Dietary Vitamin D Intake and Vitamin D Status in Canine Cancer Patients

by

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ABSTRACT

Dietary vitamin D intake and vitamin D status in canine cancer patients

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In humans, low dietary vitamin D intake and a low vitamin D status have been linked to increased risk of cancer development. This association is starting to be explored in canines. This thesis is an investigation of the dietary vitamin D intake and vitamin D status of dogs with cancer, specifically osteosarcoma, lymphoma and mast cell tumours, in comparison to healthy dogs. Plasma 25(OH)D concentration was significantly higher in healthy dogs than in those with osteosarcoma and lymphoma, but not mast cell tumours. There was an independent effect of cancer, dietary vitamin D intake and plasma ionized calcium on plasma 25(OH)D concentrations. The independent effect of cancer suggests that dietary vitamin D intake is not responsible for observed differences in plasma 25(OH)D concentrations. Further research is needed to investigate whether decreased plasma 25(OH)D concentrations are a factor in cancer development, or a consequence of cancer.

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List of Abbreviations

1,25(OH)$_2$D:	**1,25-dihydroxyvitamin D**
24,25(OH)$_2$D:	**24,25-dihydroxyvitamin D**
25(OH)D:	**25-hydroxyvitamin D**
AAFCO:	**The Association of American Feed Control Officials**
BCS:	**Body condition score**
Canis lupus familiaris:	**Canine**
CHOP:	**Cyclophosphamide (C), Doxorubicin (H, hydroxydaunorubicin), Vincristine (O, Oncovin), and Prednisone (P)**
D2:	**Vitamin D2**
D3:	**Vitamin D3**
DHA:	**Docosahexaenoic acid**
DM basis:	**Dry matter basis**
EPA:	**Eicosapentaenoic acid**
ICa:	**Ionized calcium**
LC-MS:	**Liquid chromatography–mass spectrometry**
LC-MS/MS:	**Liquid chromatography-tandem mass spectrometry**
LSA:	**Lymphoma**
MCT:	**Mast cell tumour**
MCS:	**Muscle condition score**
MTT:	**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**
NRC:	**National Research Council**
OSA:	**Osteosarcoma**
PTH:	**Parathyroid hormone**
PTHrP:	**Parathyroid hormone-related protein**
RIA:	**Radioimmunoassay**

Chapter 1

Introduction and Literature review

Introduction

One in four companion dogs will be diagnosed with cancer and half will die of this disease (Bronson, 1982, Adams et al, 2010). Cancer is primarily a disease of ageing and therefore as advances in veterinary medicine to extend the lives of our pets, the risk of disease development increases. Although there have been improvements in disease treatment, the death toll attributable to cancer is still unacceptably high.

The important role nutrition plays in the development and progression of cancer has been emphasized in human research. A report by the World Cancer Research Fund estimates that 1/3 of human cancer cases can be attributed to nutrition and associated factors (World Cancer Research Fund/American Institute for Cancer Research, 2007). Emerging evidence also suggests nutrition can be linked to survival of human cancer patients (World Cancer Research Fund/American Institute for Cancer Research, 2007). Despite this, there remains very little research focused on nutrition in cancer development and progression in dogs.

The few studies that exist cover topics including risk factors for cancer development (Sonnenschein et al, 1991, Perez Alenza et al, 1998), prognostic indicators (Shofer et al, 1989), cancer cachexia (Michel et al, 2004, Weeth et al, 2007), and the potential for nutritional intervention (Ogilvie et al, 2000). However, the conclusions of these studies are often confounded by experimental design limitations. This leaves clinicians with little evidence-based knowledge when developing nutritional requirements

for patients with cancer.

This project was designed to address the current gap in research linking nutrition with cancer development in dogs. Though many specific nutrients may be relevant to cancer research, one that has been a major focus in humans is vitamin D. For instance, enough evidence links a low intake of foods containing vitamin D with colorectal cancer development to warrant a "suggestive" risk categorization by the World Cancer Research Fund (2011). Low levels of blood 25-hydroxyvitamin D (25(OH)D), the metabolite that defines vitamin D status, are also associated with increased cancer risk (Garland et al, 2007, Murphy et al, 2014). Associations found in the human literature may translate to dogs. Several studies have recently provided evidence in support of a vitamin D—cancer relationship in dogs (Wakshlag et al, 2011, Selting et al, 2014), warranting further research in this area. This research requires understanding of the relationship between vitamin D intake and blood 25(OH)D concentration. Unfortunately, this relationship is not well understood in dogs. As a result, this project aimed to compare the relationship between vitamin D intake and blood 25(OH)D concentration in healthy dogs to that of dogs with cancer.

Literature review

1.1 Current knowledge of nutrition and cancer in dogs

Only a small number of studies have explored the nutrition-cancer relationship in dogs. Among these studies, similar flaws in experimental design weaken the conclusions made. Future research in this area can be strengthened by several basic adjustments. Well-designed research into nutrition—cancer relationship in dogs is important and will benefit clinicians, owners, and the broader bio-medical community.

1.1.1 Risk factors and prognostic indicators

Approximately 1/3 of all human cancer cases could be prevented with proper nutrition, and emerging evidence suggests nutrition has a significant impact on cancer prognosis (World Cancer Research Fund/American Institute for Cancer Research, 2007). In humans, extensive and constantly evolving nutrition–cancer literature allows for meta-reviews that summarize diets, whole foods and nutrients that alter the risk of cancer in humans. For instance, convincing evidence indicates the consumption of foods containing dietary fibre decreases the risk of colorectal cancer, while consumption of red meat increases risk (World Cancer Research Fund/American Institute for Cancer Research, 2011). Unfortunately, no large body of evidence exists for consensus statements or guidelines on nutritional risk or prognostic factors for cancer in dogs. Several researchers have drawn on human studies to begin identifying these factors (Shofer et al, 1989, Sonnenschein et al, 1991, Perez Alenza et al, 1998, Wakshlag et al, 2011, Selting et al, 2014).

Potential risk factors (Sonnenschein et al, 1991, Perez Alenza et al, 1998) and prognostic indicators (Shofer et al, 1989) for mammary cancer in dogs have been investigated. A similar study design was used in all studies. Briefly, owners of dogs with mammary tumours completed questionnaires that included questions about their animal's main diet and body condition. Homemade diets, high intake of red meat and low intake of chicken were significant risk factors for the development of mammary tumours (Perez Alenza et al, 1998). Obesity one year prior to diagnosis was associated with mammary tumours in one study (Perez Alenza, 1998), but not in another (Sonnenschein et al, 1991). Obesity at one year of age was associated with mammary tumours (Perez Alenza et al, 1998), and a thin body condition at 9-12 months of age was associated with decreased risk of mammary cancer development in spayed dogs only (Sonnnenschein et al, 1991). For dietary prognostic factors a low fat, high protein diet was optimal for survival in dogs with lymphoma (Shofer et al, 1989).

Although the following studies will be discussed in detail later in the review, both have identified a potential association between blood vitamin D concentrations and cancer. Wakshlag et al (2011) investigated vitamin D in dogs with mast cell tumours. Vitamin D intake and blood vitamin D levels were measured in dogs affected with mast cell tumours and healthy dogs. The mast cell tumour group had significantly decreased blood vitamin D concentrations when compared to the healthy group, but dietary vitamin D intake was not significantly different between groups. Selting et al (2014) reported that relative risk of hemangiosarcoma increased as blood vitamin D levels decreased.

1.1.2 Cancer cachexia

Companion animal researchers have also focused on cancer cachexia. Cachexia often develops in human cancer patients. Chronic diseases like cancer can dramatically alter metabolic rate and fuel choice resulting in continual depletion of body protein stores (Lowry and Perez, 2006). The proposed criteria for diagnosing cancer cachexia in humans include reduction in food intake and/or abnormal metabolism leading to weight loss and/or muscle loss (Fearon et al, 2011). An estimated 50% of human cancer patients will lose weight unintentionally; this number can change depending on cancer type (Tisdale, 2009). A recent review outlined the importance of cachexia in veterinary oncology (Freeman, 2012).

Abnormal metabolism has been reported in dogs with cancer. Lactate and insulin levels were significantly higher in dogs with lymphoma when compared to healthy dogs (Vail et al, 1990). These changes were still present after remission was induced by doxorubicin chemotherapy (Ogilvie et al, 1992). Reports were similar for lipid profiles; most lipoproteins were significantly higher in dogs with lymphoma before and after remission (Ogilvie et al, 1994). Dogs with osteosarcoma had higher resting energy expenditure, decreased rates of protein synthesis, increased urinary nitrogen loss, and increased glucose flux compared to healthy dogs (Mazzaferro et al, 2001). Researchers concluded the observed hyperlactatemia, hyperlipidemia, and other metabolic changes mirrored what is commonly observed in cachectic human cancer patients.

These studies culminated in a review paper (Ogilvie, 1998), which suggested a diet low in simple carbohydrates, with high amounts of protein and fat, might help reverse metabolic changes associated with cancer cachexia, and so improve prognosis in dogs with cancer. One study has compared a high fat diet to a high carbohydrate diet in dogs with lymphoma (Ogilvie et al, 1993). Changes in energy expenditure were measured as a marker of cancer cachexia, and remission and survival data were collected as indicators of prognosis. Diet was not associated with changes in energy expenditure. Statistical analysis was not performed on remission and survival data, and so no conclusions were drawn. A basal diet with the profile suggested by the review paper (Ogilvie, 1998) was used in an interventional study with dogs with lymphoma (Ogilvie et al, 2000). The control group received the basal diet supplemented with soybean oil, and the test group received the basal diet supplemented with fish oil (EPA/DHA) and arginine. Several parameters (e.g. blood concentrations of glucose and lactic acid, energy expenditure, body weight) were measured as markers of cancer cachexia, and remission and survival data were collected as indicators of prognosis. Increases in serum EPA and DHA were observed in dogs fed the fish-oil diet. Increases in serum EPA and DHA were associated with changes in only one marker of cancer cachexia: normalized blood lactic acid concentrations. Increases in serum DHA were associated with longer disease free interval and survival time in dogs with stage III lymphoma.

