Circulating Vitamin D Metabolite and Inflammatory Marker Concentrations in Dogs with Cancer

by

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ABSTRACT

CIRCULATING VITAMIN D METABOLITE AND INFLAMMATORY MARKER

CONCENTRATIONS IN DOGS WITH CANCER

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Low circulating 25-hydroxyvitamin D (25[OH]D) concentrations have been observed in canine diseases, sparking interest in defining new 25(OH)D reference ranges in dogs and making it imperative to understand what factors might be associated with 25(OH)D measurement. Inflammation may confound 25(OH)D measurement, and is an enabling characteristic of cancer, a disease linked to low 25(OH)D concentrations in dogs. Therefore, this thesis explored relationships between vitamin D and inflammation in dogs with cancer with three studies:

1) Plasma 25(OH)D and inflammatory marker concentrations were measured in dogs with cancer before treatment. Dogs with B-cell lymphoma were the only cancer group with decreased plasma 25(OH)D concentrations compared with healthy dogs($p=0.03$), and these dogs also had increased concentrations of multiple inflammatory markers, including C-reactive protein (CRP)($p<0.001$).

2) Plasma 25(OH)D and inflammatory marker concentrations were measured during cancer treatment in dogs with lymphoma. Lower pre-treatment plasma 25(OH)D concentrations, and higher plasma CRP concentrations were observed in dogs with B-cell lymphoma compared to healthy dogs ($p=<0.001$ and 0.001). At week 25 of chemotherapy treatment, plasma 25(OH)D concentrations were increased($p=0.006$),

and plasma CRP concentrations were decreased($p=0.002$) in dogs with B-cell lymphoma compared to their pre-treatment concentrations, and were no longer different from the healthy group.

3) An explanation for lower pre-treatment 25(OH)D concentrations observed in dogs with B-cell lymphoma, namely that inflammation causes upregulation of the CYP24A1 enzyme (responsible for conversion of 25(OH)D to 24,25(OH)2D), was investigated. Circulating concentrations of 25(OH)D and 24,25(OH)2D, and the 25(OH)D/24,25(OH)2D ratio (representative of CYP24A1 activity) were measured. Plasma 25(OH)D and 24,25(OH)2D concentrations were lower in dogs with B-cell lymphoma than in healthy dogs ($p=0.02$ and 0.04), but the 25(OH)D/24,25(OH)2D ratio was not different between groups, suggesting upregulation of the CYP24A1 enzyme is not responsible for decreased plasma 25(OH)D concentrations.

Findings support a relationship between circulating 25(OH)D and inflammatory marker concentrations in dogs with B-cell lymphoma and warrant investigation into concentrations of other vitamin D metabolites in affected dogs. Findings provide clinicians with evidence-based information for owners of dogs with lymphoma who are interested in learning more about vitamin D in relation to their dog’s cancer diagnosis.

DEDICATION

For Kev. You are always with us and always will be.

For my family, two-legged and four-legged. Life would be meaningless without you.

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LIST OF ABBREVIATIONS

24,25(OH)2D	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AAG	Alpha-1-acid glycoprotein
APP	Acute phase protein
B-cell	B-cell lymphoma
CRP	C-reactive protein
HP	Haptoglobin
MCT	Mast cell tumour
OSA	Osteosarcoma
SAA	Serum amyloid A
T-cell	T-cell lymphoma

1 General Introduction

Vitamin D, traditionally recognized for its importance in skeletal health, has received renewed focus from canine health researchers over the past decade. Researchers have reported low circulating concentrations of the vitamin D metabolite, 25-hydroxyvitamin D (25(OH)D), in dogs with various diseases (Willcox et al., 2016; Parker et al., 2017; Miller et al., 2020; Parker et al., 2020; Allenspach et al., 2017; Wennogle et al., 2019; Wennogle et al., 2021; Erdogan et al., 2019; Dvir et al., 2019). These reports have sparked interest in defining new reference ranges for 25(OH)D and investigating ways to increase the concentrations of this metabolite (Wakshlag et al., 2011; Selting et al., 2016; Weidner & Verbrugghe, 2017), which makes it imperative to understand what factors might be associated with, and potentially confound, the measurement of circulating 25(OH)D concentrations in dogs. Inflammation may confound 25(OH)D measurement, and is an enabling characteristic of cancer, one of the diseases linked to low 25(OH)D concentrations in dogs (Wakshlag et al., 2011). Therefore, this thesis aims to explore potential relationships between vitamin D and inflammation in dogs with cancer. This chapter introduces the thesis by discussing the background and context, followed by the research questions and the order of the thesis.

1.1 Background

Dogs have played a central role in the history of vitamin D. Around a century ago, Sir Edward Mellanby found that cod liver oil, butter or suet was important for the prevention of rickets in puppies (Mellanby, 1918; National Research Council, 2006). He concluded the oil contained a vitamin or closely associated factor that could prevent the disease. Elmer McCollum performed further work with cod liver oil and concluded that the compound responsible for preventing rickets was a new vitamin, and named it vitamin D (McCollum et al., 2002). Research on vitamin D in dogs continued through the 20th century, with much of the work focused on examining the vitamin D requirements of puppies. Studies were often focused on vitamin D's role in skeletal health. As time

continued, research also began to focus on the roles of the vitamin in extra-skeletal health.

This research has become especially popular in the past ten years. Circulating 25(OH)D concentrations have now been reported in dogs with a wide range of diseases, from infectious diseases to kidney disease to cancer, with enough work to warrant publication of a notable amount of literature reviews (Mellanby, 2016; Corbee, 2020; Chacar et al., 2020; Hurst, Homer, & Mellanby, 2020; Zafalon, Ruberti et al., 2020; Zafalon, Risolia, et al., 2020; Stockman et al., 2021). Focus has usually been placed on measurement of the 25(OH)D metabolite of vitamin D as it is the generally accepted marker of vitamin D status (Ross et al., 2011). The 25(OH)D metabolite is less regulated and has a longer half-life than other vitamin D metabolites and seems to be stable over time in most healthy dogs (Hurst, Homer, Gow et al., 2020; Laing et al., 1999). Interest in defining new reference ranges for 25(OH)D and investigating ways to increase the concentrations of this metabolite (Wakshlag et al., 2011; Selting et al., 2016; Weidner & Verbrugghe, 2017), has been tempered by researchers emphasizing the importance of first investigating factors that may influence circulating 25(OH)D concentrations (da Fonseca et al., 2020; Corbee, 2020).

Systemic inflammation may confound 25(OH)D measurement (Ghashut et al., 2014; Silva & Furlanetto, 2015) as pro-inflammatory cytokines have shown the ability to dysregulate enzymes involved in vitamin D metabolism (Zehnder et al., 2002; Hummel et al., 2014). Evidence of a relationship between lower 25(OH)D concentrations and the presence of inflammation has been documented in cross-sectional, longitudinal and mechanistic work in humans (Reid et al., 2011; Duncan et al., 2012; Zehnder et al., 2002). This work has been replicated to a lesser degree in dogs, with similar results (Wennogle et al., 2019; Clements et al., 2020). These results are interesting and relevant for researchers interested in factors that influence 25(OH)D concentrations. Further investigation into links between circulating 25(OH)D and inflammation in dogs is warranted. Investigations into dogs with chronic inflammatory diseases where lower

25(OH)D concentrations have been observed, such as cancer (Gerber et al., 2004; Wakshlag et al., 2011), may be especially fruitful.

Inflammation is an enabling characteristic of cancer (Hanahan & Weinberg, 2011) and studies have reported increased concentrations of inflammatory markers in dogs with cancer (Nielsen et al., 2007; Chase et al., 2011; Calvalido et al., 2016). Inflammatory profiles seem to differ between cancer types (Tecles et al., 2005; Chase et al., 2012). Differences between cancers have also been observed with 25(OH)D concentrations, as lower 25(OH)D concentrations have been reported in some cancers (Wakshlag et al., 2011), but not others (Willcox et al., 2016). Interestingly, evidence suggests inflammatory marker concentrations may decrease with successful canine cancer treatment (Nielsen et al., 2007). This presents several opportunities for research and forms the foundation for the work explored in this thesis:

1) If lower 25(OH)D concentrations are associated with systemic inflammation, then concurrent exploration of 25(OH)D and inflammatory marker concentrations in several cancers may identify which cancers and/or inflammatory markers are associated with low 25(OH)D.

2) If lower 25(OH)D concentrations are a consequence of the inflammation associated with cancer, then 25(OH)D concentrations should increase and inflammatory marker concentrations should decrease upon successful cancer treatment.

3) If lower 25(OH)D concentrations observed in dogs with cancer are a consequence of the dysregulation of vitamin D metabolism by pro-inflammatory cytokines, then the concentrations of other vitamin D metabolites may be increased and/or the ratio between 25(OH)D and other vitamin D metabolites may be decreased in dogs with cancer.

1.2 Research Questions

The research questions explored in this thesis are:

1) Are decreased circulating 25(OH)D concentrations associated with increased concentrations of inflammatory markers in dogs with cancer?

2) Do circulating 25(OH)D concentrations increase and inflammatory marker concentrations decrease with cancer treatment in dogs?

3) Is there increased conversion of circulating 25(OH)D to other vitamin D metabolites in dogs with cancer?

1.3 Order

To address these questions, this thesis contains the following literature reviews and original research:

Chapter 2: Current knowledge of vitamin D in dogs

Published: Crit Rev Food Sci Nutr. 2017 Dec 12;57(18):3850-3859.

Objective: A literature review published early in the PhD program that summarized the current knowledge of vitamin D in dogs at the time of publication. This review was submitted for publication in 2016. There have been numerous studies of vitamin D in dogs published since so an additional literature review (below) was written.

Chapter 3: Vitamin D and inflammation: A relationship worth investigating in dogs with cancer?

Objective: A literature review that 1) updates that relevant terminology and conclusions of the previous literature review, and 2) connects the new information to the topic of this thesis: vitamin D and inflammation in dogs with cancer.

Chapter 4: Plasma 25-hydroxyvitamin D and the inflammatory response in canine cancer

Published: Vet Comp Oncol. 2021 Jun;19(2):232-241.

Objective: To determine plasma 25(OH)D and inflammatory marker concentrations (including acute phase proteins and cytokines) in healthy dogs and dogs with B-cell and T-cell lymphoma, osteosarcoma and mast cell tumour.

Chapter 5: 25-hydroxyvitamin D concentrations and the inflammatory response in dogs with B-cell and T-cell lymphoma during chemotherapy treatment

Objective: To determine plasma 25(OH)D and inflammatory marker concentrations in healthy dogs and dogs with B-cell and T-cell lymphoma during chemotherapy treatment.

Chapter 6: Circulating 25(OH)D and 24,25(OH)2D concentrations, and the 25(OH)D/24,25(OH)2D ratio, in dogs with B-cell lymphoma

Submitted: To Be Determined

Objective: To determine concentrations of plasma 25(OH)D and another vitamin D metabolite (24,25-dihydroxyvitamin D [24,25(OH)2D]), and the 25(OH)D:24,25(OH)2D ratio, in dogs with B-cell lymphoma and healthy dogs.

2 Current knowledge of vitamin D in dogs

Published in Critical Reviews in Food Science and Nutrition (Crit Rev Food Sci Nutr. 2017 Dec 12;57(18):3850-3859).

2.1 Abstract

There is emerging interest in linking vitamin D status to physiological health and disease states in the dog, as evidenced by the recent increase in publications in this area. This research has most likely been spurred by the studies exploring vitamin D and disease in humans. However, there are important differences in vitamin D intake and metabolism between humans and dogs that should be accounted for. The understanding of basic vitamin D metabolism and the relationship between vitamin D intake and vitamin D status in dogs remains even more limited than current knowledge in humans. This review will summarize current knowledge of vitamin D in the dog, including metabolism and dietary recommendations. Emphasis is placed on the limitations to current knowledge. Studies investigating links between vitamin D and disease will be discussed in light of this knowledge. Suggestions for future research, including the development of reference ranges to define blood vitamin D sufficiency, are provided.

2.2 Introduction

Vitamin D, historically well known for its role in calcium and phosphorus regulation, has become a hot and controversial topic in human medicine as evidence for the vitamin's involvement in extra-skeletal health continues to expand. Interest in vitamin D has recently translated to the veterinary world, with researchers linking vitamin D to several diseases in dogs. Although still in its infancy, this research is exciting, as evidence indicates that vitamin D may be influential in: reducing disease risk, increasing treatment efficacy and improving disease outcome, and may also have use as a bio-marker of disease prognosis.

There are several main foci of vitamin D research in humans: correlation of vitamin D intake with disease (Munger et al., 2004; Annweiler et al., 2012); correlation of the marker of vitamin D status, 25-hydroxyvitamin D (25(OH)D) (Holick, 1995), with disease (Ananthakrishnan et al., 2012); mechanisms of action of the most biologically active metabolite, 1,25-dihydroxyvitamin D (1,25(OH)2D), in target cells/tissues (Feldman et al., 2014); and the relationship between vitamin D intake and vitamin D status (Ross et al., 2011). Each area of research is equally important to a full understanding of vitamin D's potential roles in disease pathophysiology. However, understanding the relationship between vitamin D intake and vitamin D status allows results to be translated into meaningful real-world applications, such as the development of vitamin D intake recommendations by government organizations. The Institute of Medicine released a comprehensive review of vitamin D literature in 2011 (Ross et al., 2011) that highlighted the many limitations to the knowledge of the relationship between vitamin D intake and vitamin D status, including effects of factors like age, body composition and genetics. These limitations cover the knowledge of the relationship in humans only.

The same areas of vitamin D research in humans must be mirrored in dogs to effectively understand vitamin D's role in canine disease physiology. As with humans, understanding of the relationship between vitamin D intake and vitamin D status is essential to translate results into real world applications. In dogs, these real-world applications include the vitamin D requirements for dog foods set by the National Research Council (NRC), the American Association of Feed Control Officials (AAFCO) and the European Pet Food Industry Federation (FEDIAF). However, as "non-traditional" vitamin D research in extra-skeletal disease in dogs is still in its infancy, some areas of vitamin D research have been overlooked. In particular, the relationship between vitamin D intake and vitamin D status in adult dogs is virtually unknown. Currently, dogs receiving an AAFCO compliant dog food for adult maintenance may receive anywhere from 500–5000 IU vitamin D/kg DM (AAFCO, 2014), or 552–3200 IU vitamin D/kg DM according to FEDIAF guidelines (FEDIAF, 2014), depending on the level of vitamin D the manufacturer has chosen to include. As a result, there may be

large variation in vitamin D intakes between dogs. Many manufacturers report only the amount of vitamin D added to the diet as a premix, and do not account for the endogenous vitamin D content of ingredients used. As a result, the vitamin D content of the final product reported by the manufacturer may be inaccurate. These are important considerations for researchers choosing to enroll client-owned animals as study participants.

This review will summarize current knowledge of the relationship between vitamin D intake and vitamin D status in dogs, and discuss recent studies focused on vitamin D and disease in dogs in light of this knowledge. The goal of this literature review is not to discount current work examining vitamin D and disease in dogs, but to articulate a strong argument for basic vitamin D research in dogs, especially research focused on the relationship between vitamin D intake and vitamin D status, and to emphasize the need to include vitamin D intake as a study variable.

2.3 Metabolism

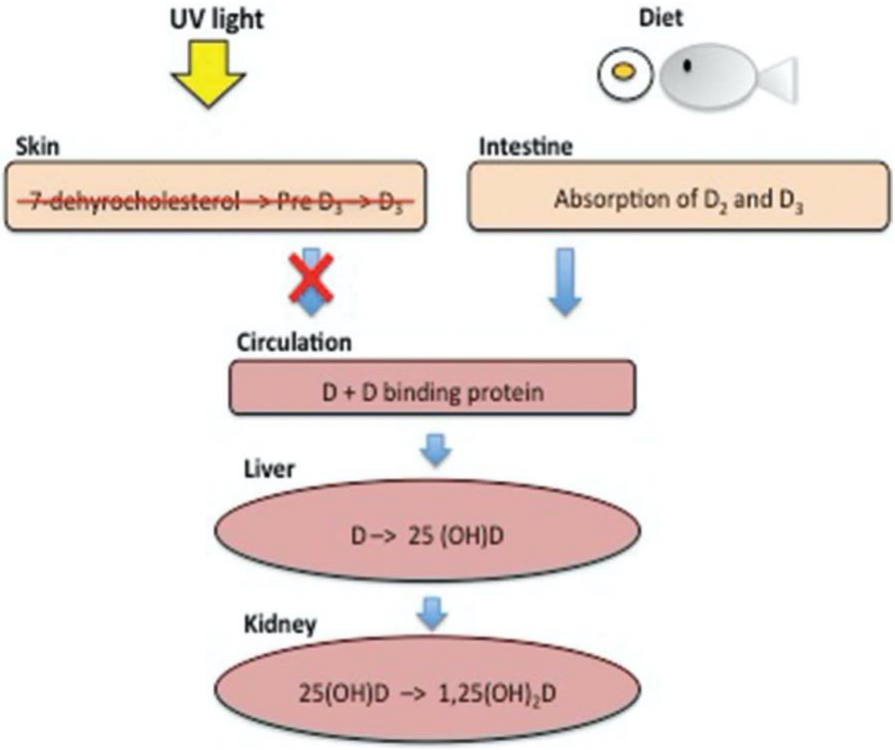

Figure 2.1: Basic vitamin D metabolism in dogs. Diet is the only source of vitamin D for dogs, as production of vitamin D in dog skin is insignificant (Hazewinkel et al., 1987; How et al., 1994).

D = Vitamin D, D2 = Vitamin D2, D3 = Vitamin D3, 25(OH)D = 25-hydroxyvitamin D, 1,25(OH)2D = 1,25-dihydroxyvitamin D.

There are two forms of vitamin D. Vitamin D2, also known as ergocalciferol, is usually the form created by plants. Vitamin D3, also known as cholecalciferol, is the form created in the skin of humans during exposure to UV light (Holick, 2007). Humans may also ingest vitamin D through diet, which could be in the form of D2 or D3. Both forms are utilized in the body, however, evidence in humans suggests that metabolites from D2 are much less potent than those from D3 (Trang et al., 1998). Vitamin D is stored predominantly in adipose tissue, but can also be found in other tissues, such as muscle (Heaney et al., 2009). After ingestion or skin production, vitamin D is transported to the liver by carrier proteins, specifically vitamin D binding protein. In the liver, Vitamin

D undergoes transformation by cytochrome P450 enzymes (i.e. cytochrome P450 27A1) and becomes 25(OH)D.

25(OH)D is one of the most stable metabolites of vitamin D, with a half-life estimated to range from 10 days to 3 weeks (Mawer et al., 1971; Vicchio et al., 1993). Circulating 25(OH)D concentrations are reflective of vitamin D obtained from the diet and skin production and have been generally accepted as a marker of vitamin D status (Holick, 1995). 25(OH)D concentration is also the marker used for associations between vitamin D status and disease status, i.e. low concentrations of 25(OH)D being associated with increased risk of colorectal cancer in humans (Feskanich et al., 2004). 25(OH)D also serves as the precursor molecule to the most biologically active metabolite of vitamin D, 1,25(OH)2D.

Primary production of 1,25(OH)2D takes place in the proximal tubules of the kidneys by action of the enzyme cytochrome P450 27B1, but also occurs in many other tissues or cell types. Parathyroid hormone and the concentration of blood calcium, phosphorus and 1,25(OH)2D itself, tightly regulate renal production of 1,25(OH)2D. This regulation, combined with a short half-life of 4-6 hours (Kumar, 1986), and blood concentrations of only 1/1000th those of 25(OH)D (Holick, 2009), make 1,25 (OH)2D less useful as a marker of vitamin D status (Holick, 2009). 1,25(OH)2D controls the body's calcium and phosphorus levels by increasing intestinal absorption and stimulating mobilization from bones.

Cytochrome P450 27B1 has also been found in extra-renal locations, such as skin, colorectal and pancreatic tissues, (Zehnder et al., 2001; Friedrich et al., 2006), suggesting 1,25 (OH)2D production also occurs in these tissues. This local production is hypothesized to be an important regulator of cell growth (Fleet et al., 2012), which provides evidence for the proposed extra-skeletal and potentially therapeutic roles of vitamin D in target tissues.

2.4 Canine vitamin D requirements

Vitamin D is produced in the skin of most mammals when 7- dehydrocholesterol is exposed to UV light and forms previtamin D (Holick et al., 1980). Pre-vitamin D undergoes thermal conversion to vitamin D3 (Holick et al., 1980). Thus, studies in humans must account for vitamin D obtained from skin production in addition to that obtained from the diet. In dogs, however, evidence suggests that UV mediated production of vitamin D is essentially insignificant (Wheatley & Sher, 1961; Hazewinkel et al., 1987; Griffiths & Fairney, 1988; How et al., 1994), meaning only dietary intake should be accounted for (Figure 2.1).

Wheatley and Sher (1961) found high amounts of cholesterol in lipid extracts of dog skin, but no intermediate products of cholesterol synthesis, such as the vitamin D precursor, 7-dehydrocholesterol. The absence of 7-dehydrocholesterol formed the basis of the hypothesis that dogs have lost the ability to produce vitamin D, and rely on dietary intake of the vitamin. Building upon the work of Wheatley and Sher (1961), How et al. (1994) found very low concentrations of 7-dehydrocholesterol present in dog skin. The 7-dehydrocholesterol that was present showed minimal UV mediated conversion to vitamin D, especially when compared to conversion rates in rat skin. In vivo studies have corroborated these findings. Researchers investigating the vitamin D status of huskies in polar latitudes observed an inverse relationship between UVB radiation from the sun and the huskies' serum 25(OH)D concentrations, instead of the expected positive association (Griffiths and Fairney, 1988). Serum 25(OH) D concentrations were more aptly explained by the huskies' dietary vitamin D intake. Hazewinkel et al. (1987) fed puppies a diet containing no supplemental vitamin D. Puppies developed rickets, and this was not prevented by exposure to UVB light. These studies represent the most commonly cited evidence for limited cutaneous vitamin D production in dogs.

Further work is necessary to conclusively say that epithelial vitamin D production is insignificant in dogs. One study has shown that puppies could achieve healthy growth rates when fed a diet with no supplemental vitamin D (Kealy et al., 1991), but the basal

vitamin D content of the diet is unclear. Additionally, the calcium and phosphorus levels in the diet were well above respective requirements. The vitamin D content may have been more important if levels of these minerals had been lower. The interaction between these nutrients is often overlooked, but remains important. Up-regulated activity of the enzyme 7-dehydrocholestrol-D7-reductase, responsible for the conversion of 7-dehydrocholesterol into cholesterol, is thought to explain the limited epithelial vitamin D production in cats (Morris, 1999). This work has not yet been completed in dogs. Still, NRC (2006), AAFCO (2014), and FEDIAF (2014) have classified vitamin D as an essential dietary nutrient for dogs. Further work, such as the study done in cats (Morris, 1999), would help establish the extent of vitamin D production in canine skin.

2.5 Current vitamin D requirements

The NRC, AAFCO and FEDIAF have developed nutritional guidelines for the dietary level of vitamin D needed to maintain health. The minimum adequate intake, minimum recommended allowance and safe upper limit of vitamin D for the NRC (2006), AAFCO (2014) and FEDIAF (2014) can be found in Table 2.1. The minimum adequate intake reflects the amount required by an animal receiving a purified diet, while the minimum recommended allowance reflects the amount required by an animal receiving a typical commercial pet food (NRC, 2006; AAFCO, 2014). AAFCO and FEDIAF compliant dog food manufacturers can choose to include any level of vitamin D within the range of the AAFCO or FEDIAF nutrient profiles.

Table 2.1: Vitamin D requirements set by the NRC (2006), AAFCO (2014), and FEDIAF (2014) for adult dogs and dogs during growth, gestation, and lactation.

		Life Stage	Minimum Adequate Intake	Minimum Recommended Allowance	Safe Upper Limit
NRC	**DM basis[a] (IU/kg)**	All	440	552	3200
	Caloric basis (IU/1000 kcal ME)	All	110	136	800
AAFCO	**DM basis[b] (IU/kg)**	All		500	5000
	Caloric basis (IU/1000 kcal ME)	All		143	1429
FEDIAF	**DM basis[a] (IU/kg)**	Adult[c]		639	2270[e] 3200[f]
		Adult[d]		552	
		Reproduction & Early Growth (<14 weeks)		552	
		Late Growth (≥14 weeks)		500	
	Caloric basis (IU/1000 kcal ME)	Adult[c]		159	800
		Adult[d]		138	
		Reproduction & Early Growth (<14 weeks)		138	
		Late Growth (≥14 weeks)		125	

NRC = National Research Council, AAFCO = the Association of American Feed Control Officials, FEDIAF = European Pet Food Industry Federation, DM basis = Dry matter basis, IU = International Units

[a] Based on a dietary energy density of 4000 kcal ME/kg

[b] Based on a dietary energy density of 3500 kcal ME/kg

[c] Based on a dog with a daily energy intake of 95 kcal/kg$^{0.75}$

[d] Based on a dog with a daily energy intake of 110 kcal/kg$^{0.75}$

[e] Legal maximum set by European Union legislation. Legal maximums do not account for energy density, and so are given on a dry matter basis only.

