 600g of dog food as fed, supplying around 1,300 IU of vitamin D daily before treatment. After an initial baseline blood collection, the dogs' diet was then supplemented with wild Alaskan Chum Salmon caught by gill net from the Yukon River. Approximately 0.45 kg of salmon per day was fed to each dog, replacing most but not all of the commercial food. The addition of salmon accounted for more than half of the total diet and dogs that were larger (eating more) received more salmon, but it was proportionally the same between all dogs. Dietary wild Alaskan salmon is reported to have 988 IU of D3 in 0.1 kg on average.[6] Therefore, the additional vitamin D levels from salmon were likely around 4,400 IU/day.[13] This means during treatment dogs were receiving approximately 5,070 IU/day of vitamin D. After 4 weeks of salmon supplementation a final blood collection was done.

Blood Collection

Initial blood collection occurred on March 22, 2019. Final blood collection took place ~4 weeks later on April 24th, 2019. Eight mL of blood was drawn by venipuncture with a 21-gauge butterfly needle from the cephalic vein into a 10-ml syringe, and then transferred into a vacutainer containing the anticoagulant EDTA. Within 30 minutes the samples were centrifuged at 3600RPM for 15 minutes, then

the plasma layer was aliquoted and divided into 8 freezer vials per dog to be stored at -80°C for later analysis.

Biochemical Analysis

Canine specific enzyme-linked immunosorbent assay (ELISA) kits were used to quantify concentrations of PTH (MyBioSource, San Diego, CA), and DBP (Blue Gene, Livermore, CA) in plasma samples according to the manufacturers' instructions. Absorbance was measured at 450nm with a microplate reader (BioTek Synergy HT). Sample concentrations were interpolated from standard curves determined with known concentrations of the respective protein that was being measured.

One sample from each dog was later sent to Michigan State University Veterinary Diagnostic Laboratory for analysis by radioimmunoassay (RIA). In 2020 a study associating vitamin D metabolites with DBP and proteinuria in dogs used both ELISA for DBP and RIA for serum 25(OH)D, consistent with our study, [14] and validated by literature. [4] The same study also used multivariable linear regression models to determine associations with various clinical variable, similar to our analysis.

Data & Statistical Analysis

All samples were analyzed using GraphPad Prism statistical software (version 9.0.0) to evaluate differences between pre- and post-salmon supplementation. Differences in serum 25(OH)D, PTH, and DPB concentrations between groups were evaluated by a paired t-test assuming Gaussian normal distribution, also consistent with literature [2], [14]. Two-way ANOVA with Sidak's multiple comparison was used to test variations based on sex and age independently due to treatment. Regression analyses were used for determining correlations between vitamin D levels with concentrations of PTH and DBP. Pearson r correlation was evaluated using 25(OH)D concentrations as the independent factor, with the PTH and DBP as the dependent factors. Significance was determined using a p-value of <0.05.

2.4 Results

Serum 25(OH)D Concentrations

There was a significant increase in serum vitamin D concentrations before and after treatment with salmon (P=0.0011, SEM-pre=12.36, SEM-post=10.69) (Figure 2.1.A). The two-way ANOVA

revealed significance in variation based on age and treatment: puppies (pre) vs adults (pre) (p=0. 011), puppies (pre) vs adults (post) (p=<0.0001), puppies (post) vs adults (post) (p=0.009), adult (pre) vs oldies (pre) (p=0.0128), adults (post) vs oldies (pre) (p=0.0001), adults (post) vs oldies (post) (p=0.0107) (Figure 2.1.B). Separation by sex yielded a significant difference (P=0.0403) (Figure 2.1.C).

Biomarkers

There was no significant difference between parathyroid hormone levels before and after treatment. However, significance was found in DBP concentrations before and after treatment with salmon (P=0.0367) with no significance in correlation coefficient (Figure 2.2).

Correlation of 25(OH)D Concentration with Biomarkers

Analysis using the Pearson r correlation statistical test revealed no significant correlation of the PTH or DBP to concentrations of serum 25(OH)D.