Recent studies have taken a different approach to exploring cancer cachexia in dogs, by measuring body condition and/or muscle condition scores of patients. A lower prevalence of an overweight and obese body condition in dogs with cancer compared to

healthy dogs was attributed to cancer cachexia (Weeth et al, 2007). When both body condition score and muscle condition score were measured, the observed prevalence of cancer cachexia was less than that reported for human cancer patients (Michel et al, 2004).

1.1.3 Limitations to current knowledge

Many of the studies looking at dietary risk factors and prognostic indicators share the same limitations in experimental design. These limitations should be considered when interpreting researchers' conclusions. Dietary questionnaires asked owners about the dog for a time range from puppyhood to cancer diagnosis, which may have resulted in recall bias (Shofer et al, 1989, Sonnenschein et al, 1991, Perez Alenza et al, 1998). Questionnaires included only owner evaluation of the dog's body condition (Shofer et al, 1989, Sonnenschein et al, 1991, Perez Alenza et al, 1998), and no validated body condition scoring system for dogs was used. Control groups sometimes consisted only of dogs with disease other than cancer (Sonnenschein et al, 1991), or dogs assumed healthy without any veterinary exam (Selting et al, 2014). One study included benign mammary tumours in the sample population (Perez Alenza et al, 1998), making comparison with studies looking at malignant tumours difficult. The most important limitations to these studies are assumptions about the nutritional content of each dog's food, without actual food analysis (Shofer et al, 1989, Sonnenschein et al, 1991, Perez Alenza et al, 1998, Wakshlag et al, 2011). While this is still an improvement over studies that do not account for the nutritional content of the food at all (Selting et al, 2014), information obtained from manufacturers is not always accurate. Macronutrient values provided on pet food

labels only reflect maximums or minimums (guaranteed analysis) (AAFCO, 2014), and not the analytically exact or average content. Micronutrient values often reflect amounts contained in a premix, and do not account for contributions from other ingredients or effects of processing and storage.

Since one research group performed much of the cancer cachexia research in dogs (Vail et al, 1990, Ogilvie et al, 1992, 1993, 1994, 2000), the experimental limitations of the studies are similar. For example, the researchers refer to the animals as "hyperlactatemic" and "hyperlipidemic", yet no laboratory reference ranges are provided for many parameters measured (Vail et al, 1990, Ogilvie et al, 1992, Ogilvie et al, 1994). Thus, the clinical significance of these findings is unclear. The researchers do not consider other factors that may affect the parameters measured. For instance, many complex factors can contribute to hyperlipidemia (Xenoulis and Steiner, 2008) that were not accounted for in the study by Ogilvie et al (1994). The Ogilvie et al (2000) fish oil/arginine study is often cited as providing evidence that a low carbohydrate, high fat, high protein diet can extend survival of lymphoma patients. However, since all diets had the same macronutrient profile, this conclusion is not justified by the scope of the study.

These studies make up the sparse evidence-based *in vivo* knowledge that exists for nutrition and cancer in dogs. Although several authors have published review papers that summarize and discuss potential therapeutic strategies for use in the treatment of cancer in dogs (Roudebush et al, 2004, Baek et al, 2009, Freeman, 2009, Raditic and Bartges, 2014), the studies referenced are often *in vitro* work or studies done in humans. Most of

these reviews conclude by emphasizing the need for well-designed *in vivo* clinical trials. Addressing key limitations of previous studies will strengthen the design of future research.

1.1.4 Future research

Researchers should ensure appropriate factors are accounted for when designing studies in this area. Information from pet food manufacturers cannot be taken at face value, necessitating that a food sample is obtained from owners and analysis completed for the nutrients of interest. Researchers interested in the impact of body condition should make use of body condition scoring systems validated for use in dogs, e.g. Laflamme (1997). Ideally, only one individual should be responsible for the scoring, as inter-individual variability may exist (White et al, 2011b). If the owner is to assess the dog, then detailed information on the chosen scoring system should be provided. The health of the control group should be confirmed by as many methods as is feasible, i.e. physical exam by a veterinarian, bloodwork and urinalysis. Most importantly, interdisciplinary collaboration between nutritionists and oncologists is key to ensure that the experimental design meets the needs of both groups and thus the results will be of clinical relevance and value.

Well-designed research in this area will primarily benefit the veterinary community and ultimately, the patient. Proper nutritional management may help prevent disease recurrence and extend survival. This is true not only for patients with a predicted long-term survival, but also for patients with poorer prognoses. Nutritional management may

help alleviate treatment side effects, such as neutropenia. Proper nutritional management may help the anorectic or cachectic patient. While these outcomes obviously benefit the owner, nutritional management strategies can also provide the owner with the emotional satisfaction that he/she is able to "help" with the animal's treatment. Owners are often looking for complementary treatment strategies, and have rated nutritional supplements as the most commonly used of these therapies (Lana et al, 2006). Results of this research would also provide clinicians with alternative suggestions to owners who choose to switch the animal's diet.

Indirect benefits of studies in this area come from the use of dogs in translational research. As with many types of disease, animal models are relied upon for cancer research. Often rodent models are chosen, as the cancer is quick to develop and easier to manipulate. However, dogs are gaining popularity as a complement to traditional rodent models (Paoloni and Khanna, 2008). A significant advantage of the dog model is that the development of the disease is spontaneous, much like many human cancers. The human genome more closely resembles the dog genome than the mouse, and thus the genetic basis of cancer development is more ideally studied in the dog. In addition, many human and dog cancers have similar pathogenesis, disease progression and biological behaviour in response to treatment (Paoloni and Khanna, 2008).

1.2 Vitamin D in humans

A gap in the literature looking at the role of nutrition in cancer development and treatment in dogs has been identified. The most obvious way to begin to address this gap

is by looking to the human literature to identify nutrients showing anti-cancer associations both *in vitro* and *in vivo* on a consistent basis. One of these nutrients is vitamin D.

1.2.1 Metabolism

Vitamin D is a steroid hormone most commonly known for regulation of the body's calcium and phosphorus stores. Emerging evidence suggests vitamin D may also provide protection against a wide range of physiological conditions in humans, including cancer. In order to correctly interpret results of these studies, an understanding of basic vitamin D metabolism is necessary.

There are 2 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 et al, 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-hydroxyvitamin D (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 (Feskanich et al, 2004). 25(OH)D also serves as the precursor molecule to the most biologically active metabolite of vitamin D, 1,25-dihydroxyvitamin 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 extrarenal tissues (Zehnder et al, 2001, Friedrich et al, 2006), suggesting 1,25(OH)2D production 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 extraskeletal and potentially therapeutic roles of vitamin D in target tissues.

1.2.2 Vitamin D and human cancer

Epidemiological studies provided some of the first evidence that vitamin D may play a role in cancer development (Garland and Garland, 1980, Boscoe and Schymura, 2006). These studies identified populations of people located in areas that received minimal exposure to UV light (i.e. low UV light intensity in northern latitudes) as having increased risk of cancer development. Since sunlight plays a major role in vitamin D synthesis in humans, it was hypothesized that this increase in risk was due to differences in vitamin D status among these populations. This spurred studies linking low vitamin D status with increased risk of cancer (Garland et al, 1989).

The association between vitamin D and risk of cancer development is dependent on cancer type. For instance, a meta-analysis released by the World Cancer Research Fund concluded there was suggestive evidence that food containing vitamin D decreases the risk of colorectal cancer (World Cancer Research Fund/American Institute for Cancer Research, 2011). A meta-analysis reviewing breast cancer risk, and released by the same organization, concluded that the results of studies were too variable to support any relationship with vitamin D (World Cancer Research Fund/American Institute for Cancer Research, 2010).

No meta-analyses currently exist for the cancers of most interest for vitamin D

research in dogs: osteosarcoma, lymphoma, and mast cell tumours. Mast cell tumours are rare in humans, however non-Hodgkin's lymphoma and osteosarcoma in humans share many similarities with the respective cancers that develop in dogs (Marconato et al, 2013, De Maria et al, 2009). Studies that have investigated links between vitamin D and lymphoma and osteosarcoma in humans are summarized below.

As with other cancers, epidemiological studies provided the basic evidence for a potential relationship between vitamin D and non-Hodgkin's lymphoma. Increased sun exposure was associated with decreased risk of non-Hodgkin's lymphoma (Hughes et al, 2004, Hartge et al, 2006, Kricker et al, 2008). Increased vitamin D production from sun exposure was hypothesized to be responsible for the protective effect (Armstrong and Kricker, 2007). However, further studies using multivariate analyses suggested that increased sunlight independently decreases risk of non-Hodgkin's lymphoma, and that neither vitamin D intake nor vitamin D status are related to risk (Hartge et al, 2006, Bertrand et al, 2011). The conclusions of studies looking specifically at the relationship between vitamin D status (by measuring 25(OH)D) and non-Hodgkin's lymphoma are mixed. Increased serum 25(OH)D showed protective effects in one study (Lim et al, 2009), but no association in another (Purdue et al, 2010). Several reviews have acknowledged the number of studies is small, and the results inconsistent, when examining the relationship between 25(OH)D and non-Hodgkin's lymphoma risk and call for further research (Kelly et al, 2009, Negri, 2010).

Other studies have explored the potential mechanisms by which vitamin D could

influence the development of non-Hodgkin's lymphoma. Expression of vitamin D receptors is low in non-Hodgkin's lymphoma cells (Hickish et al, 1993, Lopes et al, 2010, Renné et al, 2012). Still, the active metabolite of vitamin D, 1,25(OH)2D, was able to induce differentiation and inhibit proliferation of these cells, suggesting there are potential vitamin D receptor-independent mechanisms of action (Hickish et al, 1993). These anti-cancer activities may not be pertinent to the link between vitamin D status and risk of development of non-Hodgkin's lymphoma, but could impact survival. This is supported by several studies that have linked lower vitamin D levels with poorer survival in human non-Hodgkin's lymphoma patients (Drake et al, 2010, Bittenbring et al, 2014).