[f] Nutritional maximum that should not cause any adverse effects

2.6 How were vitamin D recommendations developed?

The NRC develops vitamin D recommendations by reviewing published studies that have investigated the vitamin D requirements in dogs (Zicker, 2008). The absolute minimal vitamin D requirement of puppies remains unclear as similar studies produced conflicting results (Hazewinkel et al., 1987; Kealy et al., 1991). Both studies tested if commercial dog foods, without any added vitamin D supplementation, may contain adequate vitamin D for growing dogs. Puppies were raised on either a diet with no added vitamin D and the same diet with an added 1800 IU vitamin D/kg diet (Hazewinkel et al., 1987), or a diet with no added vitamin D, and the same diet with an added 2420 IU vitamin D/kg diet (Kealy et al., 1991). Puppies fed the diet with no added vitamin D in the first study (Hazewinkel et al., 1987) developed rickets, while puppies fed the diet with no added vitamin D in the second study (Kealy et al., 1991) showed no negative effects on skeletal health or selected serum parameters (e.g. total calcium). Unfortunately, no conclusions can be drawn about the minimum vitamin D requirements of dogs from either study, as except for the fact that no vitamin D supplement was added, the vitamin D content of the basal diets derived from the selected ingredients is unclear. The vitamin D content of the internal organs included in the diet used by Kealy et al. (1991) was likely sufficient at the calcium and phosphorus concentrations used in that diet.

The minimum adequate intake suggested by the NRC (2006) (440 IU/kg DM) is somewhat supported by results from work by Tryfonidou et al. (2002). Great Dane puppies were fed diets containing »500 IU vitamin D/kg as fed, which equates to »535 IU vitamin D/kg DM using the moisture value provided in the paper, and achieved

normal growth. However, the energy density of the diets was not given, and may not have been equivalent to the energy densities of the AAFCO/NRC recommended diets. Therefore direct comparisons of the vitamin D content of the diet to AAFCO/NRC recommendations cannot be made.

Values that were 4 to 10 times the requirement were chosen as the safe upper limits in 1987 (NRC, 2006). This was adjusted in 2006, with a reference to a paper by Tryfonidou that showed impaired bone ossification with a level of vitamin D supplementation that was closer to “10 times the requirement.” However, the referenced paper is not listed in the references of the NRC chapter. The diet that is referred to (»4,000 IU/kg as fed) describes one Tryfonidou et al. study (2002), which showed depressed calcium absorption, but similar and healthy growth rates when compared with puppies receiving a diet containing »500 IU/kg as fed. The impaired bone ossification results match another Tryfonidou et al. study (2003), which used a diet with a much higher vitamin D content (»54,000 IU/kg as fed). A true SUL, supported by clear scientific evidence for reproduction/growth and for adult maintenance, is not currently known in dogs. This knowledge, and an understanding of vitamin D metabolism in adult dogs, is urgently needed in light of the increased interest in vitamin D supplementation for canine health.

2.7 Limitations to current knowledge

The latest publication released by the NRC (2006) acknowledges that no definitive conclusions can be made on the vitamin D requirement of dogs based on the literature available for review at that time. Some of the major limitations to current knowledge are highlighted in this publication (NRC, 2006) and include: (1) Efficiency with which different forms of vitamin D may be used by the dog; (2) Knowledge of the vitamin D requirements of adult dogs; and (3) Relationship between vitamin D intake and vitamin D status, including factors that may affect this relationship.

2.7.1 Efficiency with which different forms of vitamin D may be used by the dog

Although not directly relevant to the purpose of this review paper, this limitation will be briefly discussed. There are 2 forms of vitamin D. Vitamin D2, also known as ergocalciferol, is usually created by plants. Vitamin D3, also known as cholecalciferol, is the form created in the skin of most mammals during exposure to UV light (Holick, 2007). Thus, vitamin D ingested through diet may be in the form of D2 or D3, depending on the ingredient source. Both forms are utilized in the human body, however, evidence suggests that metabolites from D2 are much less potent than those from D3 in humans (Trang et al., 1998). Previous NRC publications (NRC, 1953; NRC, 1985) have stated that vitamin D2 is used as efficiently as vitamin D3 by dogs, with reference to work by C.A. Elvehjem (Arnold and Elvehjem, 1939; Michaud and Elvehjem, 1944).

However, the latest publication (NRC, 2006) states that no studies examining the differences in forms could be found in dogs, so NRC (2006) recommendations exist only for vitamin D3. This has important implications for pet food companies choosing to use vitamin D2 as the vitamin D supplement, i.e. for use in “vegan” diets. The possibility that pet food companies have conducted their own research into vitamin D2 requirements cannot be discounted, however, the proprietary nature of this research results in no gain in knowledge to the scientific community. An abstract presented at the American Association for Veterinary Nutrition 2015 Symposium, confirmed the 1939 findings, showing that two adult dogs fed homemade diets supplemented with vitamin D2 maintained normal serum ionized calcium (ICa) and 25(OH)D levels (Delaney, 2015). This suggests a similar potency between D2 and D3, however, larger scale studies would be helpful to fully establish this.

2.7.2 Knowledge of the vitamin D requirements of adult dogs

Studies examining vitamin D requirements could only be found for puppies, so the NRC (2006) chose to recommend the same requirements for adult maintenance as for growth and reproduction (Table 2.1). The same approach (Table 2.1) was taken by AAFCO (2014). Despite the acknowledgement that no studies of the vitamin D

requirements of adult dogs could be found, the NRC (2006) states that mature dogs are relatively resistant to a dietary deficiency of vitamin D. This is dismissive to the need for basic vitamin D research in adult dogs, yet may be due to the timing of the publication (2006). Evidence for the multiple roles that vitamin D may play in an adult dog's health (Gow et al., 2011; Holowaychuk et al., 2012; Kraus et al., 2014), has since been published. As this research continues to grow, it is essential to know as much as possible about the requirements of vitamin D for every life stage, and particularly mature dogs, that have increased risk of developing the very conditions that vitamin D has been associated with (Merlo et al., 2008; Wakshlag et al., 2011).

2.7.3 Relationship between vitamin D intake and vitamin D status, including factors that may affect this relationship

Although some studies referenced by the NRC measure both 25(OH)D concentrations and vitamin D intake (Hazewinkel et al., 1987; Tryfonidou et al., 2002), no work has been done to determine the relationship between the two. A recent study (Sharp et al., 2015) examined serum 25(OH)D concentrations of dogs fed various commercial and homemade diets. Serum 25(OH)D concentrations were significantly different among dogs fed diets from different manufacturers, and there was a large range of serum 25(OH)D in dogs receiving homemade diets. However, the vitamin D levels of each diet were not measured, so no conclusions could be drawn about the impact of vitamin D intake on serum 25(OH)D concentrations. Clinicians often measure a dog's serum 25(OH)D status, using the reference range provided by the laboratory as an indicator of the dog's health. For example, the often cited reference range for serum 25(OH)D concentration in dogs comes from the Michigan State University's Diagnostic Center for Population and Animal Health and is 60– 215 nmol/L (Nachreiner et al., 2014). The use of serum 25(OH) D as a marker is now being extended to a variety of disease states in the dog, such as chronic kidney disease (Galler et al., 2012), primary hyperparathyroidism (Gerber et al., 2004), inflammatory bowel disease (Gow et al., 2011), induced endotoxemia (Holowaychuk et al., 2012), and cancer (Selting et al., 2016). However, many of these researchers (Gerber et al., 2004; Galler et al., 2012;

Selting et al., 2016) failed to measure the vitamin D intake of participating animals. If the relationship between dietary vitamin D intake and serum 25(OH)D concentration is not established, then the ability of dietary vitamin D intake to prevent or alleviate disease cannot be determined. This knowledge is essential for the development of appropriate vitamin D intake recommendations, as well as for translating the results of vitamin D—disease research into clinical applications. Potential factors to affect the relationship between vitamin D intake and serum 25(OH)D (e.g. age, body condition score, breed, genetic variation) must also be taken into consideration.

2.8 How much vitamin D does a typical dog receive?

Since AAFCO requirements range from 500–5000 IU vitamin D/kg DM, the amount of vitamin D that a dog fed an AAFCO compliant commercial diet will receive is dependent on the manufacturer's choice of inclusion level within this range. The same holds true for manufactures that follow the FEDIAF guidelines (552–3200 IU vitamin D/kg DM). As a result, dogs fed different diets (even among the same brand), may receive vastly different amounts of vitamin D (Kritikos et al., 2015; Sharp et al., 2015). Dogs receiving a homemade diet, if not formulated by a board-certified veterinary nutritionist (i.e. Diplomate of the American College of Veterinary Nutrition (ACVN) or of the European College of Veterinary and Comparative Nutrition (ECVCN)), may be fed any amount of vitamin D that the owner chooses to include or not include. However, since vitamin D is only present in a few food sources, homemade diets are often deficient according to NRC, AAFCO, and FEDIAF standards (Remillard, 2008; Larsen et al., 2012; Stockman et al., 2013). Researchers must not assume that all dogs enrolled in a study are receiving similar amounts of vitamin D, even if all dogs are receiving commercial diets, or all dogs are receiving home prepared diets. Moreover, as mentioned above, the vitamin D content of the final product reported by the manufacturer may be inaccurate. Researchers interested in analyzing vitamin D intake should analyze feed samples for the most accurate measure of vitamin D content.

Dogs fed an AAFCO compliant commercial diet are likely receiving more dietary vitamin D than many humans, as dietary vitamin D intake, including supplement use, in humans is often reportedly lower than recommended levels (Moore et al., 2004; Calvo et al., 2005). This has prompted mandatory food fortification measures in some countries, e.g. milk in Canada and the United States. However, studies suggest even this is not enough (Calvo et al., 2004; Calvo et al., 2005), especially for those with certain dietary habits, such as vegetarians or individuals with lactose intolerance. This is important to remember when comparing results from human studies and canine studies. Dogs may have a higher baseline vitamin D status as a result of higher dietary vitamin D intake, which may affect the relationship between the two, and other influential factors like parathyroid hormone (PTH) and ICa.

2.9 Disease

Researchers are drawing on inspiration from human studies to investigate links between vitamin D and disease in dogs. In humans, evidence exists for a role of vitamin D in a range of health outcomes, including bone health, diabetes, cardiovascular disease, immune function and cancer (Hyppönen et al., 2001; Holick, 2004; Jenab et al., 2010; Kamen & Tangpricha, 2010). This is a novel area of research in dogs, and only a few diseases have been investigated. Focus has been placed on low vitamin D status as a potential risk factor for disease development, as a consequence of disease, and as an indicator of poor prognosis. Results may lead to improved strategies for disease prevention and disease management. No studies linking vitamin D with survival outcomes could be found.

2.9.1 Bone Health

Rationale

The latest committee formed by the Institute of Medicine in the United States chose bone health as the health outcome for development of the Dietary Reference Intakes for vitamin D (Ross et al., 2011). Although other health outcomes (e.g. cancer,

cardiovascular disease) were considered, bone health was the only outcome with enough conclusive evidence to support its use. Many of the studies referenced by the NRC (2006) for development of vitamin D intake recommendations in dogs also use bone health as a measure of adequate vitamin D intake. Vitamin D's role in bone health is well accepted, given its regulatory role in bone metabolism.

Studies

Much of the work done on vitamin D and bone health in dogs was completed by researchers at the University of Utrecht, and has been covered in an earlier section of this review. Briefly, these studies include: the development of rickets in puppies fed a diet with no supplemental vitamin D, which resolved with feeding the same diet supplemented with vitamin D (Hazewinkel et al., 1987); normal growth rates and skeletal development in dogs fed diets containing AAFCO (2014) compliant vitamin D levels (Tryfonidou et al., 2002); and impaired endochondral ossification in puppies fed a diet containing approximately 10.8 times the maximum recommended intake set by AAFCO (2014) (Tryfonidou et al., 2003).

2.9.2 Chronic Kidney Disease

Rationale

The kidneys play a central role in vitamin D metabolism as 1,25(OH)2D is produced in the proximal tubules by the enzyme cytochrome P450 27B1. Chronic kidney disease (CKD) can impair this process (Al-Badr & Martin, 2008). Suboptimal 25 (OH)D concentrations, the accepted marker of vitamin D status (Holick, 1995), are common in human CKD patients (Gonzalez et al., 2004; Ravani et al., 2009). Correcting this suboptimal status with supplemental vitamin D is recommended in human patients with CKD and concurrent hyperparathyroidism (Holick, 2007; Al-Badr & Martin, 2008).

Studies

Plasma and serum 25(OH)D concentrations are decreased in dogs with CKD when compared to healthy controls (Galler et al., 2012; Gerber et al., 2003, 2004). However, no conclusions have been reached specifically regarding vitamin D2 or D3 supplementation for these patients (Galler et al., 2012), however, supplementation of the active form of vitamin D, 1,25(OH)2D, is used for treatment (Nagode et al., 1996; Roudebush et al., 2010). Though 1,25(OH)2D is not used as a marker of vitamin D status (Holick, 2009), measurement of 1,25(OH)2D is still useful in cases where production may be affected, as is likely with CKD, and a relationship between CKD and 1,25(OH)2D should be expected. Investigation of 1,25(OH)2D concentrations between dogs with CKD and healthy controls has yielded mixed results. Affected dogs had decreased serum 1,25(OH)2D concentrations in one study (Gerber et al., 2003) but no changes in another (Gerber et al., 2004). A relationship is more likely observed when 1,25(OH)2D concentrations are related to disease severity, than to the presence of disease, and this has been demonstrated in recent work (Cortadellas et al., 2010).

2.9.3 Inflammatory Bowel Disease

Rationale

Low vitamin D status is common in human patients with inflammatory bowel disease (IBD). Many hypotheses have attempted to explain this association, addressing a low vitamin D status as both a consequence of and a risk factor for IBD.

Interested readers should look to the review published by Garg et al. (2012) for explanation of these hypotheses. Briefly, causes for low vitamin D status in human IBD patients include: mucosal disease or surgical resection causing malabsorption, reduced sunlight exposure, and/or reduced dietary intake. Other suggested hypotheses include: “leakage” of vitamin D through the gastrointestinal tract and reduced circulation of vitamin D metabolites (Pappa et al., 2006). Since a low vitamin D status may be observed prior to IBD diagnosis, Garg et al. (2012) also summarize roles for vitamin D

in IBD development and progression including: the maintenance of epithelial barrier, involvement in the innate immune response and the adaptive T-cell response, and genetic polymorphisms in vitamin D metabolism which may increase IBD risk.

Studies

Case reports linking abnormal vitamin D metabolism to ionized hypocalcemia in dogs with protein-losing enteropathies (PLE) (Kimmel et al., 2000; Bush et al., 2001) lead researchers to investigate serum 25(OH)D in dogs with IBD, dogs with IBD and hypoalbuminemia (referred to as the PLE group), and healthy dogs (Gow et al., 2011). Only dogs with PLE had significantly decreased serum 25(OH)D concentrations when compared to dogs with IBD and healthy dogs, supporting the hypothesis that an increased loss of vitamin D through the gastrointestinal tract is responsible for lower 25(OH)D concentrations in patients with gastrointestinal disease.

2.9.4 Cardiovascular Disease

Rationale

Epidemiologic and mechanistic evidence support a role for vitamin D in cardiovascular disease. Current evidence indicates a decreased risk for cardiovascular disease with increasing plasma 25(OH)D (Wang et al., 2012). The shape of this relationship and the plasma 25(OH)D concentration where risk may plateau is still under debate (Melamed et al., 2008; Wang et al., 2012). Mechanisms behind this relationship have been reviewed and include improvement of insulin sensitivity, negative regulation of renin and anti-inflammatory activities (Judd & Tangpricha, 2009; Pilz et al., 2011).

Studies

Serum 25(OH)D concentrations were significantly lower in client-owned dogs with congestive heart failure (CHF) when compared to healthy dogs (Kraus et al., 2014). Researchers found no differences in vitamin D intake, when calculated per kg of metabolic body weight, between groups. Researchers also found lower serum 25(OH)D

concentrations were associated with increased risk of cardiovascular events, when events were defined as any CHF-related medical complications, sudden death or adjustments made to cardiac medications for suspected CHF. Osuga et al. (2015) investigated whether serum 25 (OH)D concentrations were correlated with disease severity in client-owned dogs with chronic valvular heart disease (CVHD). Serum 25(OH)D concentrations were significantly lower in dogs with increased disease severity (Stage B2 and Stage C/D) than in dogs with stage B1 CVHD. There was a significant negative correlation between serum 25(OH)D and left atrial and ventricular size, suggesting vitamin D may be associated with the degree of cardiac remodeling. No comparisons were made to healthy dogs in this study, however, median serum 25(OH) D concentration for stage B1 dogs (Osuga et al., 2015) was much lower than those observed for healthy dogs in other studies (Wakshlag et al., 2011; Kraus et al., 2014), and falls below the often-cited Michigan State University's Diagnostic Center for Population and Animal Health's reference range of 60–215 nmol/L (Nachreiner et al., 2014). Still, these differences may be attributed to methods used for 25(OH)D measurement, as a commercially available enzyme-linked immunosorbent assay (ELISA) was used in the Osuga et al. (2015) study, while radio-immunoassays were used in other studies (Wakshlag et al., 2011; Kraus et al., 2014) and by Michigan State.

2.9.5 Cancer

Rationale

Studies have linked low vitamin D intake and low 25(OH)D concentrations (Holick, 1995) to increased risk of human cancers, i.e. breast and colorectal cancer (Garland et al., 2007; Jenab et al., 2010). 1,25(OH)2D shows a range of anticancer activities, such as the induction of cellular apoptosis and differentiation, inhibition of cellular proliferation, angiogenesis and metastasis, and enhancement of DNA repair (Fleet et al., 2012). The strength of the association between vitamin D and cancer seems dependent on cancer type. For instance, the World Cancer Research Fund released a meta-analysis concluding there is suggestive evidence of a link between a low intake of foods containing vitamin D and the development of colorectal cancer

(World Cancer Research Fund/American Institute for Cancer Research, 2011). The same organization released another meta-analysis concluding that the results of studies were too variable to support any relationship with vitamin D and breast cancer risk (World Cancer Research Fund/American Institute for Cancer Research, 2010).

Studies

The presence of vitamin D receptors has been shown in osteosarcoma and mast cell tumor tissue from dogs (Russell et al., 2010; Davies et al., 2012), and 1,25(OH)2D induced apoptosis, differentiation, and/or reduced cell growth in canine osteosarcoma and mast cell tumor cell lines (Barroga et al., 1998, 2000; Rassnick et al., 2008). Selting et al. (2016) reported that dogs with hemangiosarcoma had decreased vitamin D status when compared to healthy dogs. Dogs with neoplastic spirocercosis had significantly lower serum 25(OH)D concentrations compared to dogs with non-neoplastic spirocercosis and healthy dogs (Rosa et al., 2013). Wakshlag et al. (2011) measured vitamin D intake and serum 25(OH)D concentrations in healthy Labrador Retrievers, and those with mast cell tumors, and reported dogs with mast cell tumors had a significantly decreased serum 25(OH)D concentrations, but similar vitamin D intake when compared to healthy dogs. Two studies measured blood vitamin D metabolites in dogs with hypercalcemia (Rosol et al., 1992; Gerber et al., 2004). Dogs with lymphoma were one of the hypercalcemic groups in each study. Both studies reported high inter-individual variability of serum 1,25 (OH)2D concentrations. Gerber et al. (2004) included measurement of serum 25(OH)D and reported serum 25(OH)D to be significantly decreased in dogs with lymphoma when compared to healthy controls.

A recent study has explored the relationship between vitamin D intake and vitamin D status in healthy dogs and dogs with cancer, specifically lymphoma, osteosarcoma and mast cell tumors (Weidner et al., 2015). The effects of other variables (e.g. body condition score, ICa, PTH) were also investigated. Dietary vitamin D intake showed a relationship with vitamin D status, and this relationship was the same in all groups of dogs. There was also a relationship between 24,25(OH)2D and vitamin D status, which

was the same in all groups. ICa and cancer both had significant effects on vitamin D status, and interacted with each other to exert effects. Significant differences in vitamin D status between the healthy group and cancer groups were only observed at certain blood concentrations of ICa. Dietary vitamin D intake, plasma 24,25(OH)2D concentrations, plasma ICa concentrations and cancer impacted vitamin D status, further demonstrating the importance of accounting for multiple variables that may affect vitamin D status in experimental designs.

2.10 Implications for future research

These studies give researchers the beginnings of a structure on which to build future research in this area. However, for a building to be sound, there must also be a sturdy foundation in place. A better understanding of the basic vitamin D knowledge, and its limitations, specific to dogs is essential to ensure appropriate experimental design of studies. Table 2.2 summarizes the aspects of vitamin D metabolism accounted for in the above studies, and illustrates that many studies do not account for the impact of vitamin D intake. Measurement of dietary vitamin D intake is important, as this contributes to the dog's vitamin D status (Hazewinkel et al., 1987), and because, as discussed above, vitamin D intake can be quite variable among client-owned animals. Additionally, even when vitamin D intake is measured, only one study on the relationship between vitamin D intake and blood 25(OH)D concentrations could be found (Weidner et al., 2015). This knowledge is essential for the translation of research associating blood 25(OH)D concentrations with reduction in disease risk and/or improvement in disease outcome. Without knowledge of the dietary vitamin D intake that correlates with the target blood 25(OH)D concentration, the vitamin D intake recommendations (by NRC/AAFCO/ FEDIAF) and/or vitamin D supplementation recommendations (by clinicians) cannot reflect the results.

Table 2.2: Studies examining associations between vitamin D and disease in dogs. Many studies do not account for vitamin D intake. Despite measurement of both vitamin D intake and vitamin D status (25(OH)D) in some studies, only one study examined the relationship between the two variables.

Study	Health Outcome	Vitamin D Intake	25(OH)D Status
Hazewinkel et al., 1987	Growth/Bone health	No individual intake information **but** amount of vitamin D supplement added to test diet	X
Kealy et al., 1991	Growth/Bone health	No individual intake information **but** amount of vitamin D supplement added to test diet	X
Tryfonidou et al., 2002	Growth/Bone health	X	X
Tryfonidou et al., 2003	Growth/Bone health	X	X
Gerber et al., 2003	CKD		X
Gerber et al., 2004	CKD, lymphoma, primary hyperparathyroidism		X
Galler et al., 2012	CKD		X
Gow et al., 2011	IBD		X
Holowaychuk et al., 2012	Endotoxemia		X
Rosa et al., 2013	Spirocercosis (non-neoplastic and neoplastic)		X
Kraus et al., 2014	Congestive heart failure	X	X
Wakshlag et al., 2011	Cancer (MCT)	X	X
Selting et al., 2014	Cancer (Primarily hemangiosarcoma)		X
Spoo et al., 2015	None	X	X
Sharp et al., 2015	None		X
Osuga et al., 2015	Chronic valvular heart disease		X
Weidner et al., 2015	Cancer (LSA, OSA, MCT)	X	X

CKD = Chronic kidney disease, IBD = Inflammatory bowel disease, LSA = lymphoma, MCT = Mast cell tumour, OSA = Osteosarcoma, PTH = Parathyroid hormone, IGF-1 =

Insulin-like growth factor 1, VDR = Vitamin D receptor, 25(OH)D Status = Blood 25-hydroxyvitamin D concentration

2.11 Sufficiency

The purpose of many vitamin D studies in humans is to define a blood 25(OH)D level sufficient to minimize disease risk to the majority of the population. There has been a move towards categorizing blood vitamin D levels as deficient, insufficient, and sufficient (Dawson-Hughes et al., 2005), however, there are many different opinions about cutoff points and health outcomes that should be associated with each (Hollis, 2005; Dawson-Hughes et al., 2005; Holick, 2007; Godel et al., 2007; Ross et al., 2011). Currently, most researchers define sufficiency as the level of 25(OH)D where: PTH secretion is minimized, intestinal calcium absorption is stabilized and/or calcium resorption from bone is minimized (Dawson-Hughes et al., 2005; Hollis, 2005; Canadian Pediatric Society, posted 2007, reaffirmed 2015; Ross et al., 2011). The Institute of Medicine (IOM) reviewed 25(OH)D levels in relation to markers of bone health (calcium absorption, fracture risk, osteomalacia) when developing dietary intake guidelines for vitamin D (Ross et al., 2011), and concluded that all people were sufficient at 50 nmol/L. Many researchers suggest a much higher set point, of at least 75 nmol/L, and sometimes higher (Dawson-Hughes et al., 2005; Hollis, 2005; Holick, 2007; Godel et al., 2007). However, the IOM emphasizes that many cut-points are not backed by scientific consensus, and encourages further research.

