Nutritional Management of Canine Cancers and the Role of Nutritional Supplements

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A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Clinical Studies

Guelph, Ontario, Canada

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ABSTRACT

NUTRITIONAL MANAGEMENT OF CANINE CANCERS AND THE ROLE OF NUTRITIONAL SUPPLEMENTS

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This thesis is an investigation of the dietary decisions made for pets by owners of dogs with cancer and owners of healthy dogs. In addition, an in vitro investigation of the effects of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on canine osteosarcoma cells both as a single agent and in combination with doxorubicin was conducted. Owners of dogs with cancer were found to make different dietary decisions for their pets compared to owners of healthy dogs, especially with regards to diet type and use of nutritional supplements. DHA and EPA had antagonistic effects on doxorubicin and had variable effects as a single agent treatment. Further research is needed to determine the safety and efficacy of nutritional supplements. Greater emphasis is needed in veterinary practices on the importance of client and clinician communication to ensure dogs with cancer are provided a safe and healthy diet.
ACKNOWLEDGEMENTS

This thesis could not have been possible without the support and assistance of many people. Firstly, the deepest of thanks to my advisor, Dr. Adronie Vergbrugghe. This project would not have been possible without her never-ending support and guidance. I am grateful that she offered me the opportunity to participate in this project, an experience which has undoubtedly made me a stronger person by challenging me in all the right ways. Throughout each of these challenges, I always knew that Adronie was just an email away if I ever needed advice or guidance in finding a solution, even while on maternity leave. I couldn’t have asked for a more passionate or dedicated advisor and for that I extend my deepest gratitude.

I would also like to acknowledge the rest of my advisory committee, Tony Mutsaers, Sarah Abood, Byram Bridle and Paul Spagunolo. Each of you had a wealth of knowledge to offer me during our meetings and your questions and comments throughout my progress constantly pushed me to think deeper and work harder. I appreciate the time all of you took to provide assistance and guidance when I needed it, and for all your kind words and support throughout my course of study. In particular, I would like to extend extra special thanks to Tony Mutsaers my co-advisor for providing assistance and expertise throughout my time in the lab, and to Sarah Abood for always having a can-do attitude and helping me to get things done while conducting my survey. Neither project could have been completed without you.

Next, I would like to thank Nada Halfdez, for support and assistance in conducting statistical analysis of my survey data; Arata and Andrew from the Mutsaers lab, for teaching me everything I needed to know about working with cell culture; David Ma and Lynn for instructions and advice in working with the fatty acids; Kaya, Vicky as well as the Mona Campbell Centre for Animal Cancer and PHC staff members for their assistance in recruiting participants for my survey. Lastly, a big thank you to everyone from the Nutrition Lab group for your support and encouragement – in particular thank you to Nicole Weidner from whom I "inherited" this project. From passing along resources, to venting about those dang beta-glucans, I knew I could always count on your experience for guidance, and I surely wouldn’t be here writing this without you.

Finally, an acknowledgement to everyone who supported me from outside of the academia world; to Mom and Dad, Alessandra and Angelina, thank you for believing in me, supporting me, and for your compassionate understanding throughout my schooling. You guys have always been the best cheerleaders and the best support system I could have asked for, so thank you, from the bottom of my heart. And to everyone else (I think you know who you are) this one’s for you. Thank you for keeping me sane and for anchoring me to the real world when I’ve gotten lost in the endless PubMed time warp, your support and encouragement was essential in getting me here.
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ABBREVIATIONS