2.5 Discussion

Supplementation using wild Alaskan salmon as a dietary source of vitamin D provided significant increases in serum 25(OH)D in sled dogs after 4 weeks; furthermore, separation of dogs by confounding factors, like age and sex, yielded significance (Figure 2.1). These results highlight the importance of sex and developmental state on vitamin D status in canines. Although we did not see significant changes in PTH hormone concentrations, there was a significant increase in DBP concentrations after salmon supplementation (Figure 2.2).

When circulating vitamin D status is low, the parathyroid hormone is triggered which creates an inverse relationship between the vitamin D concentration and the PTH concentration. When vitamin D is sufficient, the PTH levels plateau. The PTH plateau seen after 100ng/mL of 25(OH)D has been reported in past literature and is suggested to be used as a more physiological relevant definition of healthy vitamin D status in dogs.[8] Although we did not find significance in our correlation data when comparing concentrations of DBP and PTH independently to changes in concentrations of 25(OH)D, a larger sample size could support the plateau seen after 100ng/mL of serum 25(OH)D. A similar correlation is found in humans. However, it occurs at a lower level of serum 25(OH)D.[13] Past literature suggests a healthy range

of PTH concentrations between 12-34 pg/mL;[15] however, our dogs averaged 0.42 pg/mL pre- and post-treatment. This low detection is cause for question and may need validation from a different method than ELISA, like RIA. Optimal health for vitamin D in dogs is seen at sufficient levels ranging between 100-120 ng/mL, anything less than 30 ng/mL is suggested to be VDD in dogs.[8] Based on this range, the mean vitamin D levels in the dogs in our study were found to be sufficient before or after treatment; this could be indicative of the lack of change in PTH concentration. While the mean 25(OH)D levels in dogs used in this study indicate that the dogs were sufficient prior to salmon supplementation, when looking at the dogs individually, eight out of the fourteen dogs in this study were considered insufficient prior to salmon and only two out of the fourteen after the salmon supplementation. Interestingly, the only two dogs that were not able to reach sufficient vitamin D levels after salmon were both 10-month-old pups. Without a larger sample size for each age group, we can only speculate why this happened, but it may be an indication of increased vitamin D needs in growing dogs. Old dogs in this study also reported lower serum vitamin D levels. This is consistent with literature in both dogs and humans. Vitamin D status tends to decrease with age.[16] It is important to note, that with only a 4-week supplementation all of the previously insufficient old dogs reached sufficiency, indicating that this population could likely benefit from a salmon-oil supplement.

Only one other study has investigated vitamin D levels of sled dogs specifically. The researchers found this group to be overall insufficient or deficient in vitamin D levels before and during an 8-day race. Values ranged between 57-87 ng/mL through the race.[2] While the dogs in this study were regularly exercising, their race season was complete, and they were not undergoing any strenuous events. Athletes are suggested to be at increased risk for developing VDD, therefore the subgroup of sled dogs we are studying may represent the suggested increase of nutrients needed by endurance athletes.[17]

Correlations between vitamin D status and DBP levels have not previously been observed in dogs.[14] This relationship should be further established to determine the physiological relevance of DBP levels. Of the few studies that measured DBP in dogs, their reported values were magnitudes higher than the values reported here. Whether that has to do with the method of analysis or breed specificity cannot

be determined at this time. Despite the low levels, there was a significant increase in DBP after supplementation which mirrors the increase in circulating D3 from the salmon. It's been shown that there are differences in bioavailability of vitamin D due to the source. [18] The increase in vitamin D due to salmon specifically could be a result of the affinity of the DBP for the D3 from fish fat stores. It's been suggested that binding efficacy for D3 over D2 is preferred for the DBP. [19]