Selting et al. (2016) used suppression of PTH secretion to define the sufficiency level for serum 25(OH)D in dogs. Other measurements, such as the level of serum 25(OH)D where variation in phosphorus levels are minimized, were also used as markers of sufficiency, for reasons that are not explained in the publication. Authors concluded that 25(OH)D sufficiency in dogs be defined as serum concentrations of 100–120 ng/mL. If converted into nmol/L, this sufficiency range would be 115– 139% of the maximum vitamin D range set by the Michigan State University's Diagnostic Center for

Population and Animal Health (Nachreiner et al., 2014). The authors did not measure vitamin D intake in any of the participating animals, nor did they perform any bloodwork, urinalysis or medical imaging to ensure the health of enrolled animals. These results must be interpreted with extreme caution, and should not be used to justify supplementation to achieve blood vitamin D levels suggested by the authors. Currently, reference ranges for blood 25(OH)D are lab specific. Furthermore, reference ranges for individual labs may be quite large. For instance, the often-cited Michigan State University's Diagnostic Center for Population and Animal Health's range is 60–215 nmol/L (Nachreiner et al., 2014). Further work is necessary before any consensus statements on blood 25(OH)D concentrations that define sufficiency in dogs can be made.

3 Vitamin D and inflammation: A relationship worth investigating in dogs with cancer?

Author's note: The literature review in the previous chapter was submitted for publication in 2016. There have been numerous studies of vitamin D in dogs published since. This prompts another review and critical assessment of the literature to 1) update relevant terminology and conclusions of the previous literature review, and 2) connect this new information to the topic of this thesis: vitamin D and inflammation in dogs with cancer.

3.1 Abstract

This literature review summarizes new findings related to the previous literature review, including sections on: vitamin D in dog food; the relationship between dietary vitamin D intake and circulating 25-hydroxyvitamin D (25(OH)D) concentrations; other factors that affect circulating 25(OH)D concentrations; and 25(OH)D concentrations and disease. Importantly, in the past 5 years, researchers have increasingly emphasized the need to identify other variables that may influence circulating 25(OH)D concentrations. A key variable suggested to influence 25(OH)D concentrations in humans is inflammation. There is a limited amount of work investigating links between vitamin D and inflammation in dogs, but relationships have been observed in several cross-sectional, longitudinal, and mechanistic studies. These relationships should be explored in canine cancer. Cancer is a chronic, inflammatory condition. Researchers have observed that the inflammatory profile of cancer can differ depending on cancer type (potentially due to differences in pathogenesis or pathophysiology), and that concentrations of inflammatory markers can decrease with successful treatment. Exploration of links between 25(OH)D and inflammation in dogs with cancer would enable investigation of whether decreased 25(OH)D concentrations are associated with a particular inflammatory profile, and whether 25(OH)D concentrations increase as inflammatory marker concentrations decrease with successful cancer treatment.

3.2 Introduction

Since publication of the previous review (Weidner & Verbrugghe, 2017), vitamin D has continued to gain attention from canine health researchers. Circulating 25(OH)D concentrations have now been reported in dogs with a wide range of diseases, from infectious diseases to kidney disease to cancer, with enough work in this area to warrant the publication of a notable amount of literature reviews (Mellanby, 2016; Corbee, 2020; Chacar et al., 2020; Hurst, Homer, & Mellanby, 2020; Zafalon, Ruberti et al., 2020; Zafalon, Risolia, et al., 2020; Stockman et al., 2021). Researchers have continued to express interest in defining new reference ranges for 25(OH)D and have investigated ways to increase the concentrations of this metabolite (Young and Backus, 2016, 2017). This makes it imperative to understand what factors might be associated with, and potentially confound, the measurement of circulating 25(OH)D concentrations in dogs.

Research has suggested that systemic inflammation may confound 25(OH)D measurement (Ghashut et al., 2014; Silva & Furlanetto, 2015), potentially through the dysregulation of vitamin D metabolic pathways by pro-inflammatory cytokines (Zehnder et al., 2002; Hummel et al., 2014). This finding is important to investigate in dogs, as inflammation plays a crucial role in the development and/or progression of many of the diseases where lower circulating concentrations of 25(OH)D have been observed.

This may be particularly relevant for researchers investigating dogs with cancer. Inflammation has been included as one of the enabling characteristics of cancer, playing an important role in the development and progression of the disease (Hanahan & Weinberg, 2011). Several studies have reported increased concentrations of inflammatory markers in dogs with cancer (Nielsen et al., 2007; Chase et al., 2012; Calvalido et al., 2016). Interestingly, variable inflammatory profiles have been noted for different cancer types (Tecles et al., 2005; Chase et al., 2012). Similar differences between cancers have been reported with 25(OH)D concentrations, where lower 25(OH)D concentrations are reported in some cancers (Wakshlag et al., 2011), but not

others (Willcox et al., 2016). Additionally, inflammatory marker concentrations have been reported to decrease with successful canine cancer treatment (Nielsen et al., 2007). This presents several opportunities for research in this area:

1) If lower 25(OH)D concentrations are associated with systemic inflammation, then concurrent investigation of 25(OH)D and inflammatory marker concentrations in several cancers may identify which cancers and/or inflammatory markers are potentially associated with low 25(OH)D.
2) If lower 25(OH)D concentrations are a consequence of the inflammation associated with cancer, then 25(OH)D concentrations should increase and inflammatory marker concentrations should decrease upon successful cancer treatment.
3) If lower 25(OH)D concentrations observed in dogs with cancer are a consequence of the dysregulation of vitamin D metabolism by pro-inflammatory cytokines, then measurement of additional vitamin D metabolites and the ratio between metabolites would be of value.

The goals of this literature review are to provide background knowledge, update the conclusions of the previously published literature review, and demonstrate why the studies contained in this thesis investigating circulating concentrations of 25(OH)D and inflammatory markers in dogs with cancer are warranted.

3.3 Current knowledge of vitamin D in dogs – An update

Research published in the last few years is relevant to the conclusions drawn in the previous literature review. For instance, new research has addressed some of the difficulties associated with determining the vitamin D content of dog food. This knowledge is important to determine dietary vitamin D intake. An extension of the work reported in this thesis was published in 2018 (Kritikos et al., 2018). Commercial dog food samples were obtained from owners of dogs enrolled in the studies contained in this thesis. The vitamin D3 concentrations of commercial dog foods were analyzed and compared with Association of American Feed Control Officials (AAFCO) and National Research Council (NRC) recommendations, and manufacturer-reported concentrations.

The analyzed vitamin D3 concentrations did not differ significantly from manufacturer reported vitamin D3 concentrations. Vitamin D3 concentrations were below both AAFCO and NRC recommendations for one sample only and below the assay detection limit for another, suggesting that dog owners can usually be confident that a dog's vitamin D3 intake is adequate for AAFCO-compliant commercial dog foods. Further work related to the vitamin D content of dog foods investigated the vitamin D content of fish ingredients (Lopresti et al., 2020), which can be variable and is important knowledge for manufacturers trying to control the vitamin D concentrations of the final product. Vitamin D content varied depending on species, cut, year of collection and farm status (i.e. farmed vs wild-caught fish). Most recently, gas chromatography tandem mass spectrometry (GC/MS/MS) was successfully investigated as an alternative to the liquid chromatography tandem mass spectrometric (LC/MS/MS) methods usually used for measuring vitamin D concentration in dog foods (Lehner et al., 2021). Elevated, and potentially toxic, concentrations of vitamin D were reported in 18/27 dog foods investigated. However, these foods were submitted to the diagnostic laboratory because of a suspected excess vitamin D content, so findings may not be representative of the larger dog food market. Overall, results of these studies are helpful to manufacturers, researchers, and nutritionists, interested in determining vitamin D content of foods and dietary vitamin D intake.

Interestingly, the relationship between intake and circulating 25(OH)D concentrations has also received attention. Recent work has revealed the relationship between dietary vitamin D intake and circulating 25(OH)D concentrations may be less direct than was previously thought (Young & Backus, 2016; Weidner et al., 2017; da Fonseca et al., 2020; Hurst, Homer, Gow, et al., 2020). The estimated dietary vitamin D intake of client-owned dogs did not correlate with serum 25(OH)D concentrations at the beginning of a vitamin D supplementation trial (Young & Backus, 2016). Additionally, there was no change in the treatment group's 25(OH)D concentrations while receiving a vitamin D supplement at five times the NRC's recommended allowance over 10 weeks (Young & Backus, 2016). Dietary vitamin D intake did not have a significant effect on

circulating 25(OH)D concentrations in a cross-sectional study of healthy dogs and dogs with cancer (Weidner et al., 2017). Similarly, there was no correlation between the amount of vitamin D consumed and circulating 25(OH)D concentrations in adult dogs and puppies (da Fonseca et al., 2020). Finally, though circulating 25(OH)D concentrations remained stable in healthy dogs consuming the same diet with a standardized vitamin D content over a 1-year period, there was considerable variation in 25(OH)D concentrations between individual dogs (Hurst, Homer, Gow, et al., 2020). Several researchers have now suggested that circulating 25(OH)D concentrations may not directly reflect vitamin D intake and emphasized the importance of investigating other factors that may influence circulating 25(OH)D concentrations (da Fonseca et al., 2020; Corbee., 2020), sparking interest in these factors.

A few studies have investigated the effects of age, sex, body condition score and breed on circulating 25(OH)D concentrations. These are usually tertiary objectives of a larger study, and nothing conclusive has been established. Age was not associated with 25(OH)D concentrations in adult dogs (Weidner et al., 2017) in one study, however lower 25(OH)D concentrations were recently reported in puppies compared to adults, despite the puppies having a higher vitamin D intake (da Fonseca et al., 2020). Sex and neuter status have been suggested to influence 25(OH)D concentrations (Sharp e al, 2015), though this has not been replicated in other work (Weidner et al., 2017). Body condition score has not been observed to influence 25(OH)D concentrations (Wakshlag et al., 2011; Weidner et al., 2017). This might be limited by the lack of underweight and obese dogs in the populations studied. Finally, differences in 25(OH)D concentrations were observed between golden retrievers and German shepherds, suggesting the possibility of breed specific factors that may influence vitamin D metabolism (Sharp et al., 2015). Further studies that are designed to account for confounding variables (e.g. a standardized diet, confirmation of health, age/breed controlled, etc.) are still required before firm conclusions can be drawn (Hurst, Homer, Gow, et al., 2020).

Researchers have also expanded the body of work linking low circulating vitamin D concentrations to disease. The list of conditions investigated now includes dogs with

cancer (Willcox et al., 2016; Weidner et al., 2017), chronic kidney disease (Parker et al., 2017; Miller et al., 2020; Parker et al., 2020), chronic enteropathies (Allenspach et al., 2017; Wennogle et al., 2019; Wennogle et al., 2021), gallbladder mucocele (Jaffey et al., 2020), infectious disease (Erdogan et al., 2019; Dvir et al., 2019), hypercalciuric calcium oxalate urolithiasis (Groth et al., 2019), cranial cruciate ligament rupture (Clements et al., 2020; Clements et al., 2021), immune mediated disease (Mick et al., 2019), exocrine pancreatic insufficiency (Barko et al., 2018), acute polyradiculoneuritis (Laws et al., 2018) and prostatic hyperplasia (Beining et al., 2021). The author of a recent review concluded that the "most evidence is available for effectiveness of calcitriol [most active metabolite of vitamin D] supplementation in dogs with IRIS stage 3 and 4 chronic kidney disease" (Corbee, 2020). An in-depth look at these studies is beyond the scope of this review. Readers may look to Zafalon, Risolia, et al. (2020), Chacar et al. (2020), and Parker et al. (2017) for an overview of studies linking 25(OH)D to disease, and Corbee (2020) for a discussion on the clinical relevance and practical implications of these findings.

Emerging evidence also suggests that the term vitamin D status should not be used as interchangeably with 25(OH)D concentrations (Corbee, 2020), as was done in the previously published review. Several studies have now been unable to establish a relationship between dietary vitamin D intake and circulating 25(OH)D concentrations (Weidner et al., 2017; da Fonesca et al., 2020). The existence of a relationship has not been ruled out, but it's possible that other variables may play influential roles in influencing circulating 25(OH)D concentrations, and so 25(OH)D concentrations may not be the most sensitive indicator of vitamin D status in dogs (Corbee, 2020). The remainder of this thesis will take these findings into account and the term vitamin D status will not be used, unless presented with a new definition.

The knowledge related vitamin D in dogs has grown since the publication of the previously published literature review in the previous chapter. Investigators are better equipped to estimate dietary vitamin D intake in dogs, with advancements in knowledge of the vitamin D content of ingredients, the final dog food product, and measurement

methodologies. Efforts have also been made to investigate the relationship between vitamin D intake and circulating 25(OH)D concentrations, however the relationship remains unclear, highlighting the need to identify other factors that may influence 25(OH)D concentrations. Factors like age, sex, breed, and body condition score have been investigated, but nothing conclusive has been established. Lower circulating 25(OH)D concentrations have been linked to additional canine health conditions, and investigators have looked at potential mechanisms and supplementation in some of these conditions. Advancements in knowledge suggest researchers should exercise caution in using the terms “vitamin D status” interchangeably with circulating 25(OH)D concentrations. Importantly, researchers have emphasized the need to identify other variables that may influence circulating 25(OH)D concentrations.

3.4 Vitamin D and inflammation

One variable suggested to influence 25(OH)D concentrations in other species is inflammation. Inflammation is a body’s reaction to injury or infection. Inflammation can be detected through measurement of commonly accepted circulating biomarkers including white blood cell, cytokine/chemokine, and acute-phase protein (APP) concentrations (Brenner et al., 2014). White blood cells play an important role in the immune system, fighting infection and other disease by producing antibodies and attacking bacteria, viruses, and other foreign substances (Brenner et al., 2014). Cytokines and chemokines are a group of proteins secreted by immune cells that are involved in cell signalling, with some considered pro-inflammatory and others anti-inflammatory (Feghali and Wright, 1997). The group of proteins known as APP are produced by the liver and change in concentration in response to inflammation (Cerón et al., 2005). Concentrations of positive APP increase in response to tissue injury and inflammation (Cerón et al., 2005). Examples of positive APP include C-reactive protein [CRP], haptoglobin [HP], serum amyloid A [SAA] and alpha-1-acid glycoprotein [AAG] (Cerón et al., 2005). Concentrations of negative APP decrease in response to inflammation (Cerón et al., 2005). Examples of negative APP include transferrin and albumin (Cerón et al., 2005).

The link between vitamin D and inflammation in humans has received a fair bit of attention (Liu et al., 2018; Filgueiras et al., 2020; Reijven & Soeters, 2020; Smolders et al., 2021). Numerous studies have reported inverse relationships between circulating concentrations of 25(OH)D and inflammatory markers (most notably with APP concentrations, such as CRP) (Duncan et al., 2012; Ghashut et al., 2014; Liefaard et al., 2015; Kruit & Zanen, 2016; Srikanth et al., 2016; Yarparvar et al., 2020). Prospective cohort studies also assessed 25(OH)D concentrations before and after an inflammatory event, such as an elective surgery or lipopolysaccharide administration (Reid et al., 2011; Waldron et al., 2013; Smolders et al., 2021). A systematic review found that 25(OH)D concentrations decreased after the inflammatory event (elective surgery, amino bisphosphonate administration or acute illness) in six of the eight studies included (Silva & Furlanetto, 2015). No change in 25(OH)D concentrations was observed in two of the studies, but baseline concentrations were measured after symptom onset in both studies, limiting interpretation of the results.

Researchers have explored several mechanisms to explain observed relationships between lower 25(OH)D concentrations and inflammation using human cell lines (Zehnder et al., 2002; Hummel et al., 2014). Pro-inflammatory cytokines can regulate enzymes involved in vitamin D metabolism, affecting the conversion of 25(OH)D to other vitamin D metabolites (Zehnder et al., 2002; Hummel et al., 2014). For example, treatment of human colon cancer cells with interleukin-6 increased expression of CYP24A1, the enzyme responsible for converting 25(OH)D to 24,25(OH)2D (Hummel et al., 2014). It's worth noting that the circulating 25(OH)D:24,25(OH)2D metabolite ratio has recently been used as an indicator of CYP24A1 activity in humans and dogs (Groth et al., 2019; Makris et al., 2020) and may be a relevant variable for future mechanistic studies. Overall, the discovery of links between 25(OH)D and inflammation in humans has sparked interest in determining if this phenomenon occurs across species, and specifically in dogs.

Although there is limited research exploring links between vitamin D and inflammation in dogs (Titmarsh et al., 2015; Wennogle et al., 2019; Erdogan et al.,

2019), the results are intriguing and call for further investigation. A negative relationship between circulating 25(OH)D concentrations and markers of inflammation has been observed in cross-sectional studies of healthy dogs (Selting et al., 2016) and dogs with disease such as chronic enteropathy or visceral leishmaniasis (Titmarsh et al., 2015; Wennogle et al., 2019; Erdogan et al., 2019). Also, a small number of longitudinal studies measured 25(OH)D concentrations before and after an inflammatory event (Holowaychuk et al., 2012; Spoo et al., 2015; Clements et al., 2020). Circulating 25(OH)D concentrations of healthy dogs decreased after intravenous administration of a commonly used inflammatory agent, lipopolysaccharide, (Holowaychuk et al., 2012). In contrast, 25(OH)D concentrations increased during the inflammatory response triggered by intense exercise in racing sled dogs (Spoo et al., 2015). Most recently, serum total 25(OH)D concentrations decreased transiently after an acute inflammatory insult (an elective orthopedic surgery) in dogs with a spontaneous cranial cruciate ligament rupture (Clements et al., 2020). Serum total 25(OH)D concentrations were not significantly different from concentrations recorded pre-surgery when measured 60 days after surgery (Clements et al., 2020). Serum C-reactive protein concentrations were also not significantly different from concentrations recorded pre-surgery at this time (Clements et al., 2020). Finally, from a mechanistic standpoint, the most biologically active form of vitamin D, 1,25-dihydroxy vitamin D (1,25(OH)2D) or calcitriol, reduced production of the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α) and increased lipopolysaccharide stimulated production of the anti-inflammatory cytokine, interleukin-10 (IL-10) in endotoxin primed whole blood collected from healthy dogs and whole blood collected from critically ill dogs (Jaffey, Amorim, et al., 2018a, 2018b). Taken altogether, these findings warrant further investigation into links between circulating 25(OH)D and inflammation in dogs.

Cross-sectional, longitudinal and mechanistic work in humans has documented a relationship between lower 25(OH)D concentrations and the presence of inflammation. This work has been replicated to a lesser degree in dogs, with similar results. These results are interesting and may be especially relevant to individuals interested in other

factors that may influence 25(OH)D concentrations. Further investigation into links between circulating 25(OH)D and inflammation in dogs is warranted. Investigations into dogs with chronic inflammatory diseases where lower 25(OH)D concentrations have been observed, such as cancer (Gerber et al., 2004; Wakshlag et al., 2011), may be especially fruitful. Cancer is a relatively common, chronic inflammatory disease affecting dogs that may make a suitable candidate for this research.

3.5 Vitamin D and inflammation – Why investigation in canine cancer is warranted

Cancer is a common inflammatory disease affecting dogs, with one in four expected to develop cancer (Bronson, 1982; Adams et al., 2010). Inflammation plays a critical role in cancer development and progression and is considered one of the enabling characteristics of cancer (Hanahan & Weinberg, 2011). Immune inflammatory cells can act as tumour-promoting cells and release signalling molecules, such as cytokines and chemokines, that can induce tumour angiogenesis and cancer cell proliferation and invasion (Hanahan & Weinberg, 2011). Inflammatory biomarkers, such as APP, cytokines and chemokines, have been investigated for potential use in estimating cancer risk, detection and prognosis (Kehm et al., 2020; Kartikasari et al., 2021). This knowledge has spurred interest in determining whether the links reported between circulating 25(OH)D concentrations and inflammatory markers in other diseases also exist with cancer.

Relationships between 25(OH)D concentrations and inflammatory markers have been observed in humans with cancer. Prostate cancer patients had decreased serum 25(OH)D concentrations and increased serum CRP and interleukin-8 (a pro-inflammatory cytokine) concentrations, and an inverse relationship between serum 25(OH)D concentrations and the two inflammatory markers was observed (Xie et al., 2017). Humans with renal cell carcinoma had decreased serum 25(OH)D concentrations and increased CRP concentrations (Xu et al., 2019). In humans with colorectal cancer, plasma 25(OH)D concentrations decreased intraoperatively and

decreased further 1-2 days after colorectal cancer surgery compared to pre-operative concentrations (Vaughan-Shaw et al., 2020). Plasma 25(OH)D concentrations began recovering within a week of surgery and had increased beyond the pre-operative concentrations at the last sample taken (a median of 225 days after surgery). Concentrations of CRP increased after surgery and peaked at 3-5 days after surgery (Vaughan-Shaw et al., 2020). Serum 25(OH)D concentrations were also inversely associated with concentrations of the pro-inflammatory cytokine interleukin-6, and a summary inflammatory score, in the 2 years following colorectal cancer diagnosis in human patients (Wesselink, Balvers, et al., 2020). Very limited data exists on the potential mechanisms behind these observations, however the most biologically active form of vitamin D, calcitriol, was shown to suppress activation of nuclear factor kappa B (an important player in the inflammatory response) in renal cell carcinoma cells (Xu et al., 2019).

To date and to the author's knowledge, no studies have scrutinized links between 25(OH)D concentrations and inflammatory markers in dogs with cancer, despite the links observed between inflammatory markers and vitamin D in humans with cancer, and in dogs with other inflammatory diseases. Circulating 25(OH)D and inflammatory marker concentrations have only been investigated independently of each other in dogs with cancer. Results from 25(OH)D studies are somewhat mixed, with some researchers reporting decreased concentrations in affected dogs (Gerber et al., 2004; Wakshlag et al., 2011; Rosa et al., 2013; Weidner et al., 2017), and others reporting no change (Willcox et al., 2016). Studies have only been cross-sectional, so it remains unclear whether 25(OH)D are a cause or consequence of the disease and/or what mechanisms may be responsible for changes in 25(OH)D concentration. If low 25(OH)D concentrations are associated with systemic inflammation, then it's possible that inflammatory processes associated with the cancer are related to changes in 25(OH)D concentrations. Cross-sectional studies documented increased concentrations of inflammatory markers, such as APP (e.g. CRP and HP) and cytokines, in dogs with cancer (Tecles et al., 2005; Chase et al., 2012). Interestingly, there have been

differences noted in APP profiles between tumour types (Tecles et al., 2005; Chase et al., 2012). Longitudinal studies also demonstrated that concentrations of inflammatory markers, like CRP, may decrease with successful cancer treatment (Nielsen et al., 2007), so much so that they have potential as biomarkers of remission (Nielsen et al., 2007; Alexandrakis et al., 2017). If there are differences in the inflammatory profile based on tumour type, then exploring simultaneous 25(OH)D and inflammatory marker concentrations in several cancers may identify which inflammatory markers are potentially associated with low 25(OH)D.

Some of the most common cancers in dogs are lymphoma, osteosarcoma (OSA), and mast cell tumour (MCT). These cancers are commonly studied by canine cancer researchers for several reasons: their prevalence ensures that researchers can recruit a sufficient sample size; each cancer represents an appropriate animal model for the respective human disease; and/or there are standard treatment protocols that oncology care centres usually adhere to for osteosarcoma and lymphoma (Withrow and Vail, 2013).