AAFCO ......................................................... Association of American Feed Control Officials
AAHA ............................................................. American Animal Hospital Association
ALA .................................................................. α-linolenic acid
ATP ................................................................. Adenosine Triphosphate
BAK ................................................................. Bcl-2 Homologous Antagonist Killer
BAX ................................................................. Bcl-2-associated X Protein
BCL-2 .............................................................. B-cell Lymphoma 2
BCS ................................................................. Body Condition Score
BID ............................................................... BH3-interacting Domain Death Agonist
CBD .............................................................. Cannabidiol
CI ................................................................. Combination Index
COX-2 .......................................................... Cyclo-oxygenase–2
CVS ............................................................... Crystal Violet Staining
DHA ............................................................. Docosahexaenoic Acid
DPA ............................................................... Docosapentaenoic Acid
EPA ............................................................... Eicosapentaenoic Acid
FBS ............................................................... Fetal Bovine Serum
FEDIAF ........................................................ European Pet Food Industry Federation
IC50 .............................................................. Half-maximal Inhibitory Concentration
IGF-1 .............................................................. Insulin-like Growth Factor 1
IQR ............................................................... Interquartile Range
LA .............................................................. Linoleic Acid
LSA .............................................................. Lymphoma
MCS.................................................................Muscle Condition Score
MER ...............................................................Maintenance Energy Requirement
MOMP.............................................................Mitochondrial Outer Membrane Permeability
N-3 FA ..............................................................Omega-3 Fatty Acid
OSA......................................................................Osteosarcoma
PIGF...................................................................Placenta Growth Factor
PUFA .................................................................Polyunsaturated Fatty Acid
RER.....................................................................Resting Energy Requirement
THC....................................................................Tetrahydrocannabinol
TNF ......................................................................Tumor Necrosis Factor
VEGF..................................................................Vascular Endothelial Growth Factor
WSAVA...............................................................World Small Animal Veterinary Association
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CHAPTER 1:
LITERATURE REVIEW
1 Nutrition support for the canine cancer patient

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1.1 Introduction

In dogs, cancers have become prevalent diseases and account for approximately 27% of mortalities (Adams, Evans, Sampson, & Wood, 2010). Nutritional management in canine cancer patients may have important implications with respect to disease progression, alleviation of treatment-related side effects, quality of life, and survival time (Mauldin, 2007, 2012). Cancers and cancer therapy can cause changes in metabolism of carbohydrates, proteins, and fats (K. C. Fearon & Moses, 2002; Mazzaferro et al., 2001). Loss of appetite is a common clinical sign in dogs with cancers (Sogame, Risbon, & Burgess, 2018). In addition, cancers can cause altered immune function, which may also be influenced by chemotherapy, radiation therapy, and surgery. Pet owners are responsible for nutritional decisions; therefore, owners’ attitudes, food preferences, and feeding practices should be considered when designing nutrition support plans and assessing dietary supplement use following diagnosis of a cancer in their pet. The purpose of this review is to provide practical information to veterinary health care teams to guide nutritional assessment, ensure nutritional adequacy and assist early individualized nutrition support in canine cancer patients as well as to help client communication. This review presents a pro-and-con view on a variety of diet types and critiques the body of literature and level of evidence surrounding popular dietary supplements.

1.2 Nutritional assessment

Providing nutritional support to a canine patient with cancer includes evaluation of the patient’s individual needs. Nutritional assessment guidelines have been published
by the American Animal Hospital Association (AAHA) and the World Small Animal Veterinary Association (WSAVA) (Baldwin, Freeman, Grabow, & Legred, 2010; L. Freeman, Becvarova, Cave, MacKay, et al., 2011). A first step involves evaluating the dog, the diet, and the feeding environment and management. Next, an extended evaluation is warranted based on patient, diet and cancer-related risk factors (Table 1.1) (Baldwin et al., 2010; L. Freeman, Becvarova, Cave, MacKay, et al., 2011; Parr & Remillard, 2014). Obtaining a complete diet history is an essential component of this assessment. The information collected should be sufficient to determine if adequate amounts of all dietary essential nutrients are provided (Baldwin et al., 2010; Parr & Remillard, 2014). At each visit, body weight, Body Condition Score (BCS), Muscle Condition Score (MCS) and fecal score should be recorded to establish a baseline and track trends following implementation of a nutrition support plan and during cancer treatment (J. Wakshlag & Kallfelz, 2006). This is important to determine if alterations in energy intake, key nutrients, diet characteristics, and/or feeding management are needed. In general, a diet change is not needed if a dog receives a cancer diagnosis and no other nutritional risk factors are present (e.g. has normal BCS and is fed a complete and balanced diet).

1.3 Nutritional status and body composition

1.3.1 Weight loss

In species other than dogs, cancers pose various metabolic complications that may be associated with the development of cachexia (K. C. H. Fearon, Glass, & Guttridge, 2012). In people, cancer cachexia is described as “a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot
be fully reversed by conventional nutritional support and leads to progressive functional impairment”(K. Fearon et al., 2011). This catabolism occurs due to changes in appetite and alterations in glucose, fat, and protein metabolism(K. C. Fearon & Moses, 2002). In dogs, there is no consensus regarding the definition and clinical relevance of cancer cachexia.