Future research should establish a link between specific vitamin D intake levels and associate that with overall vitamin D status. Our research fails to note the exact value of vitamin D ingested by each individual but shows that salmon provides a significant dietary source for increasing serum 25(OH)D. Overall status of vitamin D may be attributed to a variety of factors. Future directions should focus in on those factors and establish a physiologically relevant definition of vitamin D health in dogs. This information could aid commercial dog food manufacturers to better understand the needs and range of vitamin D to supply in their diets and tailor the diets for different life stages. Furthermore, the relationship between increased intake for athletic dogs should be researched to determine if these athletes, similar to humans, may have higher requirements of vitamin D. Often supplementation with vitamin D does not improve vitamin D status. The fact that this study showed an improvement in vitamin D status in a short duration and allowed most of the dogs to reach sufficient levels supports the use of a bioavailable vitamin D source as a supplement. Confounding factors are suggested to effect vitamin D metabolism in dogs; these include age, body condition scores, breed, genetic variation, and sex hormones. Future research should be conducted to establish the impact of sex hormones on vitamin D status and metabolism. Our research shows a significant variation of plasma 25(OH)D between sex, and past literature has seen significant variation based on intact status.[8] The link between sex hormones and vitamin D status should be further examined.

2.6 Acknowledgements

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2.7 Conflicts of Interest

There are no conflicts of interest to report.

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2.9 Figures

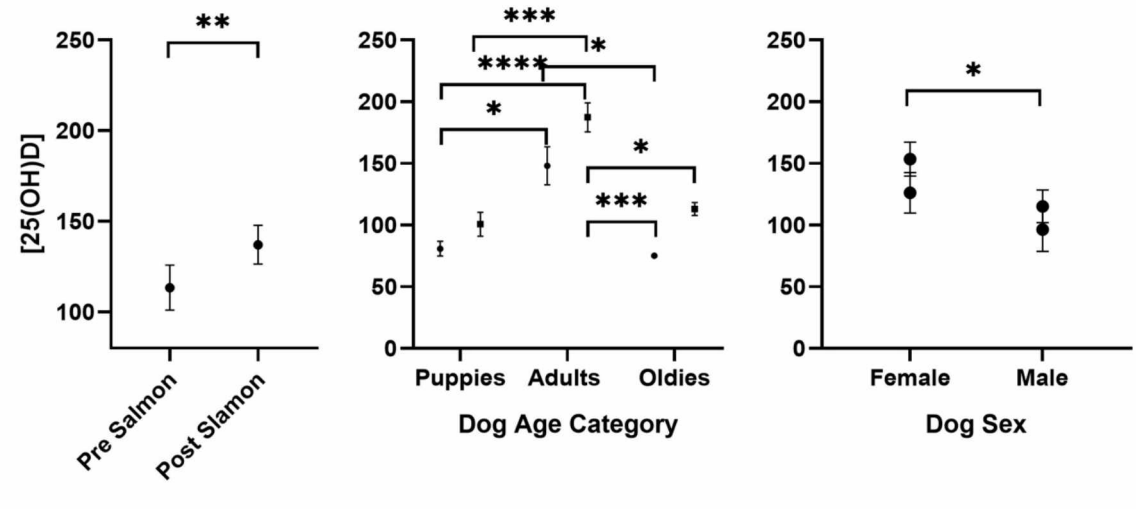

Figure 2.1. Differences in serum 25(OH)D before and after 4-week supplementation with wild Alaskan salmon. Mean values reported with SEM bars, asterisk represent significance. Dogs were separated by age and sex for analysis. Puppies were 10-month-old (female n= 1, male n=3), adults were 2-4 years old (female n=5, male n=2), and oldies were 7 years or older (female n=2, male n=1). Separations by age yielded significant variation between age and treatment. Separations by sex (female n=8, male n=6) also yielded significant variation.