Concentrations of 25(OH)D and inflammatory markers have been investigated independently in dogs affected with these three cancers. There have been mixed results with circulating 25(OH)D (Gerber et al., 2004; Wakshlag et al., 2011; Willcox et al., 2016). Decreased 25(OH)D concentrations have been reported in dogs with lymphoma (Gerber et al., 2004) and MCT (Wakshlag et al., 2011) but not OSA (Willcox et al., 2016). Work previously completed by this author showed decreased plasma 25(OH)D concentrations for all three cancers when compared with healthy dogs, but only at low concentrations of plasma ionized calcium (Weidner et al., 2017). With regards to inflammation, dogs with lymphoma, MCT and OSA presented with increased inflammatory markers, however differences in the inflammatory profile between tumour types have been noted (Tecles et al., 2005; Chase et al., 2012). As an example, HP was not elevated in dogs with MCT (Chase et al., 2012) but was in dogs with lymphoma and leukemia (Tecles et al., 2005). Interestingly, there may also be differences in serum cytokine and peripheral blood cell profiles based on lymphoma immunophenotype (T-

cell vs B-cell) (Calvalido et al., 2016). Immunophenotype is a helpful indicator of prognosis for dogs with lymphoma. Dogs with T-cell lymphoma (T-cell) usually have a poorer response to therapy and survival than dogs with B-cell lymphoma (B-cell) (Teske et al., 1994; Ruslander et al., 1997; Appelbaum et al., 1984).

The difference in 25(OH)D concentrations and inflammatory profiles observed between these three cancer types means they may be good candidates for simultaneous study of 25(OH)D and inflammatory marker concentrations of several cancers. Studies such as this may be able to determine which cancers and/or inflammatory markers are potentially associated with low 25(OH)D. Furthermore, because canine lymphoma and osteosarcoma have standardized treatment protocols (Withrow and Vail, 2013), and inflammatory marker concentrations (CRP) have been shown to decrease with successful treatment of canine lymphoma (Nielsen et al., 2007), then researchers would be able to investigate whether 25(OH)D concentrations change in relation to changes in inflammatory marker concentrations. Finally, researchers may be able to investigate the mechanisms responsible for any observed differences in circulating 25(OH)D concentrations by measuring additional vitamin D metabolites, such as 24,25(OH)2D, and the ratio between metabolites, to determine whether pro-inflammatory cytokines are regulating enzymes involved in vitamin D metabolism.

3.6 Conclusion

This literature review aimed to update the conclusions of the previously published literature review. There have been advances made in knowledge related to vitamin D in dog food; the relationship between dietary vitamin D intake and circulating 25(OH)D concentrations; other factors that affect circulating 25(OH)D concentrations; and 25(OH)D concentrations and disease. Researchers have increasingly stressed the need to better understand what variables may influence circulating 25(OH)D concentrations. A commonly noted variable of interest is inflammation. A limited amount work has investigated links between vitamin D and inflammation in dogs, but intriguing results

have been observed in cross-sectional, longitudinal, and mechanistic studies. Investigation into these findings in dogs with cancer addresses the need to identify factors that may influence circulating 25(OH)D concentrations and would allow researchers to answer questions related to how disease processes may affect vitamin D metabolism. Answers to these questions may be relevant to dogs with other inflammatory conditions and may have practical implications for the dog owner, who might be interested in vitamin D supplementation, and clinician, who might be interested in the diagnostic utility of 25(OH)D measurement or may have to explain the current state of research to owners interested in supplementation. This research is especially necessary as interest in the use of 25(OH)D as a marker of canine health continues.

4 Circulating 25-hydroxyvitamin D concentrations and the inflammatory response in dogs with cancer

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4.1 Abstract

Decreased circulating 25-hydroxyvitamin D (25(OH)D) and increased inflammatory marker concentrations have been reported separately in canine cancer. Correlations between the two exist in humans, but little work has examined links in dogs. This study aimed to determine plasma 25(OH)D and inflammatory marker concentrations in healthy dogs and dogs with cancer and to assess correlations in each group. Newly diagnosed dogs with B-cell lymphoma (B-cell, n=25), T-cell lymphoma (T-cell, n=9), osteosarcoma (OSA, n=21), and mast cell tumour (MCT, n=26) presenting to a tertiary oncology center, and healthy dogs (n=25), were enrolled. Plasma samples were analyzed for 25(OH)D, C-reactive protein (CRP), haptoglobin (HP), serum amyloid A (SAA), alpha-1-acid glycoprotein (AAG), and 13 chemokines and cytokines. Dogs with B-cell had decreased plasma 25(OH)D (p=0.03), and increased plasma CRP, AAG, HP, KC-like and MCP-1 concentrations (p<=0.001, 0.011, <0.001, 0.013 and 0.009, respectively) compared to healthy dogs. Plasma CRP, HP, and SAA concentrations were increased in dogs with OSA compared to healthy dogs (p=0.001, 0.010, and 0.027, respectively). No differences were noted in dogs with T-cell and MCT. Negative correlations were observed between plasma 25(OH)D concentrations and: AAG concentrations in dogs with T-cell (Rs=-0.817, p=0.007); GM-CSF concentrations (Rs=-0.569, p=0.007) in dogs with OSA; and IL-7 concentrations (Rs=-0.548, p=0.010) in dogs with OSA. Decreased 25(OH)D concentrations and increased concentrations of multiple inflammatory markers were observed in B-cell patients, supporting an association between 25(OH)D and inflammation. The cross-sectional study design meant the timing of changes could not be determined. Prospective cohort studies are warranted.

4.2 Introduction

Vitamin D has quickly gained research attention in companion animal disease. Low circulating 25-hydroxyvitamin D (25(OH)D) concentrations, the accepted marker of vitamin D status (Ross et al., 2011), have been reported in dogs with a range of diseases (Galler et al., 2012; Gow et al., 2011; Kim et al., 2017; Titmarsh et al., 2015; Wakshlag et al., 2011). This spurred interest in defining new reference ranges for 25(OH)D (Selting et al., 2016) and investigating ways to increase 25(OH)D (Young & Backus, 2016; Young & Backus, 2017). However, it is imperative to understand what factors may be associated with, and potentially confound, measurement of circulating 25(OH)D concentrations in dogs.

Systemic inflammation may confound 25(OH)D measurement (Ghashut et al., 2014; Silva & Furlanetto, 2015), potentially through the regulation of 25(OH)D concentrations by circulating cytokines, with pro-inflammatory cytokines shown to induce conversion of 25(OH)D to 1,25(OH)2D (Edfeldt et al., 2010; Hummel et al., 2014; Srikanth et al., 2016; Zehnder et al., 2002). Systemic inflammation is commonly detected through elevated white blood cell count, and alterations in cytokine/chemokine and acute-phase protein (APP) concentrations. Cytokines and chemokines are a group of proteins involved in cell signalling, with some considered pro-inflammatory and others anti-inflammatory. Concentrations of positive APP (e.g. C-reactive protein [CRP], haptoglobin [HP], serum amyloid A [SAA] and alpha-1-acid glycoprotein [AAG]) increase in response to tissue injury and inflammation, while concentrations of negative APP (e.g. transferrin, albumin) decrease. Studies in humans have reported associations between inflammatory markers (most notably with APP concentrations, such as CRP) and decreased 25(OH)D concentrations (Duncan et al., 2012; Ghashut et al., 2014; Liefaard et al., 2015; Srikanth et al., 2016).

Links between altered 25(OH)D concentrations and markers of inflammation have only recently been investigated in healthy dogs (Selting et al., 2017; Spoo et al., 2015)

and dogs with disease (Kim et al., 2017; Titmarsh et al., 2015). Decreased serum 25(OH)D concentrations were reported in dogs with sepsis when compared to healthy dogs (Jaffey, Backus, et al., 2018). The most biologically active metabolite of vitamin D, calcitriol, decreased leukocyte production of the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α) in dogs (Jaffey, Amorim, et al., 2018a, 2018b, 2018c) and increased lipopolysaccharide stimulated leukocyte production of the anti-inflammatory cytokine, interleukin-10 (IL-10) in endotoxin primed whole blood collected from healthy dogs (Jaffey, Amorim, et al., 2018a) and whole blood collected from critically ill dogs (Jaffey, Amorim, et al., 2018b).

Inflammation also plays an important role in the development and/or progression of cancer (Hanahan & Weinberg, 2011). Inflammation has been included as one of the enabling characteristics of cancer, based the ability of immune inflammatory cells to act as tumour-promoting cells (Hanahan & Weinberg, 2011). Signalling molecules, such as cytokines and chemokines, released by these tumour-promoting cells, can induce tumour angiogenesis and cancer cell proliferation and invasion (Hanahan & Weinberg, 2011). Multiple reports describe increased concentrations of inflammatory markers in dogs with cancer (Chase et al., 2012; Mischke et al., 2007; Nielsen et al., 2007; Ogilvie et al., 1993; Tecles et al., 2005; Yuki et al., 2011). Studies have shown decreased 25(OH)D concentrations in dogs with certain cancers (Gerber et al., 2004; Rosa et al., 2013; Selting et al., 2017; Wakshlag et al., 2011; Weidner et al., 2017). Despite these separate reports, the authors are unaware of studies investigating links between inflammatory markers and 25(OH)D in canine cancer.

Cancer is widespread in dogs, with one in four dogs estimated to develop cancer at some point in their lifetime (Adams et al., 2010; Bronson, 1982) Lymphoma, mast cell tumour (MCT), and osteosarcoma (OSA) are among the most common cancers in dogs. Data on circulating 25(OH)D concentrations in these cancers are mixed. Decreased serum 25(OH)D concentrations were observed in dogs with lymphoma (Gerber et al., 2004) and MCT (Wakshlag et al., 2011), but not OSA (Willcox et al., 2016), while a

recent study reported decreased plasma 25(OH)D concentrations for all three cancers when compared to healthy dogs, but only at low concentrations of plasma ionized calcium (Weidner et al., 2017). With regards to inflammation, dogs with lymphoma, MCT, and OSA have presented with increased inflammatory markers, however tumour type differences have been noted (Chase et al., 2012; Tecles et al., 2005). For instance, HP was elevated in dogs with lymphoma and leukemia (Tecles et al., 2005), but not in dogs with MCT (Chase et al., 2012). Importantly, a recent study also identified differences in serum cytokine and peripheral blood cell profiles based on lymphoma immunophenotype (T-cell vs. B-cell) (Calvalido et al., 2016). Immunophenotype is an important prognostic indicator for lymphoma patients. T-cell lymphoma (T-cell) is usually associated with a poorer response to therapy and survival than B-cell lymphoma (B-cell) (Appelbaum et al., 1984; Ruslander et al., 1997; Teske et al., 1994). An exception to this is indolent T-cell lymphoma which has been associated with longer survival times, even without treatment (Valli et al., 2013). If low 25(OH)D concentrations are associated with systemic inflammation, then exploring simultaneous 25(OH)D and inflammatory marker concentrations in several cancers may identify which inflammatory markers are potentially associated with low 25(OH)D.

The primary objectives of this study were to 1) determine plasma 25(OH)D and inflammatory marker concentrations (including acute phase proteins and cytokines) in healthy dogs and dogs with lymphoma (including B-cell and T-cell), MCT and OSA and 2) assess correlations between plasma 25(OH)D and inflammatory marker concentrations in each group. It was hypothesized that dogs with cancer would have decreased plasma 25(OH)D and increased inflammatory marker concentrations compared to healthy dogs and plasma 25(OH)D would be negatively correlated with plasma inflammatory marker concentrations. A secondary objective was to determine the peripheral blood cell counts in each group to better understand the potential source of any observed changes.

4.3 Methods

Animals

Newly diagnosed, client-owned dogs with lymphoma (n=34), OSA (n=21), and MCT (n=26) presenting to the Mona Campbell Centre for Animal Cancer at the Ontario Veterinary College Health Sciences Centre were enrolled. Cancer diagnosis was confirmed with cytology and/or histology of a fine needle aspirate, biopsy, or the surgically excised tumour. A fine needle aspirate from a neoplastic peripheral lymph node for each lymphoma patient was analyzed with flow cytometry using a BD Accuri C6 flow cytometer to determine lymphoma immunophenotype. Healthy, client-owned dogs (n=25) from the Guelph area were enrolled as controls. Dogs were deemed healthy based on a normal medical history and unremarkable physical exam, complete blood count and biochemical profile. These tests were also performed in cancer groups to rule out the presence of concurrent disease. Dogs were excluded if they: 1) were < 2 years of age, 2) received corticosteroid treatment within 2 weeks prior to trial enrolment (due to potential associations between corticosteroid use and decreased 25(OH)D concentrations observed in humans) (Skversky et al., 2011), 3) received vitamin D and/or calcium supplements or 4) had significant concurrent systemic/infectious disease. Age, gender, breed, dietary history (Weidner et al., 2017), body condition score (Laflamme, 1997) and muscle condition score (Michel et al., 2004) for each animal was recorded upon enrollment (Table 4.1). The University of Guelph Animal Care and Use Committee (AUP #1358) and the Royal Canin Ethics Review Committee (#140217_7) approved the experimental protocol, which was in accordance with institutional and national guidelines for care and use of animals. Fully informed owner consent was obtained for both the control and patient groups.

Plasma analysis

Blood samples were collected in lithium heparin tubes from healthy dogs and prior to treatment in dogs with cancer. Samples were centrifuged at room temperature at 1500 g for 7 minutes. Plasma was then aliquoted and stored at -80 °C until analysis.

Plasma 25(OH)D was analyzed using commercial RIA kits (Diasorin, Stillwater MN, Scantibodies, Santee CA, Beckman Coulter, Miami, FL, USA) at the Diagnostic Center for Population & Animal Health, Michigan State University, East Lansing, Michigan, USA. Plasma CRP, HP, AAG, and SAA were analyzed using commercial canine ELISA kits (Life Diagnostics, Inc., West Chester, PA, USA).

Thirteen plasma cytokines and chemokines were analyzed using a commercial cytokine magnetic antibody assay kit (Milliplex MAP Canine Cytokine/Chemokine Panel Multiplex Assay) (Calvalido et al., 2016). Plasma concentration of granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), interleukin(IL)-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IFN-induced protein-10 (IP-10), keratinocyte chemoattractant-like (KC-like), monocyte chemoattractant protein-1 (MCP-1), and TNF-α were measured. Dogs with values that met or fell below the lower limit of detection for the kit were included in the statistical analysis using the analyzed value.

Complete blood count was analyzed using the ADVIA2120/2120i hematology system. Serum albumin was determined as part of the serum biochemical profile by colorimetric assay using the ROCHE COBAS 6000 system.

Statistics

SPSS software, version 25 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Shapiro-Wilk tests were used to assess data normality. A chi-square test was used to look for differences in the male/female ratio between groups. Kruskal-Wallis analyses followed by pairwise comparisons using the Dunn-Bonferroni approach were used to compare: age; weight; plasma 25(OH)D, CRP, SAA, HP, AAG, and cytokine and chemokine concentrations; serum albumin; and blood count values between groups. Spearman rank-order correlation coefficients were used to determine correlations between plasma 25(OH)D and acute phase protein concentrations, and between plasma 25(OH)D and cytokine and chemokine concentrations, within each group. A p value ≤ 0.05 was considered significant.

4.4 Results

Animals

Breed, male/female ratio, dietary history as well as the age, body weight, BCS, and MCS (shown as mean ± SD) for each group are presented in Table 4.1. Based on the immunophenotype results of the lymphoma group, 25 dogs had B-cell lymphoma and 8 dogs had T-cell lymphoma. An indolent form of the disease was discussed based on the flow cytometry results for one dog with T-cell lymphoma. About 95% of the cells expressed CD8, indicating an indolent course of disease. However, there was co-expression of CD4 on a substantial proportion of cells. The pathologist concluded that this was unusual and that the prognostic features of the disease were unknown. The flow cytometry results for 1 dog were non-diagnostic. This dog was categorized as a T-cell lymphoma, that was not believed to be indolent, as the lymphocytes were medium sized and response to therapy was poor. The dog was switched from the Madison Wisconsin CHOP chemotherapy protocol after the first cycle and euthanized within 4 months.

Plasma analysis

Dogs with B-cell had significantly lower plasma 25(OH)D concentrations compared to healthy dogs ($p = 0.03$, Figure 4.1), though this was not seen in dogs with T-cell and in the MCT group. Compared to healthy dogs, OSA patients had significantly lower plasma 25(OH)D concentrations before adjustment ($p = 0.008$), but the significance disappeared after Bonferroni-adjustment ($p = 0.076$).

Table 4.2 displays median plasma APP concentrations of dogs in the study. Dogs with B-cell had significantly higher plasma CRP, AAG, and HP concentrations compared to healthy dogs ($p <= 0.001$, 0.011, and < 0.001, respectively). These effects were not noted in dogs with T-cell. Dogs with OSA had significantly higher plasma CRP, HP, and SAA compared to healthy dogs ($p = 0.001$, 0.010, and 0.027, respectively),

while dogs with MCT did not differ from the healthy group. No differences in albumin concentrations were observed between any groups (p=0.398).

Median plasma cytokine and chemokine concentrations of dogs in the study are presented in Table 4.3. Dogs with B-cell had significantly higher plasma KC-like and MCP-1 concentrations compared to healthy dogs (p = 0.013 and 0.009, respectively), and significantly higher plasma MCP-1 concentrations than dogs with MCT and OSA (p = 0.046 and <0.001, respectively). Plasma cytokine and chemokine analysis did not reveal any other significant differences.

Finally, median blood counts of dogs in the study are shown in Table 4.4. Dogs with B-cell had a significantly greater number of white blood cells and neutrophils compared to healthy dogs (p = <0.001 and 0.01, respectively), and significantly lower number of red blood cells compared to dogs with MCT (p < 0.001). The Kruskal-Wallis p-value was significant between groups for monocyte count (p = 0.033). The monocyte count was significantly higher in B-cell, T-cell, and OSA dogs compared to healthy dogs before adjustment (0.009, 0.025 and 0.017, respectively), but paired analyses did not reveal effects after Bonferroni-adjustment. No other differences were observed for complete blood count.

Spearman rank order correlation coefficients were significant and negative between: plasma 25(OH)D concentrations and AAG concentrations in dogs with T-cell (Rs = -0.817, p = 0.007); plasma 25(OH)D and GM-CSF concentrations (Rs = -0.569, p = 0.007) in dogs with OSA; and plasma 25(OH)D and IL-7 concentrations (Rs = -0.548, p = 0.010) in dogs with OSA. Spearman rank order correlation coefficients were significant and positive between: plasma 25(OH)D concentrations and lymphocyte concentrations in healthy dogs (Rs = 0.403, p = 0.046); plasma 25(OH)D concentrations and KC-Like concentrations (Rs = 0.407, p = 0.039) in dogs with MCT; and plasma 25(OH)D concentrations and red blood cell counts (Rs = 0.489, p = 0.024) in dogs with OSA.

4.5 Discussion

The present study supports an association between decreased 25(OH)D concentrations and inflammation in dogs with cancer. Results showed that B-cell patients, the only group that showed decreased 25(OH)D concentrations compared to healthy dogs, also had increased concentrations of multiple inflammatory markers (CRP, AAG, HP, KC-like, and MCP-1). Interestingly, the OSA group also had increased concentrations of several inflammatory markers (CRP, HP, and SAA), although plasma 25(OH)D concentrations were not decreased in this group. Finally, no decrease in 25(OH)D concentrations was observed for T-cell and MCT, the groups that showed no changes in inflammatory marker concentrations.

Plasma concentrations of 25(OH)D observed in healthy dogs and those with lymphoma and MCT were similar to previous reports (Gerber et al., 2004; Kukk, 2011; Wakshlag et al., 2011; Weidner et al., 2017). The authors previously reported lower plasma 25(OH)D concentrations in dogs with LSA, OSA, and MCT (Weidner et al., 2017), but only at low ionized calcium concentrations, which was not considered in the present study. One previous study in dogs with lymphoma also reported decreased serum 25(OH)D concentrations (Gerber et al., 2004). However, immunophenotype was not assessed. No evidence of differences in serum 25(OH)D concentrations were observed between dogs with OSA and healthy controls in a recent study (Willcox et al., 2016), similar to the OSA results reported here. It should be noted that the adjusted p-value for the comparison of 25(OH)D between healthy and osteosarcoma patients in the present study was 0.076, and the OSA population included a dog with a plasma 25(OH)D concentration that was considered to be a major outlier. Moreover, the association with ionized calcium was not investigated. The present study also did not find decreased plasma 25(OH)D concentrations in dogs with MCT. This is in contrast with a decrease in serum 25(OH)D between dogs with MCT and healthy controls reported by Wakshlag et al. (2011). Differences in patient populations (e.g. the Wakshlag et al. study [2011] included dogs in the MCT group that already had the tumours excised) may limit the ability to make direct comparisons between studies.

Finally, whether decreased plasma 25(OH)D concentrations occurred pre- or post-cancer development could not be determined with the cross-sectional design of the present study.

Several studies have used APP as inflammatory markers in canine cancer (Chase et al., 2012; Nielsen et al., 2007; Ogilvie et al., 1993; Tecles et al., 2005) and some researchers have suggested that specific tumour types are associated with specific changes in APP profiles (Chase et al., 2012). Although there were some similarities with previous studies, the elevated APP profile for each cancer type was not the same. Increased CRP, AAG, and HP concentrations, as observed in the present study, have been reported in dogs with lymphoma (Mischke et al., 2007; Nielsen et al., 2007; Ogilvie et al., 1993; Tecles et al., 2005; Yuki et al., 2011). Also similar to our findings, Tecles et al. (2005) found no difference in serum SAA between dogs with hematologic neoplasia (consisting of dogs with lymphoma and leukemia) and healthy dogs. Similar to the present study, increased CRP and HP concentrations were observed in dogs with sarcoma (Chase et al., 2012). Yet, while Chase et al. (2012) found no differences in SAA levels in dogs with sarcomas compared to a reference range for healthy dogs, plasma SAA concentrations were increased in dogs with OSA when compared to healthy controls dogs in the present study. Elevations in AAG were also previously reported in dogs with OSA (Ogilvie et al., 1993) and sarcoma (Chase et al., 2012; Yuki et al., 2011), but could not be confirmed in the present study. These comparisons should be interpreted with caution as only one study included a true OSA group (Ogilvie et al., 1993). The others included a few OSA patients in a much broader sarcoma group (Chase et al., 2012; Yuki et al., 2011). In dogs with MCT, increased CRP and AAG were observed, with no change in HP and decreased SAA levels (Chase et al., 2012), while no significant increased concentrations were noted in the present study. Limited research examining serum albumin concentrations in dogs with cancer could be found; however, one study of dogs with mammary tumours found decreased serum albumin concentrations only in dogs with mammary tumours that had concurrent inflammatory diseases, and not in dogs with mammary tumours alone (Tecles et al., 2005). Further

research is necessary before any conclusions about APP profiles specific to cancer types can be made. It is important to note that most studies include different distributions of tumour stage/grade among groups, which may confound any direct comparisons.

Unlike APP, only a limited number of studies have investigated cytokine concentrations in dogs with cancer. In this study, concentrations of MCP-1 and KC-like were elevated in B-cell patients only. In previous studies, circulating concentrations of MCP-1 were also elevated in dogs with lymphoma (Calvalido et al., 2016; Perry et al., 2011) and various cancers (Ishioka et al., 2013; Nikolic Nielsen et al., 2013). However, our findings contrast with the only other study that investigated cytokine concentrations between lymphoma immunophenotypes, where MCP-1 was higher in dogs with B-cell and T-cell (Calvalido et al., 2016). Similarly, KC-like was higher for dogs with B-cell in this study, but higher in male dogs with B-cell and T-cell than males in the control group in the Calvalido et al. study (2016). Interestingly, Calvalido et al. (2016) also observed increased IL-10 in dogs with B-cell, and IL-6 in dogs with T-cell. These changes were not observed here. It is possible that differences between the results reported here and those reported by Calvalido et al. (2016) could be attributed to differences in disease stage between the study populations, especially as serum MCP-1 has been positively correlated with lymphoma stage in dogs (Perry et al., 2011).

Blood cell counts were also examined in this study. The findings of increased white blood cells and neutrophil counts in B-cell patients, but not T-cell patients, when compared to healthy dogs was similar as reported by Calvalido et al. (2016). However, Calvalido et al. (2016) also observed increased lymphocyte and monocyte counts, and decreased red blood cell counts in B-cell patients, compared to healthy dogs, which were not found here. Perry et al. (2011) also showed elevated neutrophil and monocyte counts in dogs with lymphoma compared to healthy controls, although immunophenotype was not accounted for in that study.