Although the important role vitamin D plays in bone health has led to hypotheses about potential links to osteosarcoma (Mirabello et al, 2009), no published *in vivo* human studies linking vitamin D with osteosarcoma risk or survival outcomes were evident in the literature. A few *in vitro* studies have examined vitamin D and osteosarcoma in humans. Vitamin D receptors are expressed in human osteosarcoma tissue (Gallagher et al, 2012). Additionally, human primary osteoblasts and osteosarcoma cell lines express cytochrome P450 27B1, and produce 1,25(OH)2D when exposed to 25(OH)D (Atkins et al, 2007). Exposure to exogenous 1,25(OH)2D can inhibit osteosarcoma cell growth (Tsuchiya et al, 1993). However, it is unclear if locally produced 1,25(OH)2D has the same effect. The limited research on vitamin D and osteosarcoma in humans does not preclude it from being important in other species where osteosarcoma is more prevalent.

1.2.3 Mechanisms behind anticancer activity

While 25(OH)D concentrations are inversely associated with cancer risk in population based studies, it is 1,25(OH)2D that has biological anticancer activity. The link between 25(OH)D concentration and 1,25(OH)2D activity is explained by the local production of 1,25(OH)2D3. Circulating 25(OH)D is thought to drive production of local 1,25(OH)2D, which can inhibit cell growth and initiate differentiation in extra-renal tissues (Huang et al, 2002). The presence of vitamin D receptors in extra-renal tissues, e.g. breast and colon (Buras et al, 1994, Thomas et al, 1999), supports the biologic activity of 1,25(OH)2D in these tissues. This hypothesis is currently limited by the absence of studies that confirm local production and its effects *in vivo*.

Although *in vivo* mechanisms require further investigation, many *in vitro* studies have explored the molecular mechanisms responsible for vitamin D's anticancer activities. A detailed summary of proposed mechanisms is provided in the review recently published by Fleet et al (2012). Briefly, 1,25(OH)2D can inhibit cellular proliferation and induce apoptosis by: altering gene expression; inhibiting the cell cycle; altering the IGF (insulin-like growth factor) signaling pathway; and altering the Wnt/β-catenin signaling pathway (Fleet et al, 2012). 1,25(OH)2 is also thought to be important for antioxidant defense and DNA repair (Fleet et al, 2012).

Finally, researchers are now suggesting that vitamin D may be associated with cancer progression and prognosis for human cancer patients. This relationship has been seen in cancers such as non-small-cell lung cancer, colorectal cancer, and breast cancer

(Zhou et al, 2005, Ng et al, 2008, Goodwin et al, 2009). The results of these studies have been promising enough that investigators have proposed vitamin D be used as a potential prognostic tool for clinicians (Ulrich and Holmes, 2008). Results have even led to the development of a clinical protocol to improve a patient's vitamin D status quickly and safely to test the hypothesis that improvement in vitamin D status would lead to a better prognosis (Cantor, 2014).

1.3 Vitamin D in healthy dogs

Since there are certain species-specific differences in vitamin D metabolism, the following section will review key knowledge in vitamin D metabolism of dogs. This knowledge is essential when designing research and/or drawing conclusions from translational research studies.

1.3.1 Production and metabolism

As previously mentioned, vitamin D is produced in the skin of most mammals. This takes place when the molecule 7-dehydrocholesterol is exposed to UV light, forming previtamin D (Holick et al, 1980). Previtamin D then undergoes thermal conversion to vitamin D3 (Holick et al, 1980). Studies looking at vitamin D in most mammals must account for vitamin D obtained from the diet and from the skin. However, evidence suggests that UV mediated production of vitamin D is essentially insignificant in dogs (Hazewinkel et al, 1987, How et al, 1994), meaning only dietary intake must be accounted for in canine vitamin D research (Figure 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 precursor's absence spurred the hypothesis that dogs have lost the ability to produce vitamin D, and rely on dietary intake of the vitamin. Further evidence comes from the investigation of vitamin D status of huskies in polar latitudes (Griffiths and Fairney, 1988). The authors found an inverse relationship between UVB radiation from the sun and the huskies' serum 25(OH)D concentrations, instead of the expected positive association. The authors concluded that the changes in the huskies' serum 25(OH)D concentrations were only explained by comparison with 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. Building on the conclusions 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. As a result of these studies, the National Research Council (NRC, 2006) and the American Association for Feed Control Officials (AAFCO, 2014) have classified vitamin D as a dietarily essential nutrient for dogs.

1.3.2 Requirements (NRC and AAFCO)

Since vitamin D is classified as an essential nutrient, both the NRC and AAFCO have developed nutritional guidelines for the dietary level needed to maintain health. NRC recommendations are created on the underlying assumption that the dog will be

receiving a purified diet. AAFCO converts NRC recommendations into ones that ensure the nutritional needs of the dog will still be met when receiving a commercial pet food. There are many limitations to the current NRC/AAFCO recommendations for vitamin D, mainly due to limited literature exploring vitamin D requirements in dogs. This section will review the basis for current NRC/AAFCO recommendations and highlight the need for further research in this field.

The NRC (2006) recommends the same requirements for growth and reproduction as for adult maintenance. This was necessary as information on vitamin D requirements could only be found for puppies, and even then the data was limited. The minimum adequate intake, minimum recommended allowance and safe upper limit of vitamin D for both the NRC (2006) and AAFCO (2014) can be found in Table 1. Once again, the same minimum limits are set for growth and reproduction as for adult maintenance.

When discussing requirements, the NRC (2006) references one study (Kealy et al, 1991) as evidence that commercial dog foods, without any vitamin D supplementation, may contain adequate vitamin D for growing dogs. Puppies were raised from ~6 weeks to ~2 years on either a diet containing no added vitamin D or the same diet with an added 2420 IU cholecalciferol/kg diet. Both groups had similar growth rates and food intake, with no negative effects on skeletal health or selected serum parameters (i.e. calcium). However, no conclusions can be drawn about the vitamin D requirements of dogs from this study, as the vitamin D content of the basal diet is unclear. These results are in direct contrast to another study with puppies fed a diet without vitamin D supplementation and

the same diet with an added 1800 IU/kg diet (Hazewinkel et al, 1987). Although the vitamin D content of the basal diet was not measured, puppies fed the diet without vitamin D supplementation developed rickets.

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 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.

The NRC (2006) states that values 4-10 times the requirement were chosen as the safe upper limits in 1987. 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 the "10 times the requirement". However, the referenced paper is not actually listed in the references of this NRC chapter. The diet that is referred to (~4,000 IU/kg as fed) describes one Tryfonidou et al study (2002), while the 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).

Even though the majority of referenced studies look at vitamin D requirements of puppies, the NRC (2006) acknowledges no definitive conclusions are reached and further

research is needed. Especially as many factors can affect vitamin D requirements, such as calcium and phosphorus content of the diet and expected size at maturation (NRC, 2006). However, the NRC (2006) does state that mature dogs are relatively resistant to a dietary deficiency of vitamin D. This statement is somewhat dismissive of the need for vitamin D research in mature dogs. Instead, the emphasis placed on the lack of research in puppies should also be extended to mature dogs. With emerging evidence of the multiple roles that vitamin D may play in an animal's health (Gow et al, 2011, Holowaychuk et al, 2012), it is essential that we know as much as possible about the requirements of this nutrient for every life stage. This is especially true for mature dogs, that have increased risk of developing the very conditions that vitamin D has been associated with, such as cancer (Merlo et al, 2008).

In addition to the need for further research on vitamin D requirements, there are several other gaps in basic vitamin D knowledge that should be addressed in dogs. For instance, NRC (2006) recommendations currently refer only to cholecalciferol, as the efficiency with which dogs can use ergocalciferol is still not fully understood. This knowledge is necessary for pet food companies choosing to use ergocalciferol as the vitamin D supplement, i.e. for use in "vegan" diets.

Furthermore, 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. 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 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, 2014). However, many of these researchers (Gerber et al, 2004, Galler et al, 2012, Selting et al, 2014) 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 those making vitamin D intake recommendations (e.g. NRC), pet food manufacturers and clinicians. Potential factors to affect the relationship between vitamin D intake and serum 25(OH)D (e.g. age, body condition score, breed, genetic variation) should also be explored.

1.3.3 Sufficiency

The need to better understand the relationship between vitamin D intake and serum 25(OH)D status is underscored by a recent study. Selting et al (2014) attempted to define the "sufficiency" level for serum 25(OH)D in dogs. The concept of "sufficiency" has been gaining popularity in humans for many years. Health Canada has yet to release a consensus statement on this matter (Ross et al, 2011). However, many papers suggest the sufficiency should be defined as the level of serum 25(OH)D where parathyroid hormone

(PTH) levels are maximally suppressed. Selting et al (2014) used this definition, but included others (such as the level of serum 25(OH)D where variation in phosphorus levels are minimized) for reasons that are not clear. Selting et al (2014) concluded that 25(OH)D sufficiency in dogs be defined as serum concentrations of 100-120ng/mL. If converted into nmol/L, this sufficiency range would be 115-139% of the maximum vitamin D range set by 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 these dogs were healthy. Since there are no consensus statements on vitamin D intake, serum 25(OH)D concentrations, or the relationship between vitamin D intake and serum 25(OH)D, owners and/or clinicians may read these results and attempt to supplement the dog to levels that could be physiologically harmful. Vitamin D toxicity is serious and can cause reduced growth, hypercalcemia, calcification of soft tissues, and excessive mineralization of bones (Hendricks et al, 1997, Spangler et al, 1979, Tryfonidou et al, 2003).