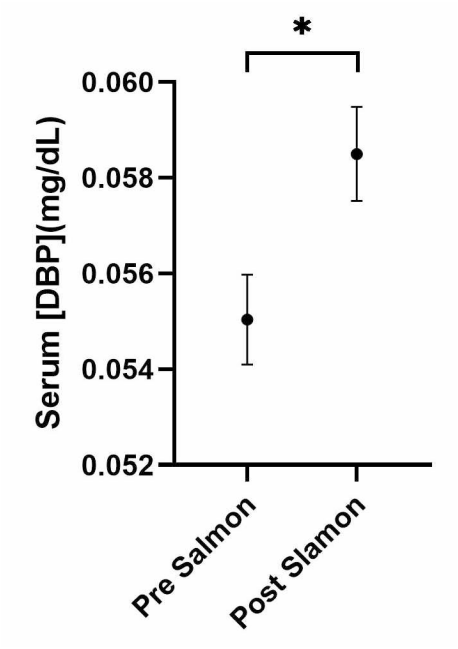

Figure 2.2. Mean serum DBP ± SEM before and after 4-week treatment with salmon (P=0.0367, SEM-pre=0.00094, SEM-post=0.00099).

Chapter 3: Conclusions/ Future Directions

Our specific objective with this research project was to compare concentrations of 25(OH)D in the plasma of Alaskan sled dogs before and after receiving a diet consisting of mostly wild Alaskan salmon, known to be rich with vitamin D. We also hoped to correlate biomarkers associated with vitamin D metabolism to the concentrations of 25(OH)D found. Our working hypothesis was that sled dogs would have increased serum 25(OH)D concentrations after 4 weeks of diet supplementation with salmon, and that lower levels of 25(OH)D would be correlated with low levels of DBP, and that PTH concentrations would be negatively correlated. A significant increase in serum vitamin D levels was found after a 4-week supplementation with chum salmon from the Yukon river of Alaska. There was also a significant increase in DBP levels after 4-week treatment of salmon supplementation; however, the DBP levels were not correlated significantly with levels of 25(OH)D found in the same individuals.

3.1 Vitamin D in sled dogs (serum 25(OH)D concentrations)

Optimal health for vitamin D in dogs is seen at sufficient levels ranging between 100-120 ng/mL, anything less than 30 ng/mL is suggested to be VDD in dogs. [26] [25] Based on current recommendations the mean plasma 25(OH)D levels in dogs in this study were within the sufficient range, however 8 out of 14 of the dogs were insufficient prior to supplementation with salmon and only 2 of the 14 after supplementation. Previous studies have failed to show increased vitamin D levels due to dietary supplementation, but those studies did not work with racing sled dogs; dogs were more commonly house pets. To date, few dog studies using supplementation have been successful in raising 25(OH)D serum levels. Studies that have been done have given many mixed results due to variations in vitamin D supplementation type, dose, and duration.[33] Using 3 types of supplementation (salmon oil, fish oil, or fortified dog biscuit), only the salmon oil was effective in significantly raising serum vitamin D levels in one study.[25] Another study using 5 times the recommended dose of vitamin D to supplement dogs for 10 weeks resulted in significant increases in vitamin D only after 9 weeks.[29] The majority of studies fail to raise serum vitamin D levels with dietary supplement; however, studies also suggest most sled dogs to be vitamin D deficient.[22] The dogs in this study were all similar breeds with little genetic variation; however, as with past research we found a significant variation when comparing sex (Figure 2.1).[25] Although we had a smaller sample size, our sample benefited by being within the same genetic pool and being co-located at the same kennel.

3.2 Biomarkers

Although commercial dog food manufacturers understand the importance of vitamin D in canine diet, they lack comprehensive understanding of the ideal dose of supplementation to maintain cellular health.[25] Currently serum 25(OH)D is the most qualified indicator of vitamin D status, however it may fail to represent storage in tissues[11]. This possibility underscores the potential importance of including biomarkers like PTH, which regulates vitamin D stores, when evaluating overall vitamin D health in an individual. [1]Some suggest that using the PTH plateau (above 100ng/mL vitamin D) can be the most physiological relevant definition for vitamin D sufficiency. In individuals with insufficient vitamin D levels (<100 ng/mL) there is an inverse relationship with PTH concentrations, when sufficient levels of vitamin D are reached the PTH concentrations level out. This conclusion is consistent with literature but was not a significant correlation in our study. Although our correlation data was insignificant for changes in PTH based on serum 25(OH)D concentrations, we do show a similar pattern compared with past literature, with the PTH levels plateauing.