There is a limited amount of work investigating the link between 25(OH)D and inflammatory marker concentrations in dogs. Low serum 25(OH)D concentrations have been negatively associated with CRP concentrations in dogs with hemoabdomen (Selting et al., 2017), and negatively associated with serum IL-2 and IL-8 concentrations, and neutrophil and monocyte counts in dogs with a chronic enteropathy (Titmarsh et al., 2015). Although concentrations of inflammatory markers were not directly investigated, decreased 25(OH)D concentrations were recently reported in dogs with sepsis (an inflammatory condition) when compared to healthy dogs (Jaffey, Backus, et al., 2018). In vitro, calcitriol exposure decreased leukocyte production of TNF-α in whole blood collected from healthy and critically ill dogs (Jaffey, Amorim, et al., 2018a, 2018b, 2018c). Interestingly, increased lipopolysaccharide stimulated leukocyte production of IL-10 was observed only in endotoxin-primed whole blood from healthy dogs (Jaffey, Amorim, et al., 2018a), and whole blood from critically ill dogs (Jaffey, Amorim, et al., 2018b), suggesting that activation of the leukocytes may influence calcitriol's ability to increase IL-10 production.

In the present study, we observed decreased 25(OH)D concentrations in association with increased concentrations of multiple inflammatory markers (CRP, AAG, HP, KC-like, and MCP-1) in B-cell patients compared to healthy dogs. Although no causation could be determined with this study, several hypotheses have been suggested to explain a relationship between systemic inflammation and decreased 25(OH)D levels. Authors of a recent literature review (Silva & Furlanetto, 2015) point to several potential mechanisms including a decrease in serum vitamin D binding protein concentrations (actin-free fraction) and increased conversion of 25(OH)D to other vitamin D metabolites. Srikanth et al. (2016) suggested that 25(OH)D concentrations could be regulated by circulating cytokines, citing in vitro work showing that pro-inflammatory cytokines induced conversion of 25(OH)D to other vitamin D metabolites (Edfeldt et al., 2010; Hummel et al., 2014; Zehnder et al., 2002).

In addition to the cross-sectional design, there are several limitations to this study that should be acknowledged. Dietary vitamin D intake information was not available for

all dogs so the impact of intake on plasma 25(OH)D concentrations could not be assessed. However, the authors did not observe an effect of dietary vitamin D intake on plasma 25(OH)D concentrations in a subpopulation of the present study which was reported previously (Weidner et al., 2017). The previous study also showed that ionized calcium may be associated with plasma 25(OH)D in dogs (Weidner et al., 2017), which was not accounted for in the present study. There are several other variables which may affect APP and cytokine concentrations, including stress, exercise, and environmental factors (Casella et al., 2013; Ceron et al., 2005; Fazio et al., 2015; Spoo et al., 2015; von Pfeil et al., 2015), which were not assessed in this study. To limit the effects of potential co-variables, efforts were made to ensure similar ages and body condition scores between cancer and healthy groups. Grading for mast cell tumour patients, a useful prognostic indicator, was not included as a variable in this study due to the already limited sample sizes. This may be a worthy avenue of investigation in future studies. Furthermore, in order to get sufficient enrollment numbers, breed-matching of groups was not feasible so any breed-specific differences in metabolism or inflammatory response could not be determined.

It should also be noted that some of the cytokines analyzed in this study had group median concentrations that met the lower limit of detection for the analyte. This was most notable for TNF-α and IL-2, where the median concentrations for all groups were equal to the lower limit of detection. This was not the case for the results where we found significance (KC-like and MCP-1). There are different approaches in the literature to handle results where values meet or fall below the lower limit of detection, including maintaining the values, halving the values and removing the values completely (Croghan and Egeghy, 2003). In this study, dogs with values that met or fell below the lower limit of detection for the kit were still included in the statistical analysis using the analyzed value. This should be noted when comparing these results to other studies. It's possible that this platform may not be ideal for analysis of certain cytokines, such as TNF-α. Investigators planning future studies in this area should be aware of this.

Currently, there is no conclusive evidence to determine whether low 25(OH)D concentrations are a cause or consequence of inflammation. In this study, 25(OH)D concentrations were decreased in B-cell patients, the cancer group with increased concentrations of multiple inflammatory markers. However, due to the cross-sectional design of this study, the exact timing of these changes could not be determined. Several authors have concluded that caution should be used when interpreting 25(OH)D measurements during an inflammatory response in humans (Duncan et al., 2012; Ghashut et al., 2014; Silva & Furlanetto, 2015). Prospective cohort studies to examine this in dogs are needed. In light of the difficult nature in designing a study pre- and post-canine cancer development, studies examining circulating 25(OH)D response after an acute inflammatory insult (such as elective surgery) in dogs are also warranted.

4.6 Tables

Table 4.1: Patient characteristics (breed, age, male/female, BCS, MCS, dietary history) of healthy dogs and those with cancer enrolled in this cross-sectional study investigating circulating 25(OH)D concentrations and markers of inflammation.

	Healthy (n=25)	B-cell (n=25, *n=23)	T-cell (n=9, *n=8)	MCT (n=26, *n=23)	OSA (n=21)
Age (years)	7.9 ± 2.2	7.7 ± 1.9	6.7 ± 1.9	7.3 ± 2.4	8.5 ± 2.4
Body Weight (kg)	32.1 ± 10.1	29.2 ± 13.4	25.8 ±12.9	30.9 ± 11.8	40.2 ± 14.2
BCS (1-9)	5.7 ± 0.9	6.2 ± 1.2*	5.7 ± 1.0*	6.13 ± 1.1*	5.7 ± 1.1
MCS (0-3)	2.5 ± 0.7	2.7 ± 0.6*	2.6 ± 0.5*	2.6 ± 0.7*	2.1 ± 0.7
Male/Female	13/12	15/10	5/4	13/13	15/6
Breeds	Boxer (2) Doberman pinscher (1) Dogue de Bordeaux (1) Golden retriever (2) Labrador retriever (4) Mastiff (2) Mixed (7) Portuguese Water Dog (1) Standard poodle (4) Weimaraner (1)	Airedale terrier (1) Beagle (1) Boston terrier (1) Bouvier des Flandres (1) Cocker spaniel (3) Corgi (1) Dachshund (1) Golden retriever (3) Labrador retriever (3) Mastiff (2) Mixed (7) West Highland White Terrier (1)	Boxer (1) Doberman Pinscher (1) English Setter (1) Golden retriever (1) Mixed (2) Norwegian Buhund (1) Portuguese Water Dog (1) Shetland Sheepdog (1)	Bernese mountain dog (1) Boxer (3) Bouvier des Flandres (1) Boston terrier (1) Cocker spaniel (1) Doberman Pinscher (1) Golden retriever (3) Labrador retriever (7) Mixed (4) Pug (1) Shar Pei (1) Shetland Sheepdog (1) Standard poodle (1)	Bernese mountain dog (1) Cane Corso (1) Cocker spaniel (1) Doberman pinscher (1) Golden retriever (2) Great pyrenees (1) Greyhound (2) Mastiff (1) Mixed (5) Pharaoh hound (1) Rottweiler (3) Siberian husky (1) Standard poodle (1)
Dietary History	Commercial diet (22) Information unable to be obtained (3)	Commercial diet (19) Information unable to be obtained (6)	Commercial diet (7) Homemade (1) Information unable to be obtained (1)	Commercial diet (22) Homemade (1) Information unable to be obtained (3)	Commercial diet (18) Homemade (3)

Age, body weight, BCS and MCS are presented as means ± SD. There were no differences in age or gender between groups (p = 0.230 and 0.617). The Kruskal-Wallis p-value was significant between groups for body weight (p = 0.048). Osteosarcoma patients had higher body weight than B-cell and T-cell lymphoma patients before

Bonferroni-adjustment (p = 0.007 and 0.013), but paired analyses did not reveal effects after adjustment (p = 0.072 and 0.133).

25(OH)D = 25-hydroxyvitamin D, B-cell = B-cell lymphoma, BCS = Body condition score, MCT = mast cell tumour, MCS = Muscle condition score, OSA = osteosarcoma, T-cell = T-cell lymphoma

*Body condition and muscle condition scores were unavailable for 2 B-cell, 1 T-cell and 3 MCT dogs.

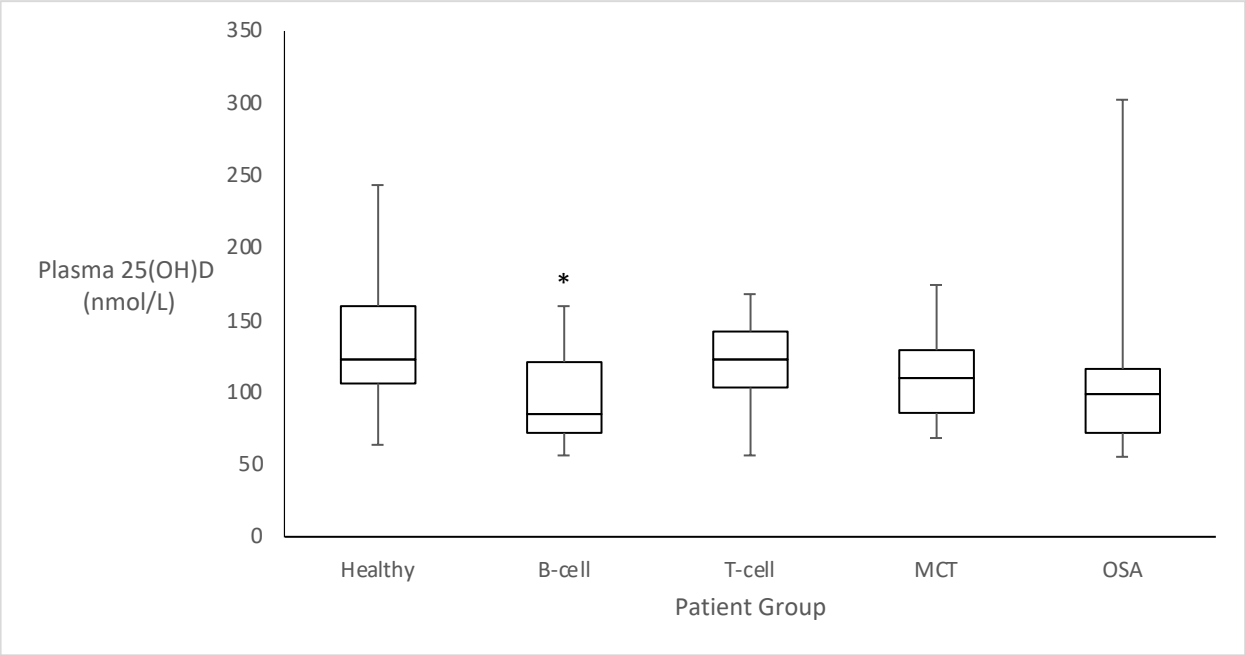

Figure 4.1: Box and whisker plot of plasma 25-hydroxyvitamin D (25(OH)D) concentrations in dogs with cancer and healthy dogs enrolled in a cross-sectional study

Asterisks represent a significant difference ($p < 0.05$ after Bonferroni adjustment) compared to the healthy group.

Healthy n=25, B-cell Lymphoma (B-cell) n=25, T-cell Lymphoma (T-cell) n=9, Mast cell tumour (MCT) n=26, Osteosarcoma (OSA) n=21

Table 4.2: Plasma acute-phase proteins (APP) concentrations in healthy dogs and those with cancer enrolled in a cross-sectional study investigating circulating 25(OH)D concentrations and markers of inflammation.

	Healthy (n=25)		B-cell (n=25)		T-cell (n=9)		MCT (n=26)		OSA (n=21)		Kruskal-Wallis p-value
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	
APP											
CRP (ug/mL)	9.4[a]	7.8 – 14.5	18.0[b]	7.8 – 332.8	11.5[ab]	7.8 – 211.3	11.8[ab]	7.8-35.3	14.4[b]	7.8 – 55.5	<0.001
AAG (mg/mL)	0.7[a]	0.2 – 2 .3	1.7[b]	0.4 – 6.2	0.8[ab]	0.4 – 4.2	0.6[ab]	0.2 – 6.3	0.8[ab]	0.3 – 2.5	0.013
HP (mg/mL)	0.2[a]	0.1 – 0.7	1.0[b]	0.6 – 3.7	0.3[ab]	0.1 – 1.5	0.5[ab]	0.1 – 2.6	0.6[b]	0.1 – 1.2	<0.001
SAA (ug/mL)	4.0[a]	3.8 – 4.7	4.1[ab]	3.8 – 9.8	4.3[ab]	3.8 – 33.3	4.1[ab]	3.8 – 55.3	4.2[b]	3.9 – 4.8	0.018
Albumin (g/L)	37[a]	30 – 44	35[a]	27 – 42	36[a]	25 – 41	37.5[a]	31 – 40	36[a]	25 – 41	0.398

Results presented as median and range. Different superscripts represent statistically significant difference of $p \leq 0.05$ from pairwise analysis after Bonferroni-adjustment.

25(OH)D = 25-hydroxyvitamin D, AAG = alpha-1-acid glycoprotein, APP = acute-phase proteins, B-cell = B-cell lymphoma, CRP = C-reactive protein, HP = haptoglobin, MCT = mast cell tumour, OSA = osteosarcoma, SAA = serum amyloid A, T-cell = T-cell lymphoma

Table 4.3: Plasma cytokine and chemokine concentrations in healthy dogs and those with cancer enrolled in a cross-sectional study investigating circulating 25(OH)D concentrations and markers of inflammation.

	Healthy (n=25)		B-cell (n=25)		T-cell (n=9)		MCT (n=26)		OSA (n=21)		Kruskal Wallis p-value
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	
Cytokine /Chemo-kine (pg/mL)											
IL-2	3.5	1.0-576.2	3.5	3.5 – 17964.6	3.5	0.1 - 343.3	3.5	3.5-2886.1	3.5	3.5-3.5	0.349
IL-6	7.8	1.4-640.6	10.1	3.7 – 999.9	8.3	3.7 – 37.9	9.6	3.2-1746.9	10.9	1.4-182.0	0.970
IL-7	63.8	5.1-649.3	14.2	0.7 – 632.1	31.1	3.8 – 150.7	32.8	7.5-4572.8	15.5	2.4-224.5	0.266
IL-8	2250.3	774.5-9356.8	2965.9	218.5 – 29459.9	2770.4	146.6 – 13125.3	3214.5	1085.8 – 16839.1	3577.3	886.7 – 9858.7	0.179
IL-10	8.5	8.5 – 2308.3	32.3	0.1 – 475.6	11.3	2.6 – 409.4	8.5	4.0 – 964.8	24.6	1.3-112.9	0.350
IL-15	58.3	9 – 10618.5	9.0	5.2 – 10492.0	49.1	6.8 – 460.4	45.7	6.8 – 18277.4	49.1	9.0 – 6656.3	0.717
IL-18	8.6	2.0—313.1	9.8	2.0 – 423.7	19.4	4.2 – 115.6	24.7	5.1 – 2902.9	16.2	1.6 – 95.1	0.369
TNF-a	6.1	2.0 – 641.4	6.1	6.1 – 124.1	6.1	6.1 – 92.3	6.1	5.9 – 1419.4	6.1	2.6 – 6.1	0.499
IFN-y	18.0	18.0-4330.9	18	18 – 333.4	18	18 – 161.6	18.0	18.0-1262.2	18	6.1 – 643.2	0.886
GM-CSF	48.6	9.2-1958.9	48.6	9.2 – 1037.0	61.7	9.2 – 751.9	53.6	9.2 – 8105.2	34.8	9.2 -428.7	0.742
KC-like	312.1[a]	44.2 – 2323.2	1193.0[b]	108.6 – 6648.2	1145.4[ab]	140.0 – 2719.2	675.6[ab]	16.1-2987.8	1277.2[ab]	22.1 – 3718.3	0.014
MCP-1	204.5[b]	21 – 1054.1	456.2[a]	21.0 – 1450.2	411.4[ab]	120.3 – 1508.3	264.9[b]	21.0 – 2489.0	210.2[b]	21 – 412.6	0.001
IP-10	22.2	3.2 – 90.6	31.7	3.1 – 166.7	64.3	3.2 – 197.4	37.9	3.2-141.9	32.4	3.2 – 106.5	0.309

Results presented as median and range. Rows with shading and different superscripts represent statistically significant difference of $p \leq 0.05$ from pairwise analysis after Bonferroni-adjustment.

25(OH)D = 25-hydroxyvitamin D, B-cell = B-cell lymphoma, GM-CSF = granulocyte macrophage colony-stimulating factor, IFN-y = interferon-γ, lL = interleukin, lP-10 = IFN-induced protein-10, KC-like = keratinocyte chemoattractant-like, MCP-1 = monocyte chemoattractant protein-1, MCT = mast cell tumour, OSA = osteosarcoma, T-cell = T-cell lymphoma, TNF-a = tumour necrosis factor-α

Table 4.4: Blood cell counts in healthy dogs and those with cancer enrolled in a cross-sectional study investigating circulating 25(OH)D concentrations and markers of inflammation.

	Healthy (n=25)		B-cell (n=25)		T-cell (n=9)		MCT (n=26)		OSA (n=21)		Kruskal Wallis p-value
	Median	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	**Median**	**Range**	
Blood cell count											
RBC (x10^12/L)	7.2[ab]	6.1 – 8.1	6.5[a]	5.4 – 8.3	7.4[ab]	6.3 – 8.5	7.4[b]	6.5-8.4	7[ab]	4.7-8.2	0.001
WBC (x10^9/L)	7.1[a]	4.1 – 15.8	11.1[b]	6.2 – 34.2	9.8[ab]	5.8 – 15.3	8.9[ab]	5-15.5	10.1[ab]	5.1-20.7	0.002
Neutrophils (x10^9/L)	4.9[a]	2.8-11.4	8.9[b]	4.1 – 26.7	6.4[ab]	4.0 – 10.5	6.6[ab]	3.6-12.9	6.1[ab]	3.2-17.2	0.002
Monocytes (x10^9/L)	0.3	.1-1.1	0.6	.1 – 1.9	0.5	0.2 – 1.4	.4	0.1-0.8	0.6	0.2-1.3	0.033
Lymphocytes (x10^9/L)	1.5	.5-2.7	1.8	0.8 – 15.8	2.1	1.1 – 3.8	1.6	0.1-2.8	1.7	0.6-3.1	0.282
Platelets (x10^9/L)	290	86-422	246	96 – 450	283	93 – 432	288	144-650	292	45-522	0.053

Results presented as median and range. Different superscripts represent statistically significant difference of $p \leq 0.05$ from pairwise analysis after Bonferroni-adjustment.

25(OH)D = 25-hydroxyvitamin D, B-cell = B-cell lymphoma, MCT = mast cell tumour, OSA = osteosarcoma, RBC = red blood cells, T-cell = T-cell lymphoma, WBC = white blood cells

5 25-hydroxyvitamin D concentrations and the inflammatory response in dogs with B-cell and T-cell lymphoma during chemotherapy treatment

5.1 Abstract

Decreased circulating 25-hydroxyvitamin D (25[OH]D) concentrations have been associated with increased inflammatory marker concentrations in dogs with cancer. Circulating 25(OH)D concentrations increase and inflammatory marker concentrations, particularly C-reactive protein (CRP), decrease with cancer treatment in longitudinal studies of human cancer patients. This study aimed to investigate similar relationship in dogs with lymphoma. Healthy dogs (n= 25) were enrolled as controls. Dogs with B-cell lymphoma (n = 14) and T-cell lymphoma (n = 5) were followed prior to and during a 25-week long chemotherapy protocol. Pre-treatment plasma 25(OH)D concentrations were lower, and plasma CRP concentrations were higher in dogs with B-cell lymphoma compared to healthy dogs ($p = < 0.001$ and 0.001). Plasma 25(OH)D concentrations increased ($p = 0.006$), and plasma CRP concentrations decreased ($p = 0.002$) by the last chemotherapy treatment in dogs with B-cell lymphoma when compared to their pre-treatment concentrations. Furthermore, plasma 25(OH)D and plasma CRP concentrations at week 25 of chemotherapy treatment were no longer different from the healthy group ($p = 0.087$ and $p = 0.696$). Pre-treatment plasma 25(OH)D and CRP concentrations were not different in dogs with T-cell lymphoma compared to healthy dogs ($p = 0.879$ and 0.995). Plasma 25(OH)D and CRP concentrations remained unchanged during chemotherapy treatment in dogs with T-cell lymphoma when compared to their pre-treatment concentrations ($p = 0.449$). Findings provide insight into what happens to circulating 25(OH)D concentrations during chemotherapy treatment in dogs and support an association between 25(OH)D and inflammation in dogs with B-cell lymphoma. Future research should investigate whether changes observed in circulating 25(OH)D concentrations in dogs with B-cell lymphoma are explained by increased conversion to other vitamin D metabolites (e.g. 24,25[OH]2D).

5.2 Introduction

Vitamin D has quickly become an area of interest to researchers in the fields of canine nutrition and oncology. Low circulating 25-hydroxyvitamin D (25(OH)D) concentrations, the currently accepted marker of vitamin status, have been observed in dogs with certain cancers (Wakshlag et al., 2011; Selting et al., 2016; Willcox et al., 2016), especially lymphoma (Gerber et al., 2004; Weidner et al., 2017; Weidner et al., 2021). Many of these studies are cross-sectional (Wakshlag et al. 2011; Selting et al., 2016; Willcox et al., 2016; Weidner et al., 2017; Weidner et al., 2021), with 25(OH)D measurements available for one time-point only, when the dog has been diagnosed but not started treatment. Many dogs with lymphoma receive treatment which lasts up to 6 months and more (Withrow and Vail, 2013), yet it remains unknown what happens to 25(OH)D concentrations after treatment begins. Research in this area has theoretical and practical implications, providing insight into potential relationships between 25(OH)D and disease processes and addressing clinicians' needs for evidence-based information for clients interested in vitamin D supplementation during cancer treatment.

A variable shown to influence 25(OH)D concentrations, with relevance to cancer research, is inflammation (Ghashut et al., 2014; Hummel et al., 2014). Inflammation plays a crucial role in cancer development and progression and is considered an enabling characteristic of cancer (Hanahan & Weinberg, 2011). There is limited, but intriguing, research exploring vitamin D and inflammation in dogs (Titmarsh et al., 2015; Wennogle et al., 2019; Erdogan et al., 2019). A negative relationship between circulating 25(OH)D concentrations and inflammatory markers has been observed in cross-sectional studies of healthy dogs and dogs with disease (Titmarsh et al., 2015; Selting et al., 2016; Wennogle et al., 2019; Erdogan et al., 2019). Additionally, 25(OH)D concentrations decreased immediately after an inflammatory insult (such as lipopolysaccharide administration or elective surgery) in several longitudinal studies with dogs (Holowaychuk et al., 2012; Clements et al., 2020). These findings may be explained by mechanistic studies with human cell lines that show pro-inflammatory cytokines can regulate enzymes involved in vitamin D metabolism, upregulating the

conversion of 25(OH)D to other vitamin D metabolites (Zehnder et al., 2002; Hummel et al., 2014). It is worth examining concentrations of inflammatory markers in cancer – vitamin D research.

Multicentric lymphoma is a common cancer in dogs (Withrow & Vail, 2013; Dorn et al., 1968; Merlo et al., 2008). Treatment usually involves a “CHOP” chemotherapy protocol, which is a combination chemotherapy regimen of the following drugs: cyclophosphamide (C), doxorubicin (H, hydroxydaunorubicin), vincristine (O, Oncovin), and prednisone (P) (Withrow & Vail, 2013). This protocol induces remission in about 80-90% of dogs, with a median survival time of 10-12 months (Withrow & Vail, 2013). An important prognostic indicator for treatment response is the disease’s immunophenotype, where B-cell lymphoma is usually associated with a better response to therapy and survival than T-cell lymphoma (Applebaum et al., 1984; Teske et al., 1994; Ruslander et al., 1997).

There are separate reports of decreased 25(OH)D concentrations and increased concentrations of inflammatory markers in dogs with lymphoma (Gerber et al., 2004; Tecles et al., 2005; Nielsen et al., 2007; Calvalido et al., 2016). In the previous chapter, dogs with B-cell lymphoma specifically had decreased 25(OH)D concentrations and increased concentrations of multiple inflammatory markers (C-reactive protein [CRP], alpha-1-acid glycoprotein [AAG], haptoglobin [HP], keratinocyte chemoattractant-like [KC-like], and monocyte chemoattractant protein-1 [MCP-1]) compared to healthy dogs (Weidner et al., 2021). There were no differences between dogs with T-cell lymphoma and healthy dogs (Weidner et al., 2021).

Interestingly, inflammatory marker concentrations have been reported to decrease with successful canine lymphoma treatment (Nielsen et al., 2007). If lower 25(OH)D concentrations are a consequence of the inflammation associated with cancer, then 25(OH)D concentrations may increase as inflammatory marker concentrations decrease with lymphoma treatment. This has been observed in humans with colorectal cancer, with reports of circulating 25(OH)D concentrations increasing and inflammatory

marker concentrations decreasing in the years following diagnosis and treatment (Vaughan-Shaw et al., 2020; Wesselink, Balvers, et al., 2020).