1.4 Cancer in dogs

The past section highlighted the key species-specific vitamin D knowledge necessary to keep in mind as we apply vitamin D – human cancer research ideas to dogs. The next section will introduce the reader to three common cancers in dogs (mast cell tumours, lymphoma and osteosarcoma), and highlight the findings of research exploring the vitamin D – cancer relationship in dogs with these cancers.

1.4.1 Osteosarcoma

Around 85% of all primary bone tumours in dogs will be diagnosed as osteosarcoma (Withrow and Vail, 2013). Around 10,000 dogs are diagnosed with the disease each year in the United States alone (Withrow and Vail, 2013). The majority of osteosarcomas affect the appendicular skeleton (Brodey and Riser, 1969). Microscopic metastatic disease will be present at the time of diagnosis in the majority of cases (Withrow and Vail, 2013). The current treatment protocol reflects this, and consists of amputation of the affected limb followed by adjuvant chemotherapy. This treatment protocol gives patients a median survival time of 207-366 days (Withrow and Vail, 2013). This is compared to a median survival time of 134 days if treated with amputation alone (Spodnick et al, 1992).

Current literature on vitamin D and osteosarcoma in dogs is limited to *in vitro and ex vivo* work. Barroga et al (1998, 2000) found that 1,25(OH)2D was able to induce cellular apoptosis and differentiation in a single *Canis lupus familiaris* (hereon referred to as canine) osteosarcoma cell line. Rassnick et al (2008) used a colorimetric assay for cellular viability (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) to investigate the anti-proliferative activities of 1,25(OH)2D in another osteosarcoma cell line. Cellular proliferation was reduced by 1,25(OH)2D, and these effects were synergistic when 1,25(OH)2D was combined with cisplatin, a chemotherapy agent. Davies et al (2012) looked at the expression of vitamin D receptors and retinoid X receptors (necessary for vitamin D biologic activity) in canine osteosarcoma tissue. The expression of receptors was correlated with markers of cellular proliferation (Ki-67),

apoptosis (survivin), and metastasis (ezrin). Vitamin D receptors were expressed in 76% of samples, and retinoid X receptors in 97%. There was a negative relationship between vitamin D receptor expression and Ki-67 levels. High Ki-67 levels indicate increased proliferation rates, which indicate a more aggressive osteosarcoma. This suggests that vitamin D receptor expression was decreased in more aggressive osteosarcomas. The authors explained these results with an example from human colonic cancers (Cross et al, 2001). Late stage carcinomas had lower vitamin D receptor expression than those in the early stage. Lower expression was attributed to the failure of vitamin D's anti-proliferative abilities, followed by loss of vitamin D receptor expression (Cross et al, 2001). Davies et al (2012) propose that this loss of expression may also occur in canine osteosarcoma. These are the only studies exploring vitamin D in canine osteosarcoma that the author is aware of, thus warranting further *in vivo* research in this area.

1.4.2 Mast Cell Tumour

Mast cell tumours are the most common cutaneous tumour to affect dogs, accounting for 7-21% of all cutaneous tumours (Bostock, 1986, Withrow and Vail, 2013). Certain breeds have a higher predilection for mast cell tumours, including Boxers, Terriers, Bulldogs, Labrador Retrievers, and Pugs (Priester and McKay, 1980, White et al, 2011a). Many prognostic indicators have been identified for mast cell tumours and include: histologic grade, clinical stage, location, cell proliferation rate, growth rate, recurrence, systemic signs, age, breed, sex, and tumour size (Withrow and Vail, 2013). Treatment of mast cell tumours is dependent on these prognostic factors (Withrow and Vail, 2013). Treatment protocols vary and may involve surgical excision, radiation and/or chemotherapy (Withrow and Vail, 2013). Prognosis is better for dogs with low or

intermediate grade tumours with complete surgical excision; approximately 80-90% and 75% respectively of these dogs will have long term survival (Withrow and Vail, 2013). Prognosis for dogs with high grade tumours is much poorer; the survival time for most dogs is less than a year (Withrow and Vail, 2013).

Both *in vitro* and *in vivo* work exists to support a relationship between vitamin D and mast cell tumours. In addition to the already mentioned work with osteosarcoma cells, Rassnick et al (2008) also looked at actions of 1,25(OH)2D against a canine mast cell tumour cell line. Cellular proliferation, evaluated using a MTT assay, was reduced in cells incubated with 1,25(OH)2D. These effects were synergistic when combined with cisplatin. Similar to the development of the osteosarcoma literature, this study was followed by another to confirm the presence of vitamin D receptors in mast cell tumour tissue (Russell et al, 2010). Vitamin D receptors were expressed in almost all mast cell tumour samples (97%).

Based on the above results, Wakshlag et al (2011) designed the first *in vivo* study with a primary objective and design similar to that of the human cancer – vitamin D studies. The researchers enrolled client-owned Labrador Retrievers affected with mast cell tumours and client-owned Labrador Retrievers deemed healthy by normal physical exam and bloodwork. Serum 25(OH)D and dietary vitamin D intake were measured in both groups. Labrador Retrievers with mast cell tumours had significantly decreased serum 25(OH)D concentrations when compared to healthy controls, but dietary vitamin D intake was not significantly different between groups.

Although the above results initially suggest that there is another mechanism responsible for observed differences in serum 25(OH)D, the researchers acknowledge that vitamin D intakes were only a calculated estimate. The actual vitamin D content of the diet was not analyzed, so it is possible that erroneous information from food manufacturers lead to inaccuracies in the vitamin D intake calculations. Many manufacturers report the amount of vitamin D present in the vitamin premix added to the food prior to extrusion or canning, and not in the final product. This does not account for vitamin D present in other food ingredients or the effects of processing. Additionally, vitamin D information is given only for product at the time of manufacturing. This does not account for storage, and vitamin D may degrade by as much as 43% during 8 months of storage (Coelho, 2003). The rate of degradation may be influenced by the conditions of the storage, such as exposure to air and light (Coelho, 2003). Analysis of the vitamin D content of dog food should be incorporated into the experimental design to obtain accurate intake results.

1.4.3 Lymphoma

Lymphoma is one of the most common cancers to affect dogs (Withrow and Vail, 2013). Lymphoma has an annual incidence rate of around 13 to 24 per 100,000 dogs (Dorn et al, 1968, Merlo et al, 2008). Multicentric lymphoma accounts for 84% of all diagnosed canine lymphomas, making it the most common form of the disease (Theilen and Madewell, 1987). Treatment of multicentric lymphoma involves a basic "CHOP" chemotherapy protocol. This protocol involves a combination chemotherapy regimen of

the following drugs: cyclophosphamide (C), doxorubicin (H, hydroxydaunorubicin), vincristine (O, Oncovin), and prednisone (P) (Withrow and Vail, 2013). Although treatment response is dependent on several prognostic factors, remission will be induced in about 80-90% of dogs undergoing CHOP chemotherapy, with a median survival time of 10-12 months (Withrow and Vail, 2013).

Although there are currently no studies with the primary objective of exploring vitamin D in canine lymphoma, past studies have included measurement of 25(OH)D and 1,25(OH)2D in conditions related to canine lymphoma. Hypercalcemia is common in dogs with lymphoma, with approximately 10-40% of patients affected (Withrow and Vail, 2013). 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. Rosol et al (1992) observed a high inter-individual variability of serum 1,25(OH)2D concentrations in lymphoma patients with hypercalcemia, with serum 1,25(OH)2D concentrations elevated in a subgroup of these patients. However, the statistical significance of these results is not known and no further details are given about the characteristics of this subgroup. Serum 1,25(OH)2D concentrations were significantly reduced in all patients when the hypercalcemia was treated. The authors suggest two mechanisms to explain the elevated serum 1,25(OH)2D concentrations in the subgroup of lymphoma patients: (1) tumours produce parathyroid hormone-related protein (PTHrP) and stimulate renal cytochrome P450 27B1; or (2) tumours produce 1,25(OH)2D directly. These hypotheses require further *in vivo* study. PTHrP does not seem to be as effective as PTH at stimulating renal production of

1,25(OH)2D (Horwitz et al, 2003). Although the ability of cancer cells to produce 1,25(OH)2D has been shown (Atkins et al, 2007), no evidence exists to prove that significant production occurs *in vivo* (Fleet et al, 2012).

Gerber et al (2004) conducted a similar study, but included measurement of serum 25(OH)D. The authors reported similar variable serum 1,25(OH)2D concentrations in patients with lymphoma. Additionally, a significant negative correlation between serum phosphorus and serum 1,25(OH)2D concentrations was observed. Serum 25(OH)D concentrations were significantly decreased in dogs with lymphoma when compared to healthy controls. The authors acknowledged the hypotheses suggested by Rosol et al (1992) to explain any elevated serum 1,25(OH)2D concentrations, and added that low serum phosphorus concentrations may drive 1,25(OH)2D production. This is supported by the negative correlation between serum phosphorus and serum 1,25(OH)2D, but the cause of low serum phosphorus concentrations is not clear. Gerber et al (2004) postulate that differences in serum 25(OH)D were due to differences in vitamin D intake between groups, however no conclusions could be drawn because this variable was not measured.

Conclusion

This review has highlighted the need for research into the role of nutrition in the development and progression of cancer in dogs. Since nutrition studies in human cancer have identified vitamin D as a nutrient of interest, we have suggested exploring a potential link between vitamin D and cancer in dogs. In order to properly translate research designs between species, the species-specific vitamin D metabolism of dogs has

been reviewed. As a result, we have also highlighted the need for better understanding of the relationship between vitamin D intake and vitamin D status in healthy dogs. The potential dynamic nature of this relationship should be explored in dogs with different disease states (i.e. cancer). Studies exploring the link between vitamin D and cancers of interest have been summarized, with potential shortcomings in study design highlighted so that future studies may address these limitations.