Past studies have failed to link changes in DBP to vitamin D status[34]. Our results show a significant increase in DBP concentrations that mirrors the increase we see in vitamin D precursor, 25(OH)D (Figure 2.2). Although, it's important to note that our levels of DBP were low compared to past literature. Previous studies had control dogs averaging DBP concentrations of 14.6 mg/dL in control dogs [34] while our dogs averaged around 0.05 mg/dL. Control dogs in previous study were compared to dogs with proteinuria who showed even higher concentrations of DBP. This biomarker should be further investigated to solidify its use as an indicator of vitamin D status.

3.3 Confounding Factors

Research suggests that athletes demand a higher amount of vitamin D due to their active lifestyle[23] - canine athletes may also have increased needs. This relationship has not been established but should be researched further. Clinical benefits of vitamin D supplementation for increasing fitness have already been demonstrated in human studies that concluded that athletes with higher serum 25(OH)D levels have reduced injury rates and better performance.[1] Sled dogs in the past have been suggested to be generally deficient in vitamin D with levels averaging ~87ng/mL[22]. It's important that future research take dog breed into account, as athletic dogs may need a higher intake. Future research should determine if dog breed is a confounding factor, as has been suggested in previous literature.[25] In house dogs with sufficient levels of vitamin D, more fat stores, and a less active life, diet may not be as essential for needed nutrients. Because sled dogs, and athletes in general, have fewer fat stores, they could need more vitamin D daily (potentially including more from their diet) to maintain active vitamin D for biological

functions.[35] In a recent study looking at 103 Japanese Akita dog samples, known for having immune-mediated diseases, levels of 25(OH)D of healthy and sick dogs were below 100 ng/mL on average (82.42 ng/mL) with sick dogs significantly lower in serum vitamin D levels.[36] This group may need an intake higher than currently recommended by dog associations.

Past literature highlights that age may be a confounding factor to vitamin D health in dogs.[27] A study done evaluating plasma 25(OH)D levels along with other vitamin D metabolites among different dog breeds at different ages revealed significant differences in vitamin D metabolism of dogs based on their developmental status.[37] Our results reveal a significant difference of serum 25(OH)D based on age (Figure 2.1), and future research should establish differences in metabolism based on age to help suggest recommendations based on dogs' development.

Other research suggests that neuter status may be a factor effecting vitamin D metabolism in dogs.[27] We did not have the sample sizes necessary to establish that association. However, we do see a significant variation in vitamin D levels based on sex (Figure 2.1); this confounding factor has been shown before in dogs[32] and in humans[21]. Therefore, the relationship between sex hormone concentration and vitamin D status should be taken into account for future research.

3.4 Limitations / Future Directions

Current limitations in vitamin D health in sled dogs may also be attributed to lack of appropriate experimental design;[26] therefore, our study with limited confounding factors may be a good start. One main limitations in vitamin D studies done on dogs is the lack of knowledge for vitamin D intake levels of dogs.[27] It's also been suggested that most dog food manufacturers lack specific analytical results of nutrient and only know amounts of vitamin D added to premix.[38] Knowing exact intake levels needed to raise serum 25(OH)D levels could be of clinical value to help prevent VDD, and to treat dogs with VDD. Certainly, understanding the source of vitamin D in our dogs' diets is critical. Studies on factors effecting vitamin D absorption, distribution, and metabolism is insufficient to accomplish this objective.[29] Prior research has used vitamin D metabolites to gain a clearer understanding of these relationships. Future research in this field should augment our understanding for vitamin D stores vs biologically active vitamin D metabolite. Biomarkers of vitamin D status should also be considered when determining overall vitamin D status. For example, because a rapid response of vitamin D activation is changes in calcium channels, ionized calcium levels are used as a biomarker in determining vitamin D status in dogs with cancer.[27] Other studies use the PTH plateau after sufficient levels of 25(OH)D (>100 ng/mL), and use this correlation as a risk factor in dogs with cancer risk.[32] This relationship with the PTH plateau has also been suggested as physiologically relevant in humans, highlighting benefit from continuing to study the relationship in our canine counterparts.

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