The objective of this study was thus to determine circulating 25(OH)D and inflammatory marker concentrations in healthy dogs, and dogs with B-cell and T-cell lymphoma before and during chemotherapy treatment. It was hypothesized that circulating 25(OH)D concentrations would be decreased, and acute phase protein concentrations would be increased, in dogs with B-cell lymphoma when compared to healthy dogs. We also hypothesized that circulating 25(OH)D concentrations would increase, while APP concentrations would decrease, with chemotherapy treatment in dogs with B-cell lymphoma. Finally, we hypothesized that there would be no difference in circulating 25(OH)D and acute phase protein concentrations between healthy dogs and dogs with T-cell lymphoma pre-treatment, and there would be no differences in concentrations during chemotherapy treatment in dogs with T-cell lymphoma.

5.3 Methods

Animals

Newly, diagnosed client-owned dogs with B-cell lymphoma (n = 14) and T-cell lymphoma (n = 5) presenting to the Mona Campbell Centre for Animal Cancer at the Ontario Veterinary College Health Sciences Centre were enrolled. Cancer diagnosis was confirmed by cytology, histology or both. A fine needle aspirate was taken from a neoplastic peripheral lymph node in each lymphoma dog prior to any treatment. Flow cytometry was performed on aspirates, with a BD Accuri C6 flow cytometer, to determine the immunophenotype of the lymphoma (B cell versus T cell) (Gibson et al., 2004). Dogs with B-cell and T-cell lymphoma were a subset of the population of 25 dogs with B-cell lymphoma and 9 dogs with T-cell lymphoma used in a previous study (Weidner et al., 2021) as data from the 25-week long chemotherapy protocol was only available for this subset of dogs.

Client-owned healthy dogs (n=25) from the Guelph, Ontario area, served as controls. Animals were considered healthy following dietary and medical history, physical exam, complete blood count (CBC), and biochemical profile. Healthy dogs were the same population used in the previous study (Weidner et al., 2021).

Exclusion criteria for the study were as follows: corticosteroid use within 2 weeks of enrolment; < 2 years of age; concurrent systemic and/or infectious disease; or the use supplements containing vitamin D and/or calcium. The University of Guelph Animal Care and Use Committee (AUP #1358) and the Royal Canin Ethics Review Committee (#140217_7) approved the experimental protocol, which was in accordance with institutional and national guidelines for care and use of animals. Age, gender, breed, body condition score (Laflamme, 1997) and muscle condition score (Michel et al., 2004) for each animal was recorded upon enrolment.

Blood Collection

Blood samples from healthy dogs were collected once, after health had been confirmed. Samples were not collected at multiple times for healthy dogs as there is evidence that 25(OH)D concentrations are stable over time in most healthy dogs (Hurst, Homer, Gow, et al., 2020; Laing et al., 1999).

Blood samples from dogs with lymphoma were collected three times. The first blood sample was collected at an initial visit, prior to any treatment. This sample is referred to as week 1 in the results and discussion sections. Dogs started treatment on the Wisconsin-Madison CHOP chemotherapy protocol (Garrett et al., 2002), a standard multidrug chemotherapy protocol for canine lymphoma. This is a 25-week long treatment protocol. Dogs returned to the clinic throughout the 25-week protocol to receive chemotherapy treatment. The second sample was collected at week 6, as this is when remission has been induced in most dogs on this protocol. The third sample

was collected at week 25 of treatment as this is the last treatment in the protocol. Samples were collected prior to the chemotherapy treatment given on that day.

All blood samples were collected into lithium heparin tubes and centrifuged at room temperature at 1500 g for 7 minutes. Plasma samples were then aliquoted into Eppendorf tubes and stored at -80 °C until analysis.

Plasma Analysis

Plasma 25(OH)D concentrations were analyzed using commercial radioimmunoassay kits (Diasorin, Stillwater MN, Scantibodies, Santee CA, Beckman Coulter, Miami, FL, USA) at the Diagnostic Center for Population & Animal Health, Michigan State University, East Lansing, Michigan, USA.

Systemic inflammation can be detected through measurement of circulating acute-phase protein (APP) concentrations (Cerón et al., 2005). Concentrations of positive APP (eg, CRP, HP, serum amyloid A [SAA] and AAG) increase in response to tissue injury and inflammation (Cerón et al., 2005). Plasma CRP, HP, AAG, and SAA were analyzed with commercial canine ELISA kits (Life Diagnostics, Inc., West Chester, Pennsylvania) according to manufacturer's instructions (Weidner et al., 2021).

Statistics

Statistical analysis was completed with SPSS Statistics 24.0. Data was checked for normality using Shapiro-Wilk tests.

Dogs with lymphoma before chemotherapy treatment compared to healthy dogs

A Kruskal-Wallis test was used to compare age, body weight, body condition score (Laflamme, 1997), muscle condition score (Michel et al., 2004), and plasma 25(OH)D and APP concentrations between the healthy, B-cell lymphoma, and T-cell

lymphoma groups. If the results of the Kruskal-Wallis test were significant, pairwise comparisons (using Dunn's procedure with a Bonferroni correction for multiple comparisons) were performed to determine which groups were different from one another. A p-value of < 0.017 was considered significant.

Dogs with lymphoma during chemotherapy treatment

Data from B-cell and T-cell lymphoma patients was not normally distributed for several variables at different timepoints, and therefore Friedman tests, followed by Wilcoxon sign rank tests, were used to compare plasma 25(OH)D and APP concentrations over chemotherapy treatment. Since multiple comparisons were tested, p-values were adjusted with a Bonferroni adjustment, and a p-value < 0.017 was considered significant.

Dogs with B-cell lymphoma at weeks 6 and 25 of treatment compared to healthy dogs

Mann-Whitney U tests were used to compare plasma 25(OH)D and APP concentrations of the B-cell lymphoma group at weeks 6 and 25 of chemotherapy treatment to the concentrations of the healthy dogs, and a p-value < 0.017 was considered significant.

Correlations in dogs with B-cell lymphoma

Spearman rank-order correlation coefficients were used to determine correlations between plasma 25(OH)D and APP concentrations at each time-point for B-cell lymphoma patients. A p-value ≤ 0.05 was considered significant.

5.4 Results

Patient Characteristics

Table 5.1 contains age, body weight, body condition score and muscle condition score (shown as mean ± SD), male/female ratio and breeds for each group. There

were no differences in age, body weight, body condition score and muscle condition score between groups (p = 0.315, 0.683, 0.165 and 0.257).

Plasma 25(OH)D concentrations were within laboratory reference ranges in all healthy dogs (25/25). In dogs with B-cell lymphoma, 3/14 dogs had plasma 25(OH)D concentrations below the laboratory reference range before treatment, and 1/14 dogs had plasma 25(OH)D concentrations below the reference range at week 6 of treatment. Plasma 25(OH)D concentrations were within the reference range for all dogs with B-cell lymphoma (14/14) by week 25 of chemotherapy treatment. In dogs with T-cell lymphoma, plasma 25(OH)D concentrations were within the reference range before treatment and at week 6 of treatment (5/5), but 1/5 dogs had a plasma 25(OH)D concentration below the reference range at week 25 of treatment.

Dogs with lymphoma before chemotherapy treatment compared to healthy dogs

Median plasma 25(OH)D and APP concentrations of healthy dogs and dogs with B-cell and T-cell lymphoma before chemotherapy treatment are shown in Table 5.2. Plasma 25(OH)D, CRP, AGP, HP and SAA concentrations were different between groups (p = 0.001, 0.002, 0.007, 0.003 and 0.031, respectively). Dogs with B-cell lymphoma had lower pre-treatment plasma 25(OH)D concentrations and higher pre-treatment plasma CRP, AGP, and HP concentrations than healthy dogs (p = <0.001, 0.001, 0.002, and 0.001, respectively). Plasma SAA was not different between the two groups (p = 0.121). Dogs with T-cell lymphoma had higher pre-treatment plasma SAA concentrations compared to healthy dogs (p = 0.015), while plasma 25(OH)D, CRP, AGP, and HP concentrations were not different (p = 0.879, 0.995, 0.517, and 0.156, respectively). No plasma APP differed between dogs with B-cell and T-cell lymphoma (CRP: p = 0.028, AGP: 0.156, HP: 0.406, and SAA: 0.193, respectively). However, dogs with B-cell lymphoma had lower plasma 25(OH)D concentrations than dogs with T-cell lymphoma (p = 0.014).

Dogs with lymphoma during chemotherapy treatment

Median plasma 25(OH)D and APP concentrations of dogs with B-cell and T-cell lymphoma before week 1, week 6 and week 25 of chemotherapy treatment are reported in Table 5.3.

B-cell lymphoma - Plasma 25(OH)D concentrations changed over treatment in dogs with B-cell lymphoma ($p = 0.004$) (Figure 5.1). Plasma 25(OH)D concentrations at week 25 of chemotherapy treatment were increased compared to week 1 concentrations ($p = 0.005$), and to week 6 concentrations ($p = 0.013$). Plasma 25(OH)D concentrations at week 6 of chemotherapy treatment were not different from week 1 ($p = 0.221$). A difference in plasma APP concentrations, specifically CRP ($p = 0.002$), over treatment was also observed. Plasma CRP concentrations at week 25 and week 6 of chemotherapy treatment were decreased compared to week 1 ($p = 0.009$ and 0.007, respectively). Plasma CRP concentrations at week 25 did not differ from week 6 ($p = 0.583$). Other plasma APP concentrations did not change over treatment (AGP: $p = 0.751$, SAA: 0.257, and HP: 0.232, respectively).

T-cell lymphoma - No significant differences in plasma 25(OH)D concentrations over treatment were observed in dogs with T-cell lymphoma ($p = 0.449$). Plasma CRP, AGP, and SAA concentrations also did not change over treatment ($p = 1.00$, 0.247, and 0.449 respectively). There appeared to be a significant difference in HP concentrations over treatment ($p = 0.041$). However, post hoc analysis with Wilcoxon signed-rank tests and a Bonferroni correction, revealed no differences between plasma HP concentrations and week 1 and 6 ($p = 0.080$), week 6 and 25 ($p = 0.080$) and week 1 and 25 ($p = 0.043$) when using the adjusted p-value.

Dogs with B-cell lymphoma at weeks 6 and 25 of treatment compared to healthy dogs

Since differences in plasma 25(OH)D and APP concentrations were observed over treatment in dogs with B-cell lymphoma, the concentrations of the B-cell lymphoma group at weeks 6 and 25 were compared back to the healthy group.

Plasma 25(OH)D concentrations in dogs with B-cell lymphoma were still decreased compared to healthy dogs at week 6 of chemotherapy ($p = 0.001$), however, concentrations were not different from healthy dogs by week 25 of chemotherapy ($p =$

0.087). Plasma CRP concentrations in dogs with B-cell lymphoma were not significantly different from healthy dogs at week 6 and week 25 of chemotherapy treatment (p = 0.377 and 0.696). Plasma AGP and plasma HP concentrations remained increased at week 6 and week 25 of treatment compared to healthy dogs (p = 0.005 and 0.006 for AGP and <0.001 and <0.001 for HP). Plasma SAA concentrations did not differ at week 6, but were increased at week 25 compared to healthy dogs (p = 0.426 and 0.002).

Correlations in dogs with B-cell lymphoma

Spearman rank order correlation coefficients were significant and negative between plasma 25(OH)D and CRP concentrations at week 6 (RS = -0.673, P = 0.008) and week 25 (RS = - 0.563, P = 0.036) in B-cell lymphoma patients. The Spearman rank order correlation coefficient for plasma 25(OH)D and CRP concentrations at week 1 was also negative, but not significant (RS = - 0.500, P = 0.069).

Spearman rank order correlation coefficients were significant and positive between plasma CRP concentrations at week 1 and week 25 (RS = 0.713, P = 0.004) and week 6 and week 25 (RS = 0.543, P = 0.045), plasma CRP concentrations at week 1 and SAA concentrations at week 1 (RS = 0.622, P = 0.018), plasma 25(OH)D concentrations at week 6 and HP concentrations at week 1 (RS = 0.593, P = 0.026), plasma AGP concentrations at week 1 and week 6 (RS = 0.662, P = 0.010), and week 6 and week 25 (RS = 0.670, P = 0.009), and plasma HP at week 25 and SAA at week 1 (RS = 0.692, P = 0.006).

5.5 Discussion

Despite interest in circulating 25(OH)D concentrations in dogs with cancer, it remains unclear what happens to 25(OH)D concentrations during cancer treatment in dogs. An inverse relationship observed between circulating 25(OH)D and inflammatory marker concentrations in dogs with cancer (Weidner et al., 2021), and the finding that inflammatory marker concentrations decrease with canine cancer treatment (Nielsen et al., 2007), suggests that 25(OH)D concentrations may increase with cancer treatment.

The main finding of this study supports this hypothesis as plasma 25(OH)D concentrations increased, and CRP concentrations decreased, in dogs with B-cell lymphoma after a 25-week long chemotherapy protocol. This may provide further support for a relationship between vitamin D metabolism and inflammation in dogs with cancer.

The findings of decreased plasma 25(OH)D concentrations and increased CRP, AGP, and HP before treatment in dogs with B-cell lymphoma compared to healthy dogs are consistent with the previous study (Weidner et al., 2021). This makes sense as the dogs with B-cell lymphoma were a subset of the populations used in that study and the healthy population was the same. It is interesting that statistically significant results were still maintained despite the decreased sample size (14 dogs with B-cell lymphoma in this study compared to 25 dogs in the initial population). Other studies have reported decreased 25(OH)D in lymphoma patients (Weidner et al., 2017; Gerber et al., 2004), however immunophenotype was not included as a variable. This is similar for APP studies, where increased CRP, AAG and HP concentrations have been reported in dogs with lymphoma, albeit not B-cell lymphoma specifically (Ogilvie et al., 1993; Tecles et al., 2005; Nielsen et al., 2007; Mischke et al., 2007; Yuki et al., 2011). Finally, similar to findings reported here, there was no difference in serum SAA between dogs with hematologic neoplasia (consisting of dogs with lymphoma and leukaemia) and healthy dogs (Tecles et al., 2005).

The finding that 25(OH)D increased over chemotherapy treatment in B-cell lymphoma patients could not be directly compared to other results, as to date no studies examining 25(OH)D concentrations over treatment in dogs with cancer could be found. Serum concentrations of another vitamin D metabolite, 1,25(OH)2D (formed by hydroxylation of 25(OH)D), decreased with treatment (surgery, radiation or chemotherapy) in dogs with lymphoma or anal sac adenocarcinomas and cancer associated hypercalcemia (Rosol et al., 1992). This may relate to the changes in 25(OH)D concentrations observed in this study, but future research with a uniform population and treatment protocol is needed. It’s also interesting to note that circulating

25(OH)D concentrations have been reported to be stable over time in most healthy dogs (Hurst, Homer, Gow, et al., 2020; Laing et al., 1999), and remained stable even when healthy dogs received a vitamin D supplement at five times the National Research Council's recommended allowance for vitamin D intake over 10 weeks (Young & Backus, 2016).

Longitudinal studies of 25(OH)D in humans receiving cancer treatment (Vaughan-Shaw et al., 2020; Wesselink, Balvers, et al., 2020) allow for some comparison. Similar to findings in this study, 25(OH)D concentrations increased past pre-treatment concentrations in humans with colorectal cancer in the years following surgical and/or chemotherapy treatment (Vaughan-Shaw et al., 2020; Wesselink, Balvers, et al., 2020). Circulating 25(OH)D concentrations decreased following surgery in colorectal cancer patients but recovered past pre-surgery concentrations after median of 255 days following surgery (Vaughan-Shaw, 2020). Circulating 25(OH)D concentrations were also higher 2 years after diagnosis compared to concentrations at diagnosis in colorectal cancer patients that received surgery, chemotherapy and/or radiation therapy (Wesselink, Balvers, et al., 2020). In contrast to findings reported here, circulating 25(OH)D concentrations in humans seem to decrease in the 6-month period following cancer treatment and/or if results focus on the effects of chemotherapy alone (Wesselink, Bours, et al., 2020; Isenring et al., 2018; Fakih et al., 2010; Santini et al., 2010). This highlights another difference from results reported in the present study, as increases beyond pre-treatment 25(OH)D concentrations were observed after approximately 6 months of chemotherapy treatment in dogs.

This study cannot conclusively determine what caused the observed increase in 25(OH)D concentrations over chemotherapy treatment. However, inflammatory marker concentrations were measured in this study to further investigate the hypothesis that systemic inflammation associated with cancer may affect vitamin D metabolism. The results from this study lend support to this hypothesis as increases in 25(OH)D concentrations occurred following decreases in CRP concentrations. Additionally, negative correlations were observed between 25(OH)D and CRP at weeks 6 and 25. A

similar trend of increasing circulating 25(OH)D concentrations and decreasing inflammatory marker concentrations has been observed in human colorectal cancer patients following cancer treatment (Vaughan-Shaw et al., 2020; Wesselink, Balvers, et al., 2020). A weak, negative correlation between 25(OH)D and CRP at all time points has also been reported (Vaughan-Shaw et al., 2020).

One mechanism proposed to explain the inverse relationship between 25(OH)D and systemic inflammation is that pro-inflammatory cell signaling molecules may regulate enzymes involved in vitamin D metabolism, such as those in the cytochrome p450 family, resulting in increased conversion of 25(OH)D to other metabolites (Wakshlag et al., 2011; Zehnder et al., 2002; Hummel et al., 2014). Results from this study, combined with other observations that 25(OH)D concentrations in dogs decreased after an inflammatory event (Holowaychuk et al., 2012; Clements et al., 2020), warrant further investigation into this mechanism. It may be of particular interest to examine activity of CYP24A1, the enzyme responsible for converting 25(OH)D to 24,25-dihydroxyvitamin D (24,25(OH)2D). Researchers have measured circulating 25(OH)D and 24,25(OH)2D concentrations, and determined the 25(OH):24,25(OH)2D ratio, as an indicator for CYP24A1 activity in humans and dogs with other disease (Makris et al., 2020; Parker et al., 2020; Groth et al., 2019). There is merit in repeating this approach in dogs with B-cell lymphoma.

It is also worth discussing alternate explanations for the observed increase in circulating 25(OH)D concentrations over treatment. There may be an impact of the chemotherapy drugs on 25(OH)D concentrations. A recent literature review summarized the potential impact of medication on circulating 25(OH)D concentrations in humans (Wakeman, 2021). However, the reviewed studies indicated either no change or a decrease in 25(OH)D concentrations with the use of chemotherapy drugs and the use of corticosteroids like prednisone (Wakeman, 2021). However, there remains limited research in this area in dogs and an interaction between medication and vitamin D metabolism that results in increased circulating 25(OH)D concentrations may exist.

No significant results for T-cell lymphoma patients were observed between the group's pre-treatment plasma 25(OH)D and APP concentrations compared to healthy dogs, and between the samples collected from the group over chemotherapy treatment. A lack of statistical significance between plasma 25(OH)D and APP concentrations was observed in a previous study (Weidner et al., 2021) and the current group was a subset of that population. However, these results should be interpreted with caution as the sample size of T-cell lymphoma patients was small. A power calculation using data from the B-cell lymphoma and healthy groups, with an error of 0.05 and a study power of 0.80, estimated that a minimum sample size of 11 T-cell patients would be required. It may be difficult to enrol a sufficient sample size of T-cell lymphoma patients as T-cell lymphomas are less common than B-cell lymphomas, so investigators that are interested in this area may need to consider a multicenter approach and enroll study participants from multiple oncology referral centres.

Other limitations of this study should be acknowledged. Samples were not taken before cancer development, so it is possible decreased 25(OH)D concentrations were already present in the lymphoma group. Only one sample was collected from the healthy group for 25(OH)D analysis when ideally samples would have been collected over time. However, there is evidence that the metabolite is stable over time in most healthy dogs (Hurst, Homer, Gow et al., 2020; Laing et al., 1999). Dietary vitamin D intake was not included as a study variable; however, dietary vitamin D intake did not explain differences in plasma 25(OH)D concentrations in a subpopulation of the present study (Weidner et al., 2017). There were similar ages and body condition scores between groups, but groups were not breed-matched. This variable should be investigated in future studies (Hazewinkel & Tryfonidou, 2002). Lymphoma stage was not included as a variable due to the limited sample size but should also be investigated further. Finally, a study design that included simultaneous measurement of multiple vitamin D metabolites may have provided more insight into the metabolism of the 25(OH)D metabolite.

In conclusion, plasma 25(OH)D concentrations increased, and CRP concentrations decreased, beyond pre-treatment concentrations in dogs with B-cell lymphoma after a 25-week long chemotherapy protocol. These findings provide support for a relationship between vitamin D metabolism and inflammation in dogs with cancer and warrant further research. Researchers should investigate the role of other vitamin D metabolites and dysregulation of enzymes, as well as chemotherapy drugs on vitamin D metabolism in dogs with cancer. Clinicians may consider using the results of this study to provide evidence-based information to owners of dogs with lymphoma who are interested in supplementing vitamin D beyond current dietary recommendations. This may not be warranted given that 25(OH)D concentrations seem to increase with treatment. However, further research that provides a picture of what is happening with other vitamin D metabolites is needed.

5.6 Tables

Table 5.1: Patient characteristics (age, body weight, BCS, MCS, male/female, breed) of healthy dogs and those with cancer enrolled in a study investigating circulating 25(OH)D and inflammatory marker concentrations.

	Healthy (n=25)	B-cell (n=14)	T-cell lymphoma (n=5)
Age (years)	7.9 ± 2.2	7.1 ± 1.9	6.8 ± 1.5
Body Weight (kg)	32.1 ± 10.1	30.0 ± 15.2	28.5 ± 13.9
BCS (1-9)	5.7 ± 0.9	6.38 ± 1.1*	6.2 ± 0.8
MCS (0-3)	2.5 ± 0.7	2.85 ± 0.4*	2.6 ± 0.5
Male/Female	13/12	7/7	3/2
Breeds	Boxer (2) Doberman pinscher (1) Dogue de Bordeaux (1) Golden retriever (2) Labrador retriever (4) Mastiff (2) Mixed (7) Portuguese Water Dog (1) Standard poodle (4) Weimaraner (1)	Airedale terrier (1) Beagle (1) Boston terrier (1) Bouvier des Flandres (1) Cocker spaniel (2) Labrador retriever (1) Mastiff (2) Mixed (4) West Highland White Terrier (1)	Boxer (1) Golden retriever (1) Mixed (1) Norwegian Buhund (1) Shetland Sheepdog (1)

Age, body weight, BCS and MCS are presented as means ± SD. Kruskal-Wallis analyses revealed no differences in age, body weight, body condition score and muscle condition score between the healthy, B-cell lymphoma, and T-cell lymphoma groups (p = 0.315, 0.683, 0.165 and 0.257).

B-cell = B-cell lymphoma, BCS = Body condition score, MCT = mast cell tumour, MCS = Muscle condition score

*Body condition and muscle condition scores were unavailable for 1 dog with B-cell lymphoma.

Table 5.2: Plasma 25-hydroxyvitamin D (25(OH)D) and acute phase protein (APP) concentrations of healthy dogs (n = 25) and dogs with B-cell (n = 14) and T-cell lymphoma (n = 5) enrolled in a study investigating circulating 25(OH)D and inflammatory marker concentrations.

	Healthy		B-cell LSA		T-cell LSA		Kruskal-Wallis p-value
	Median	Range	Median	Range	Median	Range	
25(OH)D (nmol/L)	123[a]	64 – 243	75.5[b]	56 - 124	123[a]	102 - 168	0.001
CRP (ug/mL)	9.4[a]	7.8 – 14.5	18.7[b]	7.8 - 332.8	9.0[ab]	7.8 – 137.9	0.002
AGP (mg/mL)	0.7[a]	0.2 – 2.3	1.7[b]	0.4 - 6.2	0.8[ab]	0.4 – 2.7	0.007
SAA (ug/mL)	4.0[a]	3.8 – 4.7	4.1[ab]	3.8 - 9.8	4.3[b]	4.2 – 33.3	0.003
Hp (mg/mL)	0.2[a]	0.1 – 0.7	1.0[b]	0.2 - 1.9	0.3[ab]	0.2 – 1.5	0.031

A Kruskal-Wallis test was used to compare plasma 25(OH)D and APP concentrations between groups. If the Kruskal-Wallis test was significant, pairwise comparisons (using Dunn's procedure with a Bonferroni correction for multiple comparisons) were performed. Different superscripts represent statistically significant results of $p < 0.017$.