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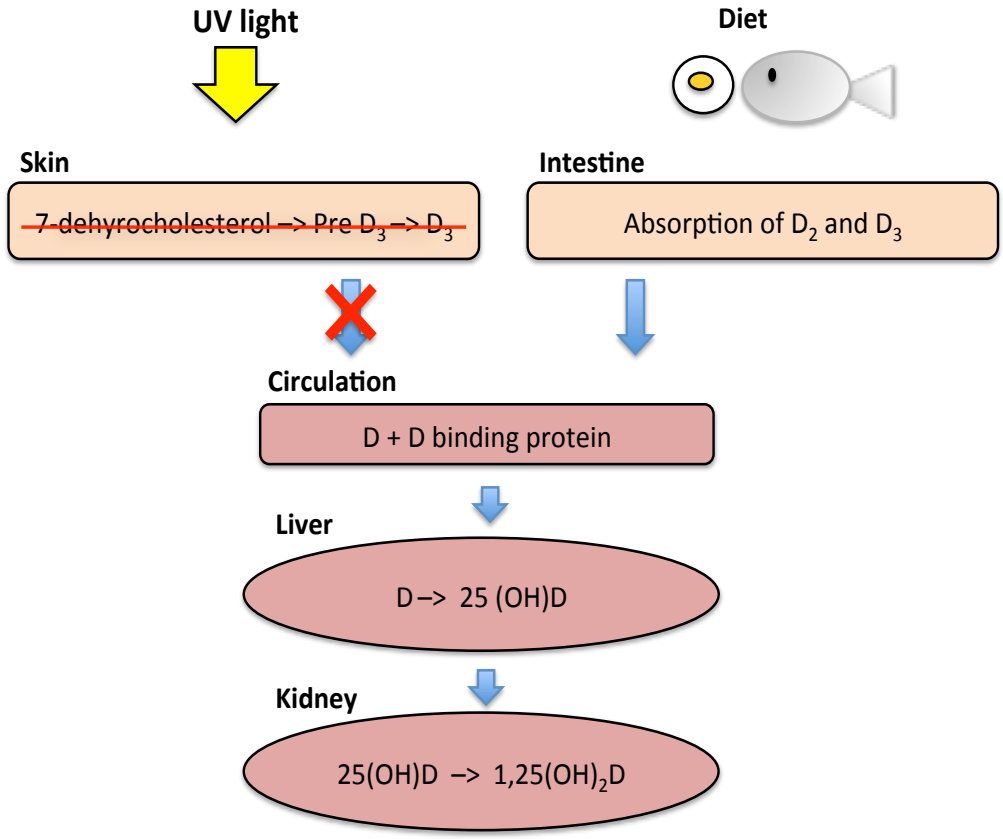
Figure 1. Basic vitamin D metabolism in dogs



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). Illustration adapted from Deeb et al (2007).

D = Vitamin D, D_2 = Vitamin D_2, D_3 = Vitamin D_3, 25(OH)D = 25-hydroxyvitamin D, 1,25$(OH)_2$D = 1,25-dihydroxyvitamin D

Table 1.1. Vitamin D requirements set by the NRC (2006) and AAFCO (2014) for dogs in all life stages

		Minimum Adequate Intake	**Minimum Recommended Allowance**	**Safe Upper Limit**
NRC	DM basis* (IU/kg)	440	552	3200
	Caloric basis (IU/1000 kcal ME)	110	136	800
AAFCO	DM basis** (IU/kg)		500	5000
	Caloric basis (IU/1000 kcal ME)		143	1429

*based on a dietary energy density of 4000 kcal ME/kg
**based on a dietary energy density of 3500 kcal ME/kg

NRC = National Research Council, AAFCO = the Association of American Feed Control Officials, DM basis = Dry matter basis, IU = International Units

Chapter 2

Dietary vitamin D intake and vitamin D status in canine cancer patients

2.1: Dietary vitamin D intake and vitamin D status in canine cancer patients

Dietary vitamin D intake and vitamin D status in canine cancer patients

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2.2: Abstract

Background: Low vitamin D status has been linked to increased risk of cancer development in humans. This association is starting to be explored in dogs.

Hypothesis/Objectives: This study aimed to explore dietary vitamin D intake and vitamin D status of dogs with cancer and healthy dogs. Those with cancer were hypothesized to have decreased plasma 25-hydroxyvitamin D (25(OH)D) when compared to healthy dogs, due to decreased dietary vitamin D intake in dogs with cancer.

Animals: Client-owned dogs with osteosarcoma (n=18), lymphoma (n=22) and mast cell tumours (n=21) were enrolled, as well as healthy, client-owned dogs (n=20).

Methods: Owners provided dietary information and a sample of the dog's food for calculation of each dog's individual dietary vitamin D intake, based on vitamin D information provided by the pet food manufacturer and vitamin D_3 analysis of the pet food sample. Blood samples were analyzed for plasma 25(OH)D, ionized calcium, parathyroid hormone, and parathyroid hormone-related protein.

Results: Median plasma 25(OH)D concentration was significantly higher in healthy dogs (126 nmol/L) than in those with osteosarcoma (94 nmol/L, $p = 0.006$) and and those with lymphoma (97 nmol/L, $p = 0.009$), but not those with mast cell tumours (107 nmol/L, $p=0.099$). There was an independent effect of cancer ($p=0.020$), dietary vitamin D intake ($p=0.009$), and plasma ionized calcium ($p=0.031$) on plasma 25(OH)D concentrations.

Conclusions and clinical importance: The independent effect of cancer suggests that dietary vitamin D intake is not responsible for observed differences in plasma 25(OH)D status. Further research is needed to investigate whether decreased plasma 25(OH)D concentrations are a factor in cancer development, or a consequence of cancer.

2.3: Introduction

One in four companion dogs will be diagnosed with cancer, and half of those diagnosed will die of this disease (Bronson, 1982, Adams et al, 2010). Cancer is primarily a disease of ageing. As advances in veterinary medicine continue to extend the lives of pets, the number of affected dogs will likely increase. Although there have been improvements in disease treatment, the death toll attributed to cancer is still unacceptably high.

The important role nutrition plays in the development and progression of cancer has been emphasized in human research. The World Cancer Research Fund estimates that one-third of human cancer cases can be prevented by proper nutrition (World Cancer Research Fund/American Institute for Cancer Research, 2007). Despite this, links between nutrition and cancer have received very little attention from the companion animal research community.

A key nutrient that has been focused on in human cancer research is vitamin D. The active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D), induces cellular apoptosis and differentiation, inhibits cellular proliferation, angiogenesis and metastasis, and enhances DNA repair (Fleet et al, 2012). There is enough evidence linking low intake of foods containing vitamin D and development of colorectal cancer to warrant a "suggestive" risk categorization in a report by the World Cancer Research Fund (World Cancer Research Fund/American Institute for Cancer Research, 2011). Studies

have linked both vitamin D intake and low 25-hydroxyvitamin D (25(OH)D) concentrations (the accepted indicator of vitamin D status [Holick, 1995]) to increased risk of other human cancers, i.e. breast and prostate cancer (Garland et al, 2007, Murphy et al, 2014). The associations found in the human literature may translate to dogs. Several studies have recently provided evidence in support of this (Wakshlag et al, 2011, Selting et al, 2014), warranting further research in this area. Building upon this foundation of vitamin D –cancer research is an effective way to begin to address the gap in current nutrition—cancer research in dogs, and provide justification for further research in this field.

Selting et al (2014) reported that dogs with hemangiosarcoma had decreased vitamin D status when compared to healthy dogs, however the dietary vitamin D intake of the animals was not measured. Measurement of dietary vitamin D intake is important, as this contributes to the dog's vitamin D status (Hazewinkel, 1987). Although, UV-mediated vitamin D production in the skin must be accounted for in human research, this production is insignificant in dogs (Hazewinkel et al, 1987, Howe et al, 1994). Wakshlag et al (2011) measured vitamin D intake and reported dogs with mast cell tumours had a significantly decreased vitamin D status, but similar vitamin D intake when compared to healthy dogs. Vitamin D information from manufacturers was relied upon for intake calculations, and Wakshlag et al (2011) acknowledged that the food's analyzed vitamin D content may differ from manufacturer information.

Osteosarcoma, lymphoma, and mast cell tumours, three of the most common types

of canine cancer, were the subject of this study. Some research exploring vitamin D and these cancers in dogs has been published. The presence of vitamin D receptors has been shown in osteosarcoma tissue from dogs (Davies et al, 2012), and 1,25$(OH)_2$D induced apoptosis, differentiation, and reduced cell growth in osteosarcoma cell lines (Barroga et al, 1998, 2000, Rassnick, 2008). In addition to the previously mentioned *in vivo* mast cell tumour study (Wakshlag et al, 2011), the presence of vitamin D receptors has been identified in mast cell tumour tissue from dogs (Russell, 2010), and the active metabolite of vitamin D acted synergistically with a chemotherapy agent to reduce cell growth in vitro (Rassnick, 2008).

The objectives of this study were to determine dietary vitamin D intake and vitamin D status of dogs with cancer and healthy dogs. We hypothesized that dogs with cancer would have lower vitamin D status, and that this would be due to a lower dietary vitamin D intake in these animals.

2.4: Materials and Methods

Animals

An observational study design was used. Newly diagnosed, client-owned dogs presenting to the Mona Campbell Centre for Animal Cancer at the Ontario Veterinary College Health Sciences Centre with osteosarcoma (n=15), lymphoma (n=29), and mast cell tumours (n=25) were enrolled. Cytology and/or histology were used to confirm cancer presence. Healthy, client-owned dogs (n=20) from the Guelph, Ontario area were

enrolled. Animals were deemed healthy by a normal: medical history, physical exam, complete blood count, and biochemical profile. This was repeated in the cancer group to rule out any other systemic or infectious disease and/or organ failure. The breed, age, gender, body weight, body condition score (BCS) (Laflamme, 1997), and muscle condition score (MCS) (Michel et al, 2004) were recorded. This information is provided in Table 1. Exclusion criteria were: younger than 2 years of age; receiving corticosteroids within 2 weeks of enrollment; receiving vitamin D and/or calcium supplements; or significant systemic or infectious disease (other than cancer in the cancer group). The experimental protocol was approved by the University of Guelph Animal Care and Use Committee (AUP #1358) and was in accordance with institutional and national guidelines for care and use of animals.

Plasma Analysis

Blood samples were collected from cancer patients and healthy dogs using lithium heparin tubes and centrifuged at room temperature at 1500 *g* for 7 minutes. Plasma samples were collected and stored at -80 °C until analysis. Analysis of 25(OH)D, parathyroid hormone (PTH), parathyroid hormone-related protein (PTHrP), and ionized calcium (ICa) was completed at the Diagnostic Center for Population & Animal Health, Michigan State University, East Lansing, Michigan, USA. 25(OH)D, PTH, and PTHrP were analyzed using commercial RIA kits (Diasorin, Stillwater MN, Scantibodies, Santee CA, Beckman Coulter, Miami, FL, USA). ICa was measured using an ion-sensitive

electrode (Nova 8 analyzer, Nova Biomedical, Waltham, MA, USA). 24,25-dihydroxyvitamin D ($24,25(OH)_2D$) was analyzed using LC-MS/MS at Heartland Assays, Ames, Iowa, USA.

Dietary Vitamin D Intake

To obtain the best estimate of each dog's vitamin D intake, owners filled out a dietary questionnaire providing information about the dog's diet for up to 3 months preceding the study. Owners also recorded the type and amount of food fed to the animal in a food log for 7 consecutive days and provided a sample of the dog's main diet. Samples were stored in airtight, opaque containers at -80°C until being sent for vitamin D_3 analysis at the Royal Canin Americas Satellite Laboratory, Guelph, Ontario, Canada using LC-MS. The analysis results were used to calculate each dog's average daily vitamin D intake per kg of metabolic body weight ($kg^{0.75}$). If a food sample could not be obtained from the owner, then vitamin D information from the manufacturer was used for pet foods, and vitamin D information from the Canadian Nutrient File (http://webprod3.hc-sc.gc.ca/cnf-fce/index-eng.jsp) was used for human foods.

Statistics

Statistical analysis was completed with SAS software, version 9.3 (SAS Institute, Inc., Cary, NC, USA). An ANOVA was used to compare age, body weight, BCS, MCS, plasma PTH, PTHrP, and ICa concentrations between groups. An analysis of covariance (ANCOVA) was used to compare the independent variables: cancer, age, gender, body weight, BCS, MCS, plasma PTH, PTHrP, ICa concentrations, and dietary vitamin D intake with the response variable: plasma 25(OH)D concentrations. Results are expressed

as mean ± SD or median ± SD. A p-value < 0.05 was considered significant.

2.5: Results:

The mean plasma ICa, PTH, and PTHrP concentrations fell within laboratory reference ranges for each group of dogs (Table 2). There were no significant differences among groups.

No effects of age, gender, body weight, BCS, MCS, plasma PTH, and PTHrP concentrations were found on plasma 25(OH)D concentrations in the ANCOVA model. Cancer (p=0.020), dietary vitamin D intake (p=0.009), and plasma ionized calcium (p=0.031) had independent effects on plasma 25(OH)D concentrations.

Median plasma 25(OH)D concentrations were significantly higher in healthy dogs (126 nmol/L) than in those with osteosarcoma (94 nmol/L, p = 0.006) and lymphoma (97 nmol/L, p =0.009), but not mast cell tumours (107 nmol/L, p=0.099) (Figure 1).

The relationship between dietary vitamin D intake and plasma 25(OH)D concentrations was logarithmic. When dietary intake was doubled (i.e. a dog receiving 8 IU/ $kg^{0.75}$ per day versus a dog receiving 4 IU/$kg^{0.75}$ per day), the plasma 25(OH)D concentration increased by approximately 6.1%. The effect of dietary vitamin D intake on plasma 25(OH)D concentrations was independent, so this relationship was the same for each group of dogs studied. Since 3 variables independently effect plasma 25(OH)D, a three dimensional graph would be necessary to display the relationship. In order to focus on the relationship between dietary vitamin D intake and plasma 25(OH)D relationship,

one variable must be held constant. Figure 2 shows this relationship at the mean plasma ICa value of all dogs, 1.34 nmol/L.

The relationship between plasma ICa and plasma 25(OH)D concentrations was quadratic. As the dog's plasma ICa value approached 1.32 mmol/L, the plasma 25(OH)D concentration was minimized. Since the effect of plasma ICa on plasma 25(OH)D was independent, this relationship was the same for each group studied. Again, the independent relationship between variables results in a complex graph. Figure 3 shows this relationship at the mean dietary vitamin D intake of all animals, 26 $IU/kg^{0.75}$.

Analysis of plasma 24,25$(OH)_2$D is still underway. An inverse relationship between plasma 24,25$(OH)_2$D concentrations and plasma 25(OH)D concentrations is expected. This relationship may show interaction with the cancer—plasma 25(OH)D and/or the dietary vitamin D intake—plasma 25(OH)D relationships.

2.6: Discussion:

The results of this study support an association between cancer and decreased plasma 25(OH)D concentrations in dogs that is not caused by differences in vitamin D intake. Whether decreased plasma 25(OH)D concentrations are associated with cancer development or are due to cancer-associated changes in metabolism can not be determined by this study design.

Plasma ICa, PTH, and PTHrP concentrations were measured to account for other variables that may affect vitamin D metabolism. Although hypercalcemia is common in

dogs with lymphoma (Withrow and Vail, 2013), no lymphoma patients in this study were hypercalcemic. PTHrP production is a common cause of humoral hypercalcemia of malignancy (Bergman, 2012). Since plasma PTHrP values were within reference range in all patients, the absence of hypercalcemia makes sense.

No association between plasma PTH or PTHrP concentrations and plasma 25(OH)D concentrations were observed. In contrast, Selting et al (2014) reported a significant inverse relationship between serum PTH and serum 25(OH)D in healthy dogs. However, these authors did not account for the same covariates (e.g. vitamin D intake) during statistical analysis, which may have affected the results. Two other studies have found no significant association between serum vitamin D metabolites and serum PTH and/or PTHrP in dogs with lymphoma. However, these comparisons were made using serum $1,25(OH)_2D$ concentrations and not serum 25(OH)D concentrations (Rosol et al, 1992, Gerber et al, 2004).

No effect of age on plasma 25(OH)D was noted. Similarly, Wakshlag et al (2011) reported only a trend towards significance of age on serum 25(OH)D. In humans, increasing age has been associated with decreased serum 25(OH)D status, attributing this to causes such as decreased sun exposure (Perry et al, 1999, Maggio et al, 2004). However, it remains unclear how physiological aging processes (e.g. decreased renal function and decreased efficiency of vitamin D production in skin) affect serum 25(OH)D status (Ross et al, 2011). No association between age and plasma 25(OH)D may have been observed because of limitations in the population age ranges. The majority of dogs

were older (mean ages in Table 1) so any possible associations with lower ages could not be captured.

No association between gender and plasma 25(OH)D status was found. No literature examining gender's effect of plasma 25(OH)D in dogs could be found. The impact of gender on plasma/serum 25(OH)D concentrations in humans varies with the study, although more studies support higher plasma/serum 25(OH)D concentrations in males (Jacques et al, 1997, Dawson-Hughes, 1997, Hagenau et al, 2009, Pazaitou-Panayiotou et al, 2012).

Similar to the results of this study, Wakshlag et al (2011) reported no effect of BCS on serum 25(OH)D concentrations. This is contrary to results from human studies, where obesity is linked to decreased serum 25(OH)D due to vitamin D deposition in adipose tissue (Wortsman et al, 2000). Similar to the age results, the lack of an observable association between BCS and plasma 25(OH)D could be due to a limited population BCS range. The majority of dogs had a BCS of 5 or higher, so possible associations with lower BCS could not be captured.

No association between MCS and plasma 25(OH)D concentrations were noted. To our knowledge, the effects of MCS on plasma 25(OH)D status have not been previously reported for dogs. MCS was only introduced recently as part of the Nutritional Assessment Guidelines for Dogs and Cats released by American Animal Hospital Association and the World Small Animal Veterinary Association (Baldwin et al, 2010,

WSAVA, 2011). MCS is a variable of interest for future studies looking at vitamin D and cancer in dogs. A substantial amount of vitamin D can be stored in muscle tissue (Heaney et al, 2009), and muscle loss may occur in patients with cancer cachexia (Freeman, 2012).

The observed median plasma 25(OH)D concentration for healthy dogs is consistent with a previous report of healthy dogs in the Guelph area (Kukk, 2011). The present study is the first, that the authors are aware of, to measure plasma 25(OH)D concentrations in dogs with osteosarcoma. The observed median plasma 25(OH)D concentrations for lymphoma patients is also consistent with a previous report (Gerber et al, 2004), although the dogs in that study were also hypercalcemic. The difference in plasma 25(OH)D concentrations for the mast cell tumour group was not considered significant, but may be explained by sample size limitations. The observed median plasma 25(OH)D levels for the mast cell tumour group and the healthy group are very similar to those reported by Wakshlag et al (2011), and the standard deviations are lower than those described by Wakshlag et al (2011). Unfortunately, the sample size for this study is smaller than that achieved by Wakshlag et al (2011).

The present study is the first to correlate various levels of dietary vitamin D intake with plasma 25(OH)D response in dogs. A non-linear relationship was observed between vitamin D intake and plasma 25(OH)D status, where increases in plasma 25(OH)D concentrations are quite dramatic at low levels of vitamin D intake, then begin to plateau at higher levels (Figure 2). These findings are supported by a study suggesting that Great Danes have the ability to up-regulate the expression of 24-hydroxylase at higher levels of

vitamin D intake (Tryfonidou, 2002). If this is true, then completion of 24,25$(OH)_2$D analysis will reveal an inverse relationship between plasma 24,25$(OH)_2$D and plasma 25(OH)D. This relationship will interact with the dietary vitamin D intake –plasma 25(OH)D relationship.