B-cell LSA = B-cell lymphoma, 25(OH)D = 25-hydroxyvitamin D, CRP = C-reactive protein, AGP = alpha-1-acid glycoprotein, SAA = serum amyloid A, T-cell LSA = T-cell lymphoma

Table 5.3: Plasma 25-hydroxyvitamin D (25(OH)D) and acute phase protein (APP) concentrations of healthy dogs (n = 25) and dogs with B-cell (n = 14) and T-cell lymphoma (n = 5) enrolled in a study investigating circulating 25(OH)D and inflammatory marker concentrations.

		Week 1		Week 6		Week 25		Friedman p-value
		Median	Range	Median	Range	Median	Range	
25(OH)D (nmol/L)	B-cell LSA	75.5[a]	56 - 124	80.5[ab]	54 – 125	109[b]	74 - 139	0.004
	T-cell LSA	123[a]	102 - 168	91[a]	82 - 179	118[a]	32 – 136	0.449
CRP (ug/mL)	B-cell LSA	18.7	7.8 - 332.8	11.3[a]	7.8 - 22.8	9.6[a]	7.8 -30.3	0.002
	T-cell LSA	9.0[a]	7.8 – 137.9	10.9[a]	7.8 – 99.5	8.6[a]	7.8 – 13.6	1.00
AGP (mg/mL)	B-cell LSA	1.7[a]	0.4 - 6.2	1.6[a]	0.4 - 3.3	1.5[a]	0.5 - 3.9	0.751
	T-cell LSA	0.8[a]	0.4 – 2.7	1.0[a]	0.4 – 1.3	0.5[a]	0.3 – 2.2	0.247
SAA (ug/mL)	B-cell LSA	4.1[a]	3.8 - 9.8	4.1[a]	3.3 -5.4	4.2[a]	4.0 - 4.7	0.257
	T-cell LSA	4.3[a]	4.0 – 33.3	4.3[a]	4.0 – 7.4	4.1[a]	4.1 – 6.8	0.449
Hp (mg/mL)	B-cell LSA	1.0[a]	0.2 - 1.9	1.1[a]	0.6 – 4.1	0.9[a]	0.1 – 2.1	0.232
	T-cell LSA	0.3[a]	0.2 – 1.5	2.2[a]	0.9 – 3.2	4.0[a]	2.0 – 4.6	0.041

Plasma 25(OH)D and APP concentrations over chemotherapy treatment were compared using Friedman tests followed by Wilcoxon sign rank tests. Since multiple comparisons were tested, p-values were adjusted for significance, and different superscripts represent statistically significant results of $p < 0.017$.

B-cell LSA = B-cell lymphoma, T-cell LSA = T-cell lymphoma, 25(OH)D = 25-hydroxyvitamin D, CRP = C-reactive protein, AGP = alpha-1-acid glycoprotein, SAA = serum amyloid A

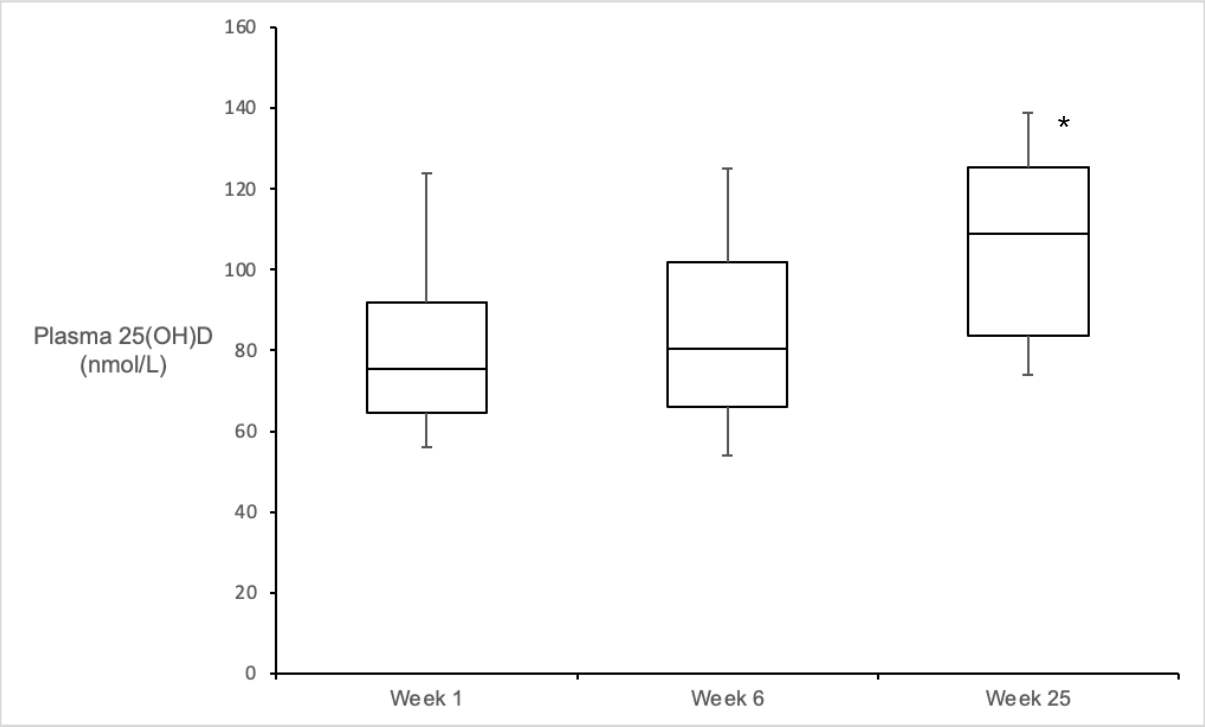

Figure 5.1: Box and whisker plot of plasma 25-hydroxyvitamin D (25(OH)D) concentrations in dogs with B-cell lymphoma (n = 14) enrolled in a study investigating circulating 25(OH)D concentrations during chemotherapy treatment

Asterisks represent a significant difference ($p < 0.017$ after Bonferroni adjustment) compared to week 1 concentrations.

25(OH)D = 25-hydroxyvitamin D

6 Circulating 25(OH)D and 24,25(OH)2D concentrations, and the 25(OH)D/24,25(OH)2D ratio, in dogs with B-cell lymphoma

6.1 Abstract

Researchers have recently emphasized the importance of measuring multiple circulating vitamin D metabolites, and the ratio between these metabolites, to better understand alterations in vitamin D metabolism in dogs with disease. The ratio between the 25(OH)D and 24,25(OH)2D metabolites is of specific interest as it provides a better reflection of CYP24A1 activity, the enzyme responsible for the conversion of 25(OH)D to 24,25(OH)2D, and so gives insight into whether dysregulation of enzyme activity is occurring. This ratio is worthy of investigation in dogs with B-cell lymphoma, as lower circulating 25(OH)D concentrations reported in affected dogs have been potentially attributed to disease and/or inflammatory processes causing dysregulation of CYP24A1 activity. The objective of this study was to evaluate circulating 25(OH)D and 24,25(OH)2D, and the ratio between these metabolites, in dogs with B-cell lymphoma and healthy dogs. The hypothesis was that dogs with B-cell lymphoma would have decreased circulating 25(OH)D concentrations, increased 24,25(OH)2D concentrations and a lower 25(OH)D/24,25(OH)2D ratio when compared to healthy dogs. Newly diagnosed client-owned dogs with multicentric B-cell lymphoma (n = 21) and healthy control dogs (n = 23) were enrolled. Plasma 25(OH)D and 24,25(OH)2D concentrations were measured and the 25(OH)D/24,25(OH)2D ratio was determined. Plasma 25(OH)D and 24,25(OH)2D concentrations were significantly lower in dogs with B-cell lymphoma compared to healthy dogs ($p = 0.02$ and 0.04, respectively). There was no significant difference in 25(OH)D/24,25(OH)2D ratio between groups ($p = 0.63$). These results suggest that CYP24A1 dysregulation is not responsible for observed decreases in circulating 25(OH)D concentrations. Future research should investigate the concentrations of other vitamin D metabolites, such as 1,25(OH)2D, and the ratio between metabolites to determine if dysregulation of enzymes in those pathways is involved in cancer-related alterations in vitamin D metabolism.

6.2 Introduction

Researchers have been investigating concentrations of circulating vitamin D in dogs with cancer for some time now (Gerber et al., 2004; Wakshlag et al., 2011; Rosa et al., 2013; Weidner et al., 2017; Weidner et al., 2021). Most research has focused on measurement of the 25-hydroxyvitamin D metabolite (25(OH)D), which has traditionally been the accepted marker of vitamin D status as it is less regulated and has a longer half-life than other vitamin D metabolites, and there is evidence that it is stable over time in most healthy dogs (Ross et al., 2011; Hurst, Homer, Gow, et al., 2020; Laing et al., 1999). Several studies have documented lower circulating 25(OH)D concentrations in dogs with cancer (Gerber et al., 2004; Rosa et al., 2013; Selting et al., 2017), but few have investigated the potential mechanisms behind these observations. There is an emerging belief that the measurement of additional vitamin D metabolites, and the ratio between these metabolites, may be important to elucidate the mechanisms behind these observations (Groth et al., 2019; Miller et al., 2020). This is worthy of investigation in dogs with cancer.

Circulating 25(OH)D concentrations are reported to be decreased in dogs with lymphoma (Gerber et al., 2004; Weidner et al., 2017), and in dogs with B-cell lymphoma specifically (Weidner et al., 2021). These studies are cross-sectional, making it unclear whether these observations are a cause or consequence of the disease. Potential mechanisms for cause and consequence have been suggested. Lower 25(OH)D concentrations may have been present prior to cancer development, thus increasing disease risk. Factors contributing to low 25(OH)D concentrations in healthy animals may include low sun exposure and/or low dietary vitamin D intake. Sun exposure is likely not a factor for dogs as research suggests that dogs are unable to synthesize adequate amounts of vitamin D in skin (How et al., 1994). Lower dietary vitamin D intake may be a potential explanation, however there have been no differences in dietary vitamin D intake observed between dogs with cancer and healthy controls in cross-sectional studies (Wakshlag et al., 2011; Weidner et al., 2017).

Circulating 25(OH)D concentrations in dogs with B-cell lymphoma were recently shown to increase after a 25-week long chemotherapy protocol, which puts the majority of dogs into disease remission (Weidner et al., 2022). This finding supports the hypothesis that decreased 25(OH)D concentrations are caused by disease-related processes and warrants further investigation. Inflammation is a disease-related process that may explain decreased circulating 25(OH)D concentrations (Ghashut et al., 2014; Silva & Furlanetto, 2015; Wakshlag et al., 2011), as pro-inflammatory cytokines have shown the ability to dysregulate enzymes involved in vitamin D metabolism, thus increasing conversion of 25(OH)D to other vitamin D metabolites (Zehnder et al., 2002; Hummel et al., 2014).There is further merit to this hypothesis as inflammation is an enabling characteristic of cancer (Hanahan & Weinberg, 2011), and inflammation has also been shown to influence 25(OH)D concentrations in dogs (Clements et al., 2020).

To lend further support to these claims, dogs with B-cell lymphoma and decreased 25(OH)D concentrations have also showed increased concentrations of multiple circulating inflammatory markers (CRP, AAG, HP, KC-like and MCP-1) (Weidner et al., 2021). It is possible that circulating 25(OH)D concentrations are decreased in this group due to inflammation-related up-regulation of CYP24A1, the enzyme responsible for converting 25(OH)D to 24,25(OH)2D. An attempt was made to investigate this connection in a previous publication by measuring the concentration of circulating 24,25(OH)2D in dogs with lymphoma (Weidner et al., 2017). However, that study did not account for the immunophenotype of lymphoma patients, and perhaps more importantly, did not determine the ratio between vitamin D metabolites. This is a significant limitation of that work, as new research suggests that the ratio between metabolites may be a more representative reflection of enzyme activity than the individual concentration of each metabolite (Groth et al., 2019; Makris et al., 2020).

Therefore, the objective of this study was to determine concentrations of the circulating vitamin D metabolites 25(OH)D and 24,25(OH)2D, and the ratio between these metabolites, in dogs with B-cell lymphoma and healthy dogs. The hypothesis was that dogs with B-cell lymphoma would have decreased circulating 25(OH)D

concentrations, increased 24,25(OH)2D concentrations and a lower 25(OH)D/24,25(OH)2D ratio when compared to healthy dogs.

6.3 Methods

Animals

The study consisted of 21 dogs with multicentric B-cell lymphoma and 23 healthy controls. The dogs enrolled and samples collected were a subset of the population and samples used in a previous study (Weidner et al., 2021).

Dogs with multicentric B-cell lymphoma were recruited from the Mona Campbell Centre for Animal Cancer at the Ontario Veterinary College Health Sciences Centre. Dogs were client-owned, newly diagnosed with multicentric B-cell lymphoma and had not yet received any treatment. The diagnosis of cancer was confirmed with cytology and/or histology of a fine needle aspirate or biopsy. A fine needle aspirate from a neoplastic peripheral lymph node was analyzed with flow cytometry using a BD Accuri C6 flow cytometer to determine B-cell lymphoma immunophenotype (Gibson et al., 2004). The presence of concurrent disease was ruled out based on normal medical history, unremarkable physical examination, complete blood count and biochemical profile.

Healthy dogs (n = 23) from the Guelph area were recruited as controls. Dogs were client-owned and considered healthy based on a normal medical history, unremarkable physical examination, complete blood count and biochemical profile.

Dogs were excluded if they: (a) were <2 years of age, (b) received corticosteroid treatment within 2 weeks prior to trial enrolment, (c) had significant concurrent systemic/infectious disease or (d) received vitamin D and/or calcium supplements. Dog age, body weight, gender, breed, body condition score (Laflamme, 1997) and muscle condition score (Michel et al., 2004) was recorded at the time of enrollment (Table 6.1).

The experimental protocol was approved by the University of Guelph Animal Care and Use Committee (AUP #1358) and the Royal Canin Ethics Review Committee (#140217_7), which followed institutional and national guidelines for the care and use of animals. Fully informed owner consent was obtained for all groups.

Blood collection and analysis

Blood was collected in lithium heparin tubes, centrifuged at room temperature at 1500 g for 7 minutes, then aliquoted and stored at −80C until analysis. Plasma 25(OH)D was analyzed with commercial RIA kits (Diasorin, Stillwater MN, Scantibodies, Santee CA, Beckman Coulter, Miami, Florida) at the Diagnostic Center for Population & Animal Health, Michigan State University, East Lansing, Michigan.

Plasma 24,25(OH)2D was analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS) at a Vitamin D External Quality Assessment Scheme-certified laboratory (Heartland Assays, Inc, Ames, Iowa) as previously described (Spoo et al., 2015). Briefly, plasma samples (200 uL) and a calibration curve were pipetted into boro-silicate test tubes and spiked with d6-24,25(OH)2D3 (Sigma-Aldrich). Proteins were precipitated using 0.2M ZnSO4 and Methanol and the 24,25(OH)2D3 extracted with hexanes. Following centrifugation, the hexane was collected and dried under vacuum. Samples were re-constituted into LCMS grade Methanol and water, and injected onto an Agilent 1290 HPLC coupled to an Agilent 6460 LC/MS/MS. Liquid chromatography separation was carried out using a PFP column (2.7 micron, 2.1 x100 mm). Electrospray ionization was used for ionization. Derivatization was not used. The 24,25(OH)2D2 mass/charge transition was 429.3>393.2; 429.3>271.2 and the 24,25(OH)2D3 mass/charge transition was 417.3>381.3; 417.3>121.1.

Vitamin D metabolite ratios have been previously calculated and investigated in dogs (Groth et al., 2019).

Statistics

Statistical analysis was completed with SPSS software, version 25 (IBM Corp., Armonk, New York). Data normality was assessed using Shapiro-Wilk tests. Mann-Whitney U tests were used to compare BCS, MCS, plasma 25(OH)D and 24,25(OH)2D concentrations, and 25(OH)D/24,25(OH)2D ratio between groups. Independent T-tests were used to compare age and body weight between groups.

6.4 Results

Patient characteristics

The age, sex, breed, body weight, BCS, and MCS for each group are presented in Table 6.1. There were no differences in mean age, body weight, BCS and MCS between groups (P = 0.737, 0.485, 0.183 and 0.576, respectively).

25(OH)D, 24,25(OH)2D concentrations and 25(OH)D/24,25(OH)2D

Median plasma 25(OH)D and 24,25(OH)2D concentrations, and the 25(OH)D/24,25(OH)2DD ratio for both groups are presented in Table 6.2. Plasma 25(OH)D and 24,25(OH)2D concentrations were significantly lower in dogs with B-cell lymphoma compared to healthy dogs (p = 0.02 and 0.04, respectively). There was no significant difference in 25(OH)D/24,25(OH)2D ratio between groups (p = 0.63).

6.5 Discussion

Lower circulating 25(OH)D concentrations have been reported in dogs with B-cell lymphoma (Weidner et al., 2021), warranting investigation into potential mechanisms. One possible explanation is that inflammation related to the disease causes dysregulation of the CYP24A1 enzyme responsible for conversion of 25(OH)D to 24,25(OH)2D (Hummel et al., 2014). This study investigated this hypothesis by measuring circulating concentrations of 25(OH)D and 24,25(OH)2D, and the 25(OH)D/24,25(OH)2D ratio (representative of CYP24A1 activity) in dogs with B-cell

lymphoma and healthy dogs. Plasma 25(OH)D and 24,25(OH)2D concentrations were significantly lower in dogs with B-cell lymphoma, however there was no difference in 25(OH)D/24,25(OH)2D ratio between groups. This result suggests that CYP24A1 dysregulation is not responsible for decreased plasma 25(OH)D concentrations.

The lower plasma 25(OH)D concentration observed in dogs with B-cell lymphoma is similar to previous reports of dogs with B-cell lymphoma (Weidner et al., 2021) and lymphoma in general (Gerber et al., 2004; Weidner et al., 2017). Unfortunately, no studies measuring 24,25(OH)2D concentrations in dogs with B-cell lymphoma could be found for comparison. The authors measured 24,25(OH)2D concentrations in dogs with lymphoma in a previous study (Weidner et al., 2017), however we did not control for lymphoma immunophenotype.

This is the first study, to the authors' knowledge, to investigate the 25(OH)D/24,25(OH)2D ratio in dogs with cancer. Plasma 25(OH)D/24,25(OH)2D ratios were not different between groups, suggesting that 25(OH)D was not being increasingly converted into 24,25(OH)2D in dogs with B-cell lymphoma. Interest in the 25(OH)D/24,25(OH)2D ratio originated in human studies, where it has been used as an indicator of vitamin D deficiency and catabolism, to determine the efficacy of vitamin D supplementation, and to investigate genetic mutations of the CYP24A1 gene (Makris et al., 2020). In dogs, those affected with hypercalciuric calcium oxalate urolithiasis had a greater ratio of 25(OH)D/24, 25(OH)2D compared to control dogs with no history of urolithiasis and no evidence of urolithiasis on screening abdominal radiographs, suggesting that decreased 24-hydroxylation of 25(OH)D to 24,25(OH)2D may contribute to calcium oxalate stone risk in some dogs (Groth et al., 2019). The 25(OH)D/24,25(OH)2D ratios of dogs with chronic kidney disease were monitored in a recent study to determine how the dogs responded to supplementation with 25(OH)D, and there were no changes over the treatment period (Parker et al., 2020). Finally, dogs with protein-losing nephropathy had higher 25(OH)D/24,25(OH)2D ratios compared to a control group (Miller et al., 2020). This finding indicates that affected dogs had decreased conversion of 25(OH)D to 24,25(OH)2D (Miller et al., 2020). It is increasingly

apparent that multiple mechanisms affecting vitamin D metabolism may be responsible for lower 25(OH)D concentrations in dogs with disease and researchers should consider including 25(OH)D/24,25(OH)2D ratio as a variable in vitamin D studies.

Since it appears that 25(OH)D is not increasingly converted into 24,25(OH)2D in dogs with B-cell lymphoma, then it is possible that 25(OH)D was instead increasingly converted to the vitamin D metabolite known as calcitriol or 1,25-dihydroxy vitamin D (1,25(OH)2D). The 1,25(OH)2D metabolite is formed when 25(OH)D is hydroxylated by the CYP27B1 enzyme. Humans with certain conditions, including some lymphomas, have increased metabolism of 25(OH)D to 1,25(OH)2D (Seymour & Gagel, 1993; Holick et al., 2011; Kohart et al., 2017; Charoenngam & Holick, 2020). Dysregulated 1,25(OH)2D production has been reported in hyper- and normo-calcemic human patients with non-Hodgkin's and Hodgkin's lymphoma (Seymour et al., 1994; Reike et al., 1989; Davies et al., 1985). A reduction in circulating 1,25(OH)2D concentrations and normalization of serum calcium concentrations was observed after cancer treatment in case reports and a small sequential study (Seymour et al., 1994; Hewison et al., 2003; Maletkovic et al., 2014; Gonciulea et al., 2021). Furthermore, after the spleen was removed from a splenic lymphoma patient who presented with hypercalcemia and increased circulating 1,25(OH)2D concentrations, serum calcium and 1,25(OH)2D concentrations normalized within 24 hours. Immunohistochemistry of the spleen revealed strong CYP27B1 enzyme expression in macrophages neighbouring the lymphoma cells, prompting investigators to suggest that abnormal 1,25(OH)2D production may be due to upregulation of CYP27B1 through cytokines produced by the lymphoma cells (Hewison et al., 2003). In fact, dysregulated calcitriol production is considered a mechanism behind hypercalcemia in human lymphoma patients, independent of, or in combination with, parathyroid-related protein (PTHrP) production (Goldner, 2016; Gonciulea et al., 2021; McMullen & Walker, 2022).

Interestingly, the finding of dysregulated 1,25(OH)2D production in human lymphoma patients seems to have been somewhat overlooked in canine literature, with very few reports of vitamin D metabolite concentrations in dogs with lymphoma. Rosol

et al. (1992) found that serum 1,25(OH)2D concentrations in hypercalcemic dogs with lymphoma decreased after chemotherapy treatment. The dogs were also normocalcemic after chemotherapy (Rosol et al., 1992), similar to the reports in humans (Seymour et al., 1994). A distinct subgroup of dogs with lymphoma and cancer associated hypercalcemia had increased serum 1,25(OH)2D concentrations, while 2 of the 28 dogs had undetectable PTHrP concentrations (Rosol et al., 1992). The authors discussed tumour production of 1,25(OH)2D and PTHrP as mechanisms behind their observations (Rosol et al., 1992). Gerber et al. (2004) measured 25(OH)D and 1,25(OH)2D concentrations in hypercalcemic dogs with lymphoma and control dogs, finding decreased serum 25(OH)D concentrations in dogs with lymphoma, but no differences in 1,25(OH)2D concentrations between groups. However, four of the twelve lymphoma dogs had elevated 1,25(OH)2D concentrations. The immunophenotype of the dogs was not investigated in both studies (Rosol et al., 1992; Gerber et al., 2004).

The aforementioned studies measured individual concentrations of 25(OH)D and 1,25(OH)2D, but not the ratio between the two metabolites. Recent work in humans illustrates the value of determining the 25(OH)D/1,25(OH)2D ratio, especially where increased 1,25(OH)2D production is suspected (Pasquali et al., 2015; Rohmer et al., 2020). The ratio may help account for any influence of differences in baseline 25(OH)D concentrations on resulting 1,25(OH)2D concentrations (Rohmer et al., 2020). For example, if a patient has low 25(OH)D concentrations then their 1,25(OH)2D concentrations may fall within the 1,25(OH)2D reference range, even in the face of increased 1,25(OH)2D production. Measuring only the 25(OH)D or 1,25(OH)2D concentration in these patients may mean that the cause of any hypercalcemia may go undetected. Measuring the ratio may be especially important for dogs, who usually have a higher baseline vitamin D intake and circulating 25(OH)D concentrations (Wakshlag et al., 2011; Weidner & Verbrugghe, 2017). Future studies measuring the 25(OH)D/1,25(OH)2D ratio in dogs with lymphoma are warranted. This knowledge would be useful in cases of lymphoma-associated hypercalcemia where no cause can be identified (Mullany et al., 2020), and may help clinicians identify appropriate

treatment strategies and/or assess the need for vitamin D supplementation (Holick et al., 2011).