The relationship between dietary vitamin D intake and plasma 25(OH)D response has been investigated in other species. A non-linear relationship between dietary vitamin D intake and plasma/serum 25(OH)D status is also reported in humans (Ross et al, 2011). Similar work has been done in cats (Morris, 1999) and revealed a linear relationship. However, levels of dietary vitamin D intake used in that study were quite low, so a full comparison is hard to make.

Vitamin D content of the diets was analyzed in the present study to ensure vitamin D intake calculations were as accurate as possible. However, these results are limited by a lack of information on the bioavailability of vitamin D in each diet. Still, the observation of a relationship between dietary vitamin D intake and plasma 25(OH)D status should be a key finding for researchers interested in designing studies in this field.

Plasma ICa concentrations tightly regulate 1,25$(OH)_2$D production, so it makes biological sense that there was an observed independent effect of plasma ICa on plasma 25(OH)D concentration. Selting et al (2014) did not measure plasma ICa concentrations, but reported no relationship between serum total calcium and serum 25(OH)D concentrations. Rosol et al (1992) and Gerber et al (2004) reported no relationship

between serum total calcium and serum 1,25$(OH)_2$D concentrations in dogs with lymphoma and healthy dogs. The relationship observed in the current study was quadratic and does not fit with what would be intuitively expected. A hypocalcemic dog is expected to have hypovitaminosis D, and conversely a hypercalcemic dog is expected to have hypervitaminosis D (NRC, 2006). Most animals fell within the narrow laboratory reference range for plasma ICa (1.25 mmol/L -1.45 mmol/L). While this relationship may be influenced by 5 dogs that fell slightly outside of the reference range, there was no reason to exclude these dogs from the dataset. However, the actual strength of this relationship should be considered with caution. Further research with a larger sample size is warranted.

Finally, cancer had an independent effect on plasma 25(OH)D status, suggesting that a cancer-associated mechanism is responsible for the differences in plasma 25(OH)D concentrations seen between healthy dogs and those with osteosarcoma and lymphoma. Since this was a cross-sectional study, there is no way to determine whether this association was present pre- or post- cancer development. If present pre-cancer development, then genetic variation in the 24-hydroxylase gene may have predisposed certain dogs to decreased plasma 25(OH)D values, and increased risk of cancer for these animals. If the decreased plasma 25(OH)D status occurred post-cancer development, then it's possible that increased inflammation altered the expression of hepatic enzymes, i.e. 24-hydroxylase, resulting in decreased plasma 25(OH)D concentrations. Analyses of other vitamin D metabolites (analysis of plasma 24,25$(OH)_2$D is currently underway) will help gain a clearer picture of the mechanisms responsible for this association. If

$24,25(OH)_2D$ is involved, results are expected to reveal a significant inverse relationship between $24,25(OH)_2D$ and plasma 25(OH)D that interacts with the cancer—plasma 25(OH)D relationship. A prospective cohort study is warranted to determine when the changes in plasma 25(OH)D status occur.

An in depth investigation into the relationship between dietary vitamin D intake and plasma 25(OH)D status is necessary, especially as plasma 25(OH)D continues to be linked with various health outcomes in dogs. The possibility of higher levels of vitamin D supplementation overcoming the observed plateau in the intake-25(OH)D relationship should be investigated, especially if vitamin D is shown to have a protective effect. It should be noted that dietary vitamin D intake is associated with plasma 25(OH)D status, so failure to account for this effect in future studies will result in fundamentally flawed conclusions.

2.7: Disclosure

Julie Bayle is a paid employee of Royal Canin.

2.8: References

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Table 1. Mean ± SD characteristics of dogs enrolled in a study to investigate dietary vitamin D intake and vitamin D status in dogs with cancer.

	Age (years)	Weight (kg)	BCS (1-9)	MCS (0-3)
Healthy	7.6 ± 2.3	32.3 ± 10.0	5.8 ± 1.0	2.5 ± 0.7
Osteosarcoma	8.6 ± 2.4	38.2 ± 13.9	5.72 ± 1.1	2.3 ± 0.7
Lymphoma	7.5 ± 2.2	30.4 ± 12.4	6.0 ± 1.2	2.6 ± 0.7
Mast cell tumour	6.9 ± 2.2	30.9 ± 10.9	6.0 ± 1.0	2.7 ± 0.6

There were no significant differences between groups for these parameters.

Healthy n=20, osteosarcoma n=18, lymphoma n=22, mast cell tumour n=21

BCS = Body condition score, MCS = Muscle condition score

Table 2.2. Mean ± SD plasma ICa, PTH and PTHrP concentrations for dogs enrolled in a study to investigate dietary vitamin D intake and vitamin D status in dogs with cancer.

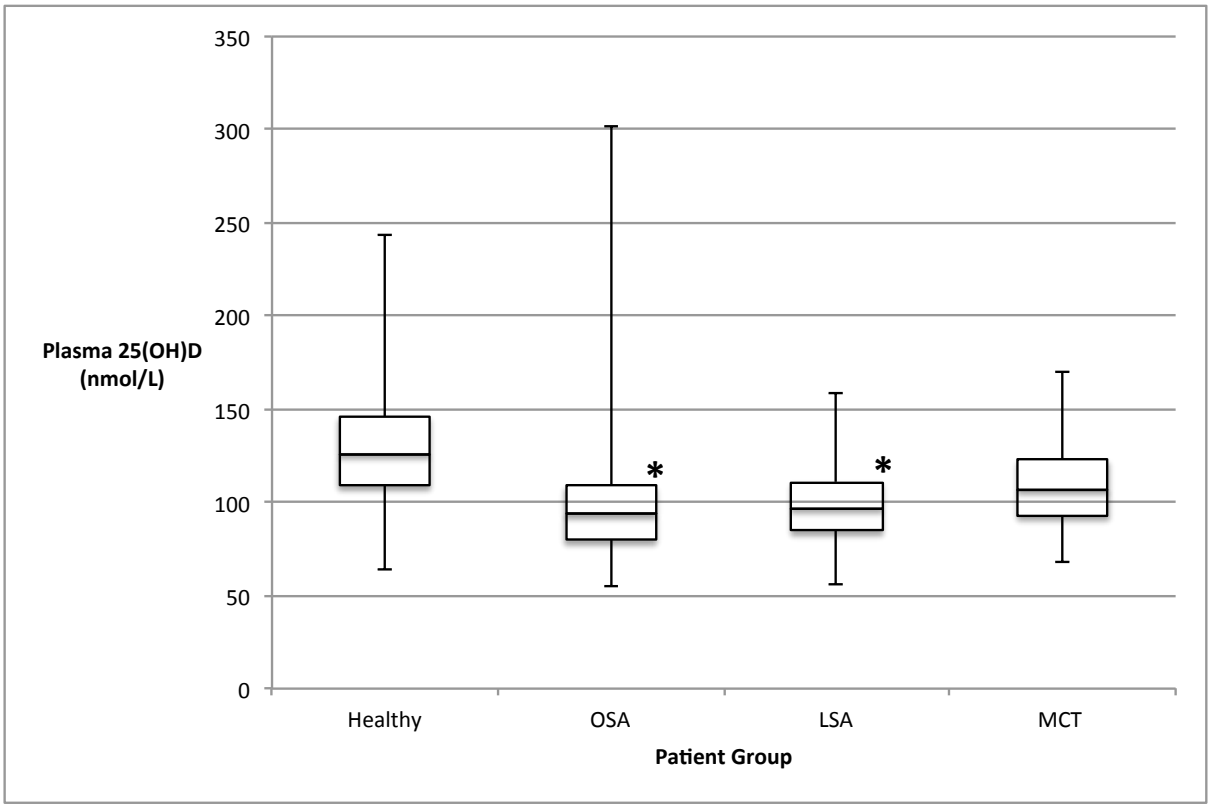
	ICa (mmol/L)	PTH (pmol/L)	PTHrP (pmol/L)
Reference Range	1.25 - 1.45	0.5 - 5.8	<1.0
Healthy	1.33 ± 0.05	1.2 ± 0.9	<1.0
Osteosarcoma	1.35 ± 0.06	1.26 ± 0.6	<1.0
Lymphoma	1.33 ± 0.03	1.45 ± 1.5	<1.0
Mast cell tumour	1.33 ± 0.05	1.5 ± 1.3	<1.0

There were no significant differences between groups for these parameters.

Healthy n=20, osteosarcoma n=18, lymphoma n=22, mast cell tumour n=21

ICa = Ionized calcium, PTH = Parathyroid hormone, PTHrP = parathyroid hormone-related protein

Figure 2.1. Box plot representing plasma 25(OH)D concentrations (nmol/L) of dogs enrolled in a study to investigate dietary vitamin D intake and vitamin D status in dogs with cancer.

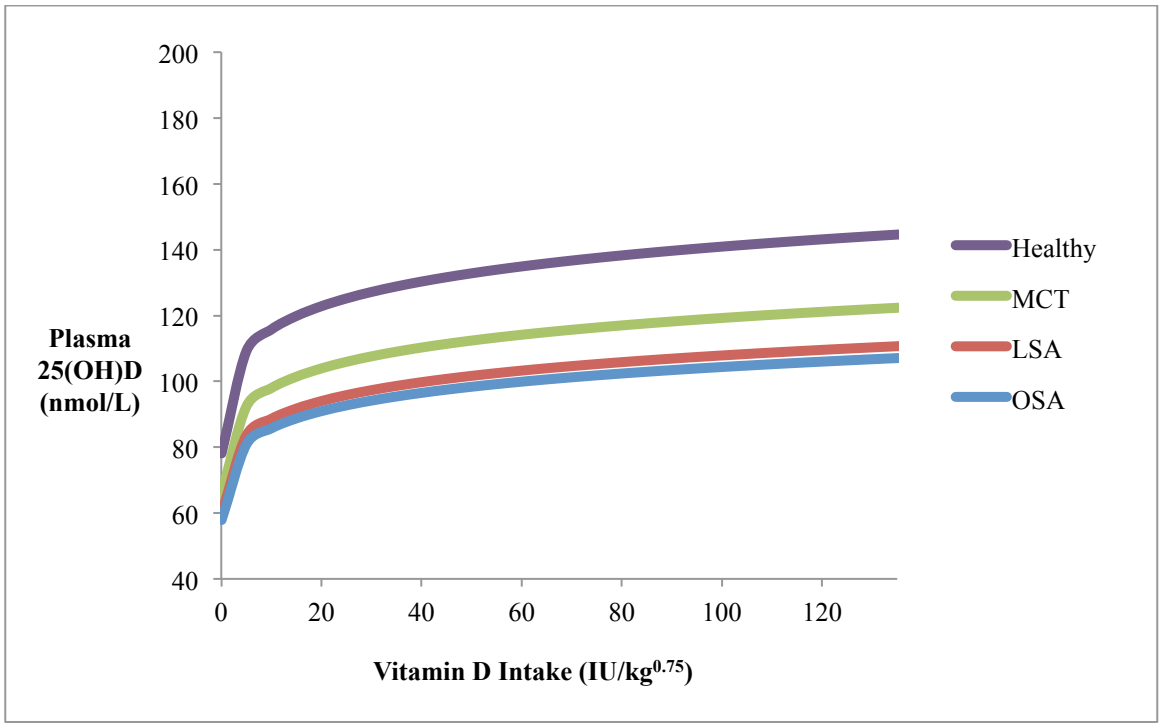


* indicates a significant difference ($p < 0.05$) when compared to healthy dogs

Healthy n=20, osteosarcoma n=18, lymphoma n=22, mast cell tumour n=21

OSA = osteosarcoma, LSA = lymphoma, MCT = mast cell tumour, 25(OH)D = 25-hydroxyvitamin D

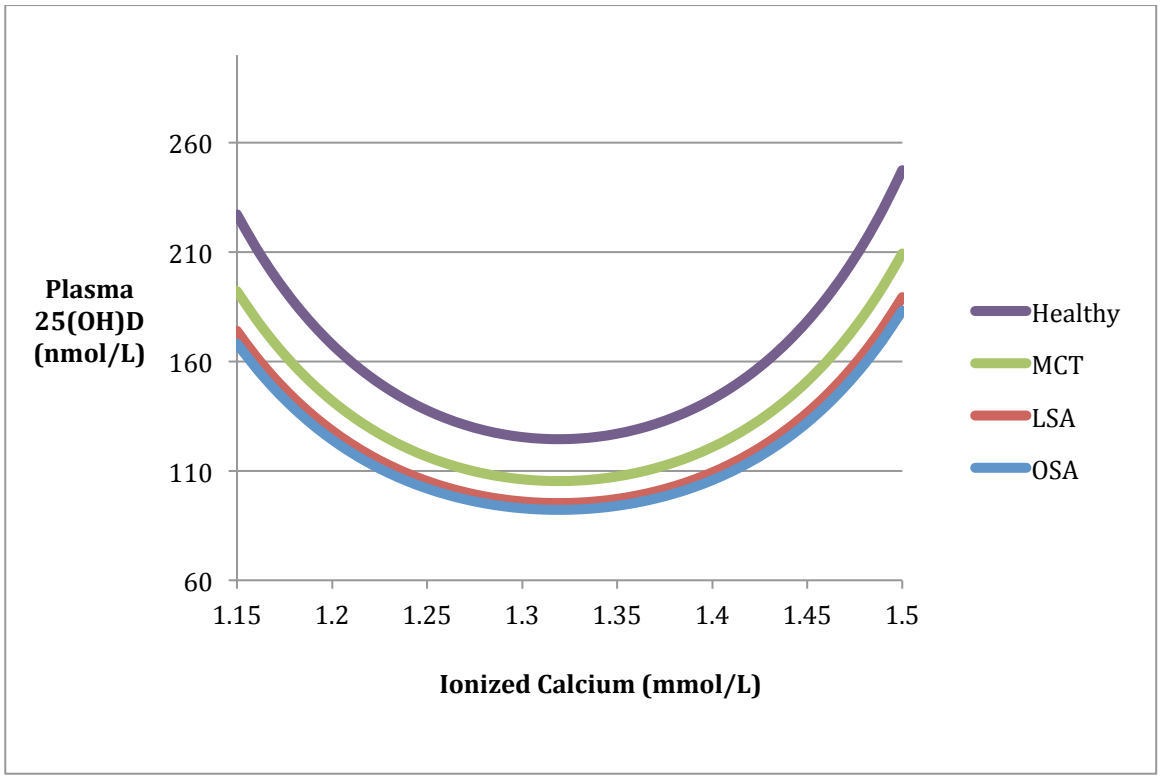
Figure 2.2. Relationship between vitamin D intake (IU/kg$^{0.75}$) and plasma 25(OH)D (nmol/L) concentrations at a plasma ICa concentration of 1.34 mmol/L in dogs enrolled in a study to investigate dietary vitamin D intake and vitamin D status in dogs with cancer.



Healthy n=20, osteosarcoma n=18, lymphoma n=22, mast cell tumour n=21

OSA = osteosarcoma, LSA = lymphoma, MCT = mast cell tumour , 25(OH)D = 25-hydroxyvitamin D, ICa = ionized calcium, IU = International Units

Figure 2.3. Relationship between plasma ICa (mmol/L) and plasma 25(OH)D concentrations (nmol/L) at a dietary vitamin D intake of 26 IU/kg$^{0.75}$ in dogs enrolled in a study to investigate dietary vitamin D intake and vitamin D status in dogs with cancer.



OSA = osteosarcoma, LSA = lymphoma, MCT = mast cell tumour

Healthy n=20, osteosarcoma n=18, lymphoma n=22, mast cell tumour n=21, 25(OH)D = 25-hydroxyvitamin D, ICa = ionized calcium, IU = International Units

Chapter 3

Conclusion

Conclusion

The results of this work have supported the need for research into the role of nutrition in canine cancer development and/or progression, specifically focusing on the role of vitamin D. The conclusions drawn from this work were highlighted in the discussion of the previous chapter. Briefly and most importantly, both dietary vitamin D intake and cancer had independent effects on plasma 25(OH)D concentrations in canines. There was a positive non-linear relationship between dietary vitamin D intake and plasma 25(OH)D concentrations in all canines. Canines with cancer had reduced plasma 25(OH)D concentrations compared to healthy canines. Further research is necessary before the full clinical significance of these findings can be known.

Since a significant, independent effect of dietary vitamin D intake on plasma 25(OH)D concentrations was observed in this study, future studies investigating 25(OH)D concentrations should also incorporate measurement of dietary vitamin D intake. In past studies, researchers concluded lowered serum/plasma 25(OH)D concentrations were associated with a disease state, without measurement of dietary vitamin D intake (Gerber et al, 2004, Galler et al, 2012, Selting et al, 2014). These associations may have been explained by differences in dietary vitamin D intake between groups.

This study is the first, that these researchers were aware of, to examine a broad range of dietary vitamin D intakes and plasma 25(OH)D response in canines. A positive,

non-linear relationship was observed, which is consistent with the relationship reported in humans (Ross et al, 2011). However, sample size limitations restricted the ability to determine effects of other variables (i.e. age, BCS, MCS) on this relationship. In humans, the report on dietary reference intakes for vitamin D concluded that further research on the vitamin D intake—25(OH)D concentration relationship was needed, especially research focused on potential covariates (Ross et al, 2011). The same research is needed in canines. A full understanding of this relationship is essential for development of accurate vitamin D requirements and clinical application of research focused on 25(OH)D concentrations and canine health.

Dogs with osteosarcoma and lymphoma had significantly lower plasma 25(OH)D concentrations than healthy dogs in this study. The difference between dogs with mast cell tumours and healthy dogs was not statistically significant, which may be due to sample size limitations. This was a cross sectional study, so whether lower plasma 25(OH)D concentrations were present pre- or post- cancer development is not known. Several study designs are necessary to fully understand vitamin D's role in canine cancer development and/or progression, and to determine how vitamin D may be used for cancer prevention and/or as a treatment modality. Prospective cohort study designs will allow investigators to determine whether decreased plasma 25(OH)D concentrations occur pre- or post-cancer development. A prospective cohort study investigating associations between vitamin D and treatment response and/or survival in dogs with cancer enrolled in this study is currently underway. Ultimately, a blinded, randomized clinical trial will be

necessary to determine if vitamin D plays a role in cancer development and/or progression in canine cancer patients.

The mechanisms responsible for the observed associations should be explored. Measurement of other vitamin D metabolites ($24,25(OH)_2D$, $1,25(OH)_2D$) in dogs with cancer and healthy dogs will provide a clearer picture of vitamin D metabolism and identify pathways of interest. Measurement of $24,25(OH)_2D$ for dogs enrolled in the current study is underway. If pathway alterations (e.g. up-regulation of 24-hydroxylase) are responsible for observed changes, then these pathways may be targeted in studies exploring therapeutic intervention. Additionally, metabolites and pathways of interest can be manipulated *in vitro* to determine the molecular mechanisms responsible for vitamin D—cancer links. These studies should be followed up with *in vivo* work to ensure *in vitro* results are reproducible in the animal.

This research project was developed to address the gap in literature examining links between nutrition and cancer development and progression in dogs. Further research in this area is key for developing novel strategies for cancer prevention and treatment in dogs. Whole foods, macronutrients and micronutrients have been linked to cancer development and progression in humans (American Institute for Cancer Research, 2007). These links may serve as a helpful guide for researchers interested in designing similar studies in dogs. Researchers should ensure that research efforts are interdisciplinary, combining the expertise of both oncologists and nutritionists, for results of utmost clinical relevance and value.

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