Researchers suggest caution regarding vitamin D supplementation in humans with lymphoma due to the potential of increased 1,25(OH)2D production (Holick et al., 2011). Careful monitoring of circulating 25(OH)D and calcium concentrations is suggested if pursued (Holick et al., 2011). Since many dogs already receive amounts of vitamin D that meet or exceed their recommended allowance through commercial dog food (Kritikos et al., 2018), and there is the potential for vitamin D toxicosis in dogs (Mellanby et al., 2005), one important clinical implication of this research may be that clinicians should consider cautioning owners against additional vitamin D supplementation of dogs and/or consider monitoring circulating 25(OH)D, 1,25(OH)2D and calcium concentrations if supplementation is pursued.

This study has several limitations. The cross-sectional design makes it unclear whether changes in 25(OH)D and 24,25(OH)2D concentrations occurred pre- or post-cancer development. The study included a limited number of dogs. Little is known about expected 25(OH)D/24,25(OH)2D ratios in dogs, making it difficult to estimate appropriate sample sizes. Although a significant difference was detected with two groups of 19 dogs in the hypercalciuric calcium oxalate urolithiasis study (Groth et al., 2019), it's possible that a larger sample size is required to detect differences in dogs with cancer. Dietary vitamin D intake was not included as a study variable; however, the authors did not observe an effect of dietary vitamin D intake on plasma 25(OH)D concentrations in a previous study that included these dogs (Weidner et al., 2017). An effort was made to include similar ages and body condition scores between groups, however there was a lack of breed-matching between groups. Breed-specific idiosyncrasies in vitamin D metabolism are possible (Hazewinkel and Tryfonidou, 2002) but require further investigation. Lymphoma stage was not included as a variable in this study due to the limited sample size but may be important to investigate in future work, as stage is a surrogate measure of overall tumor burden. A study design that included simultaneous measurement of multiple vitamin D metabolites would have strengthened

the study results. Finally, it is possible that 25(OH)D/24,25(OH)2D does not directly represent CYP24A1 activity in dogs, and investigation into methods of determining the in vivo activity of enzymes involved in vitamin D metabolism would be valuable.

In conclusion, lower plasma 25(OH)D and 24,25(OH)2D concentrations were observed in dogs with B-cell lymphoma but there was no difference in 25(OH)D/24,25(OH)2D ratio between groups. These results suggest CYP24A1 dysregulation, and therefore inactivation of vitamin D, is not a cause of lower 25(OH)D concentrations in dogs with B-cell lymphoma. Future studies should investigate concentrations of other vitamin D metabolites, especially 1,25(OH)2D, and the ratio between metabolites to determine if enzyme dysregulation in these pathways is involved in cancer-related imbalance of vitamin D metabolism.

6.6 Tables

Table 6.1: Patient characteristics (age, sex, breed, body weight, BCS, MCS) of healthy dogs and those with B-cell lymphoma enrolled in a cross-sectional study investigating circulating vitamin D metabolite concentrations.

	Healthy (n = 23)	**B-cell lymphoma (n = 21)[a]**
Age (years)	7.4 ± 2.6	7.6 ± 2.0
Male/Female	12 /11	12/9
Breeds	Doberman Pinscher (1) Dogue de Bordeaux (1) Golden Retriever (2) Labrador retriever (4) Mastiff (2) Mixed (8) Standard poodle (4) Weimaraner (1)	Airedale Terrier (1) Beagle (1) Boston terrier (1) Bouvier des Flandres (1) Cocker Spaniel (2) Dachshund (1) Golden Retriever (3) Labrador Retriever (2) Mastiff (2) Mixed (6) West Highland White Terrier (1)
Body weight (kg)	32.5 +/- 10.3	29.9 +/- 13.8
BCS (1 – 9)	5.7 +/- 1.0	6.2 +/- 1.2
MCS (0 – 3)	2.5 +/- 0.7	2.6 +/- 0.7

Age, body weight, BCS and MCS are presented as means ± SD. There were no differences in age, body weight, BCS or MCS between groups (p = 0.737, 0.485, 0.183 and 0576).

[a]Body condition and muscle condition scores were unavailable for two dogs with B-cell lymphoma.

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; 24,25(OH)2D = 24,25-dihyroxyvitamin D; BCS = body condition score; MCS = muscle condition score.

Table 6.2: Median plasma 25(OH)D and 24,25(OH)2D concentrations, and the 25(OH)D/24,25(OH)2D ratio of healthy dogs and those with B-cell lymphoma enrolled in a cross-sectional study investigating circulating vitamin D metabolite concentrations.

	Healthy (n = 23)		**B-cell lymphoma (n = 21)**		**p-value**
	Median	Range	Median	Range	
25(OH)D (ng/mL)	48.9[a]	25.6 – 97.4	34.4[b]	22.4 – 63.7	0.021
$24,25(OH)_2D$ (ng/mL)	25.7[a]	14.4 – 59.2	19.7[b]	8.8 – 40.5	0.040
25(OH)D/ $24,25(OH)_2D$	1.8	1.2 – 3.1	1.9	1.1 – 2.7	0.630

Superscripts represent statistically significant difference of $P \leq .05$ between groups.

Abbreviations: 25(OH)D = 25-hydroxyvitamin D; 24,25(OH)2D = 24,25-dihydroxyvitamin D

7 General Discussion and Conclusion

This chapter concludes the thesis by summarizing key research findings; discussing the impact of these findings, including opportunities for future research and take-home messages for stakeholders; and reviewing the limitations of the work.

7.1 Key Findings

The work in this thesis explored circulating 25(OH)D concentrations and inflammatory markers in dogs with cancer. This work was prompted by researchers emphasizing the need to identify other variables that may influence circulating 25(OH)D concentrations in dogs (da Fonseca et al., 2020; Corbee, 2020). Inflammation has been suggested to influence 25(OH)D concentrations in humans (Ghashut et al., 2014; Silva & Furlanetto, 2015). A limited amount of work has investigated links between circulating 25(OH)D and inflammation in dogs with other diseases (Titmarsh et al., 2015; Wennogle et al., 2019; Erdogan et al., 2019), but potential relationships between the two in cancer are unclear.

Study 1 (Chapter 4): Plasma 25-hydroxyvitamin D and the inflammatory response in canine cancer

The first study was performed to determine if there was a relationship between circulating 25(OH)D and inflammatory marker concentrations in dogs with cancer. Dogs with B-cell lymphoma were the only group with decreased plasma 25(OH)D concentrations compared with healthy dogs, and these dogs also had increased concentrations of multiple inflammatory markers (CRP, AAG, HP, KC-like and MCP-1). Furthermore, there was no decrease in 25(OH)D concentrations in dogs with T-cell lymphoma and mast cell tumours, both groups that showed no changes in inflammatory marker concentrations. This study supported an association between decreased 25(OH)D concentrations and inflammation in dogs with cancer, especially in dogs with B-cell lymphoma.

Study 2 (Chapter 5): 25-hydroxyvitamin D concentrations and the inflammatory response in dogs with B-cell and T-cell lymphoma during chemotherapy treatment

The second study was performed to determine what happens to circulating 25(OH)D and inflammatory marker concentrations during cancer treatment in dogs with lymphoma. The inverse relationship observed between circulating 25(OH)D and inflammatory marker concentrations in dogs with B-cell lymphoma observed in the first study (Weidner et al., 2021), and work showing inflammatory marker concentrations decrease with canine cancer treatment (Nielsen et al., 2007), suggested 25(OH)D concentrations may increase with cancer treatment. Pre-treatment plasma 25(OH)D concentrations were lower, and plasma CRP concentrations were higher in dogs with B-cell lymphoma compared to healthy dogs. Plasma 25(OH)D concentrations increased, and plasma CRP concentrations decreased by the last chemotherapy treatment in dogs with B-cell lymphoma when compared to their pre-treatment concentrations. Furthermore, plasma 25(OH)D and plasma CRP concentrations at week 25 of chemotherapy treatment were no longer different from the healthy group. Pre-treatment plasma 25(OH)D and CRP concentrations were not different in dogs with T-cell lymphoma compared to healthy dogs. Plasma 25(OH)D and CRP concentrations remained unchanged during chemotherapy treatment in dogs with T-cell lymphoma when compared to their pre-treatment concentrations. The findings from dogs with T-cell lymphoma should be interpreted with caution due to a limited sample size, but the findings from dogs with B-cell lymphoma provide further support for an association between circulating 25(OH)D concentrations and inflammation in dogs with B-cell lymphoma.

Study 3 (Chapter 6): Circulating 25(OH)D and 24,25(OH)2D concentrations, and the 25(OH)D/24,25(OH)2D ratio, in dogs with B-cell lymphoma

The third study was performed to investigate the mechanisms behind the lower circulating 25(OH)D concentrations observed pre-treatment in dogs with B-cell lymphoma. A potential explanation was that cancer-related inflammation causes

upregulation of the CYP24A1 enzyme, which is responsible for conversion of 25(OH)D to 24,25(OH)2D. This was investigated by measuring circulating concentrations of 25(OH)D and 24,25(OH)2D, and the 25(OH)D/24,25(OH)2D ratio (representative of CYP24A1 activity) in dogs with B-cell lymphoma and healthy dogs. Plasma 25(OH)D and 24,25(OH)2D concentrations were lower in dogs with B-cell lymphoma, but there was no difference in 25(OH)D/24,25(OH)2D ratio between groups. These results suggest that upregulation of the CYP24A1 enzyme is not responsible for decreased plasma 25(OH)D concentrations observed in B-cell lymphoma patients.

7.2 Implications

Taken together, the key findings of this thesis provide support for a potential relationship between circulating 25(OH)D concentrations and inflammation in dogs with cancer, specifically in dogs with B-cell lymphoma. The mechanism behind the changes observed in 25(OH)D concentrations in B-cell lymphoma patients remains unclear and warrants further investigation. However, there are still practical implications of these findings for researchers, clinicians, the pet food industry, diagnostic laboratories, and dog owners.

Implications for Researchers and Opportunities for future research

The findings contained in this thesis have added to the limited body of literature exploring vitamin D and inflammation in canine cancer. Furthermore, the study exploring 25(OH)D concentrations over chemotherapy treatment is the first report on this topic in a canine population that the authors are aware of. The work in this thesis will hopefully provide justification for scientists to continue research in vitamin D and inflammation in dogs and to develop a better understanding of vitamin D metabolism in dogs with B-cell lymphoma. There are several worthy implications for researchers to consider:

More work on inflammation and circulating 25(OH)D in dogs

The findings in this thesis supported an inverse relationship between circulating 25(OH)D concentrations and inflammation in dogs, warranting further research in this area. Unfortunately, the dogs with cancer were enrolled during or after diagnosis, meaning it is not clear whether decreased 25(OH)D concentrations occurred before or after the presence of cancer and/or cancer-related inflammatory processes. Currently, there is no consensus on whether low circulating 25(OH)D concentrations are a cause or consequence of inflammation (Hiemstra and de Jongh, 2020; Smolders et al., 2021). Prospective cohort studies that investigate when inflammation-related changes in vitamin D metabolism occur, the mechanisms behind the observed changes, and/or the clinical relevance of these changes are warranted. Designing a study pre- and post-canine cancer development may not be feasible for many researchers, so studies examining circulating 25(OH)D response before and after an acute inflammatory insult (such as the recent one using orthopedic surgery [Clements et al., 2020]) in dogs are warranted. There may also be value in looking to other experimental approaches taken in humans, particularly mendelian randomization methods. Mendelian randomization uses genetic variants to look at the causal affect between a variable and an outcome and may be less likely to be affected by confounding variables (Davies et al, 2018).

Methods used for quantification of circulating vitamin D concentrations

The two vitamin D metabolites measured in the work contained within this thesis were 25(OH)D and 24,25$(OH)_2$D. These analyses were completed in 2013 and 2014. The 25(OH)D analyses were completed at Michigan State University's Diagnostic Center for Population & Animal Health (now called the Veterinary Diagnostic Laboratory) using radioimmunoassay, which was the method of analysis used by the laboratory for 25(OH)D concentrations at that time. Michigan State was chosen for the 25(OH)D analysis as it was the laboratory that analyzed 25(OH)D concentrations in clinical cases from the Ontario Veterinary College, was used for analysis in another publication (Wakshlag et al., 2011), and allowed for measurement of other variables of

interest (e.g. ionized calcium and parathyroid hormone). The advantages to the use of radioimmunoassay for 25(OH)D measurement include good sensitivity for 25(OH)D and the requirement of only a small sample volume (Hurst, Homer, & Mellanby, 2020). However, radioimmunoassay kits are limited by the analytes they can measure (e.g. kits for $24,25(OH)_2D$ are not available) and a potential lack of specificity for the metabolite of interest (Hurst, Homer, & Mellanby, 2020). The lack of availability of a $24,25(OH)_2D$ kit affected this research project as it meant samples needed to be sent to another laboratory (Heartland Assays) for $24,25(OH)_2D$ analysis. The $24,25(OH)_2D$ analysis was completed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS addresses the limitations of radioimmunoassays and is considered the gold-standard technique for analyzing vitamin D metabolites (Hurst, Homer, & Mellanby, 2020). LC-MS/MS allows for simultaneous analysis of multiple metabolites in one sample and can differentiate between metabolites bound to the vitamin D binding protein and those circulating freely (Hurst, Homer, & Mellanby, 2020). In the future, researchers should consider using LC-MS/MS for quantification of vitamin D metabolites. The use of a standard analysis method, and the inclusion of details relevant to the LC-MS/MS analysis in the methods section, will allow for more robust comparisons to be made between study results. The literature review by Hurst, Homer, & Mellanby (2020) provides a great discussion on this topic and is an important read for researchers designing studies in this area.

Measure multiple vitamin D metabolites and forms in studies

Two of the studies included in this thesis measured only one vitamin D metabolite, 25(OH)D. This has been the traditional approach taken in studies examining vitamin D and disease, however it does not paint the full picture of vitamin D metabolism in the body (Corbee et al., 2020). The finding that plasma 25(OH)D concentrations were decreased in B-cell lymphoma patients, but were not being increasingly converted to 24,25(OH)2D, suggests that increased conversion to other metabolites may be occurring. The finding that plasma 25(OH)D concentrations increased with chemotherapy treatment illustrates the importance of measuring concentrations of these

other metabolites. If we had included these measurements in the second study, we may have been able to determine if any dysregulation of enzymes related to vitamin D metabolism was occurring during treatment. It may also be interesting to examine concentrations of free 25(OH)D, which is the amount of 25(OH)D in circulation that is not bound to vitamin D binding protein. Researchers have recently shown that free 25(OH)D concentrations could be measured in dogs (Hurst et al., 2020, Clements et al., 2020), and a recent literature review has discussed the merits of its measurement in dogs with clinical conditions (Hurst, Homer, & Mellanby, 2020). Thus, incorporating simultaneous measurement of multiple vitamin D metabolites, determining the ratio between metabolites, and/or examining concentrations of free and total forms of vitamin D, into the study design would provide researchers with a more thorough picture of vitamin D metabolism, allowing for a deeper understanding of what is occurring. This may provide insight into which metabolic pathways might be dysregulated and help isolate potential mechanisms behind these observations, which could better inform the design of therapeutic interventions targeting these pathways.

Investigate 1,25(OH)2D and the 25(OH)D/1,25(OH)2D ratio in lymphoma patients

A metabolite of specific interest for researchers interested in vitamin D metabolism in canine lymphoma patients may be 1,25(OH)2D. Our finding that circulating 25(OH)D is not increasingly converted into 24,25(OH)2D in dogs with B-cell lymphoma, suggests it's possible that 25(OH)D was instead increasingly converted to 1,25(OH)2D. Increased metabolism of 25(OH)D to 1,25(OH)2D has been observed in humans with non-Hodgkin's and Hodgkin's lymphoma (Reike et al., 1989; Seymour and Gagel, 1993; Seymour et al., 1994; Holick et al., 2011; Kohart et al., 2017; Charoenngam & Holick, 2020). These findings seem to have been somewhat overlooked in canine literature. It may be especially interesting to include measurement of the 25(OH)D: 1,25(OH)2D ratio in dogs with B-cell lymphoma, as recent work in humans illustrates the value of the 25(OH)D/1,25(OH)2D ratio, especially where increased 1,25(OH)2D production is suspected (Pasquali et al., 2015; Rohmer et al., 2020). Research in this area may be especially important for dogs, who usually have a

higher baseline vitamin D intake and circulating 25(OH)D concentration (Wakshlag et al., 2011; Weidner et al., 2017). This knowledge would be useful in cases of lymphoma associated hypercalcemia where no cause can be identified (Mullany et al., 2020), and may help clinicians identify appropriate treatment strategies and/or assess the need for vitamin D supplementation (Holick et al., 2011).

Investigate potential mechanisms

Future research should also examine the mechanisms behind changes in vitamin D metabolism. A mechanism suggested to explain the inverse relationship between 25(OH)D and pro-inflammatory marker concentrations is that pro-inflammatory cell signaling molecules can regulate key enzymes related to vitamin D metabolism, e.g. those in the cytochrome p450 family (Wakshlag et al., 2011; Zehnder et al., 2002; Hummel et al., 2014). This could be investigated in dogs with by examining the effects of pro-inflammatory cytokines and chemokines on vitamin D metabolism in canine cell lines. It would also be worth exploring whether chemotherapy drugs commonly used in canine oncology have effects on vitamin D metabolism.

Implications for Clinicians

Clinicians may consider using the findings from this thesis as evidence-based information for owners of dogs with lymphoma who are interested in supplementing vitamin D beyond current dietary recommendations and/or in addition to a commercial diet that the dog is currently receiving. Clinicians could share with the owner(s) that supplementation may not be warranted given: it is unclear whether circulating 25(OH)D concentrations observed in dogs with B-cell lymphoma are a cause or consequence of the disease; and that with chemotherapy treatment, circulating 25(OH)D concentrations increase to be not significantly different from healthy dogs. However, further research that provides a picture of what is happening with other vitamin D metabolites is needed.

Clinicians should also note that researchers suggest caution of vitamin D supplementation in humans with lymphoma due to the potential of increased

1,25(OH)2D production (Holick et al., 2011). Increased 1,25(OH)2D production is thought to increase intestinal calcium absorption and calcium mobilization from bones, which can lead to hypercalciuria and hypercalcemia (Holick et al., 2011). Careful monitoring of circulating 25(OH)D and calcium concentrations is suggested if supplementation is pursued (Holick et al., 2011). In fact, since many dogs already receive amounts of vitamin D that meet or exceed their recommended allowance through commercial dog food (Kritikos et al., 2018), and there is the potential for vitamin D toxicosis in dogs (Mellanby et al., 2005), clinicians may even consider cautioning owners against additional vitamin D supplementation of dogs and/or consider monitoring circulating 25(OH)D, 1,25(OH)2D and calcium concentrations if supplementation is pursued.

Implications for dog food industry

There are currently no specific veterinary therapeutic diets available for dietary treatment of dogs with cancer. This may reflect the limited research available on nutrition related to cancer in dogs, and the wide variability of cancer types and/or aggressiveness that can alter the nutritional needs of the patient. This thesis did not attempt to determine what amount of vitamin D should be included in diets for dogs with cancer, so should not be used to inform the development of any diets.

However, findings may provide some justification for pet food manufacturers to continue to formulate diets that fall within the current recommendations provided by the Association of American Feed Control Officials. Most dogs enrolled in the studies in this thesis were on commercially available pet foods (Kritikos et al., 2018; Weidner et al., 2021) and had circulating 25(OH)D concentrations that fell within laboratory reference ranges.

The finding that circulating 25(OH)D concentrations increased during chemotherapy treatment may be of interest to the dog food industry as a potential research target. Supporting and/or contributing to future research in this area may help

identify whether there is an optimal amount of vitamin D to include in diets of dogs with lymphoma. At the very least, it may help determine whether diets with amounts of vitamin D at the upper end of the recommended range are appropriate for patients with lymphoma, especially those with cancer-associated hypercalcemia.

It may also be prudent for companies to consider creating educational materials about the current state of knowledge related to vitamin D and/or vitamin D content of their food to address owner interest in vitamin D supplementation. Dog owners, and occasionally clinicians, are not always aware of the vitamin D content of dog foods, and since vitamin D information is not required to be included on the guaranteed analysis, dog owners may be under the impression that the food does not contain any vitamin D. Vitamin D may be present on the list of ingredients, but this is not always the case, and/or it may be present as “cholecalciferol” or “ergocalciferol”, which owners may not recognize as vitamin D. Additionally, some dog owners may believe dogs produce vitamin D in their skin, like humans, and so it is not in dog food. Finally, one study has been used to suggest that commercially available pet foods do not contain sufficient amounts of vitamin D (Sharp et al., 2015). However, there were important methodological limitations of this study that are often not acknowledged (e.g. the amount of vitamin D in each diet was not actually measured in this study, so no conclusions could be drawn about the impact of vitamin D intake on serum 25(OH)D concentrations) (Sharp et al., 2015). Thus, educational materials that address the current state of knowledge around vitamin D would be useful.

Implications for Diagnostic laboratories

Researchers have suggested caution be used when interpreting 25(OH)D measurements during an inflammatory response in humans (Ghashut et al., 2014; Silva & Furlanetto, 2015). Since the findings in this thesis and other work in dogs (Clements et al., 2020) provide some support for a relationship between increased inflammatory marker concentrations and decreased 25(OH)D concentrations, diagnostic laboratories

might consider including this information on documents that outline information about the analysis, such as a laboratory website.

Diagnostic laboratories that have the methodology available for simultaneous measurement of multiple vitamin D metabolites should consider highlighting the availability of these services to researchers interested in the vitamin D metabolite analysis.

Implications for Dog owners

Similar to the implications listed for clinicians above, dog owners should consider that additional vitamin D supplementation may not be warranted for dogs with B-cell lymphoma as dogs fed an Association of American Feed Control Officials-compliant dog food will likely already receive amounts of vitamin D that meet or exceed their recommended allowance (Kritikos et al., 2018); it's unclear whether circulating 25(OH)D concentrations observed in these dogs are a cause or consequence of the disease; and circulating 25(OH)D concentrations may increase with treatment.

7.3 Limitations

As mentioned above, the enrolment of dogs with cancer during or after diagnosis limited the ability to determine whether decreased 25(OH)D concentrations occurred before or after the presence of cancer and/or cancer-related inflammatory processes. Prospective cohort studies that investigate this are warranted. Several other limitations to this thesis should be acknowledged. Dietary vitamin D intake was not included as a variable as this information was not available for all dogs. However, several studies have been unable to establish a relationship between dietary vitamin D intake and circulating 25(OH)D concentrations (Weidner et al., 2017; da Fonesca et al., 2020). Other variables may affect APP and cytokine concentrations, such as stress, exercise, and environmental factors (Cerón et al., 2005; Fazio et al., 2014; Spoo et al., 2015) and these were not investigated in this thesis. Future studies with larger sample sizes

should consider investigation into these potential confounders. To limit the effects of other potential co-variables, an effort was made to include similar ages and body condition scores between cancer and healthy groups. However, to achieve sufficient enrolment numbers, breed-matching of groups was not feasible, meaning breed-specific differences in metabolism or inflammatory response could not be evaluated. Furthermore, grading/staging of cancer patients, which can be useful prognostic indicators, were also not included as variables in this thesis because of sample size limitations. These may be worthy avenues of investigation in future studies.

Finally, the lack of significant results for T-cell lymphoma patients observed throughout the thesis should be interpreted with caution as the sample size of T-cell lymphoma patients was small. A power calculation, with an error of 0.05 and a study power of 0.80, using data from the B-cell lymphoma and healthy groups, suggested a minimum sample size of 11 T-cell patients. Investigators designing a study in this area should consider a multicenter approach and enroll study participants from multiple oncology referral centres due to the lower prevalence of T-cell lymphoma.

7.4 Conclusion

The research in this thesis was completed to address an identified need for studies investigating circulating concentrations of 25(OH)D and inflammatory markers in dogs with cancer. The first study supported an association between decreased 25(OH)D concentrations and inflammation in dogs with cancer, and more specifically B-cell lymphoma. The second study further supported this association as circulating 25(OH)D concentrations increased and inflammatory marker concentrations decreased after a 25 week-long chemotherapy protocol. The third and final study investigated a potential explanation for these observations, finding that upregulation of the CYP24A1 enzyme was not responsible for decreased plasma 25(OH)D concentrations observed in B-cell lymphoma patients. Taken together, these studies provide support for a relationship between circulating 25(OH)D concentrations and inflammation in dogs with

B-cell lymphoma and will serve as a foundation for future research that develops a better understanding of vitamin D metabolism in dogs with cancer and addresses potential mechanisms to explain relationships between inflammation and any alterations in vitamin D metabolism in these dogs.

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