To the authors’ knowledge, studies in dogs characterizing cancer cachexia don’t exist, although a few studies investigated weight loss in dogs with cancers. One study in dogs with a variety of cancers demonstrated weight loss following cancer diagnosis in 68% of dogs with 31% losing up to 5%, 14% losing between 5-10% and 23% losing over 10% of body weight(Michel, Sorenmo, & Shofer, 2004). Loss of lean body mass and fat mass, and the time during which weight loss occurred were not reported. It is also unknown if the observed weight loss was due to cancer treatment, disease progression, or a combination of both. The same limitations exist for another study that found weight loss in 54.9% of dogs with lymphoma (LSA) and 52.3% of dogs with osteosarcoma (OSA) between diagnosis and death(Romano, Heinze, Barber, Mason, & Freeman, 2016). The percentage of underweight (≤4 out of 9) dogs with LSA doubled from diagnosis to final visit(Romano et al., 2016). Although the median survival time was shorter in underweight dogs compared to dogs with an ideal or overweight BCS(Romano et al., 2016), the documented weight loss could not be classified as cancer cachexia. Weight loss was also reported in 43% of dogs with intestinal cancers(Sogame et al., 2018).
1.3.2 Obesity

Obesity (BCS ≥ 8 out of 9) and overweightness (BCS of 6-7 out of 9) have been proposed to impact the onset of cancers in dogs (H.-Y. Lim et al., 2015). Overweight and obese female dogs are at greater risk of developing mammary cancer, especially at a younger age (H.-Y. Lim et al., 2015; H. Y. Lim et al., 2015; Rajagopaul et al., 2016). This could be due to increased aromatase expression affecting hormone receptor signaling, and thereby impacting mammary carcinoma development (H.-Y. Lim et al., 2015). In addition, lymphatic invasion in mammary tumors is increased in obese and overweight dogs, therefore, there is a higher risk of metastasis (H. Y. Lim et al., 2015).

Conversely, 10% or more body weight gain in dogs with LSA or OSA during cancer treatment increased survival time compared to dogs that gained less than 10%, maintained or lost weight (Romano et al., 2016). From the first recorded BCS to the last, the proportion of dogs categorized as overweight increased by 6% (Romano et al., 2016). Still, survival time did not differ between dogs that had an ideal BCS and dogs that were overconditioned (Romano et al., 2016). Unfortunately, this study only included a small number of obese dogs, which limited the researchers’ ability to determine if there was a difference in dogs that were overweight versus obese.

1.4 Nutritional management

1.4.1 Diet type and dietary supplement use

1.4.1.1 Conventional diets

Conventional extruded and wet diets were the primary diet for 71% of canine cancer patients presented to a tertiary referral oncology service (Rajagopaul et al., 2016). Most
of these conventional diets are considered to meet or exceed the minimum nutrient recommendations proposed by the Association of American Feed Control Officials (AAFCO) (AAFCO, 2019) in North America and/or the nutrient guidelines set by the European Pet Food Industry Federation (FEDIAF) in Europe (Ahlstrøm et al., 1984). Therefore, owners who choose to feed a conventional extruded or wet diet should be providing these foods so they make up at least 90% of the dog’s daily caloric intake (L. Freeman, Becvarova, Cave, Zealand, et al., 2011). Although these diets are nutritionally balanced for healthy adult dogs, nutritional challenges are still possible for some cancer patients. Cancers mostly affect aging dogs and although differences in digestion, metabolism, exercise, BCS, MCS, energy requirements, etc. may occur between adult and senior dogs (Swanson, Kuzmuk, Schook, & Fahey, 2004), nutrient profiles particularly for senior dogs do not exist (AAFCO, 2019).

Some owners are distrustful of conventional pet food (Bischoff & Rumbeiha, 2012) due to reports of contamination and/or recalls (Remillard, 2008). A study found 51% of owners of canine cancer patients exhibited a distrust in conventional diets, 59% believe processed conventional pet foods are unhealthy and could contribute to cancer, and 63% instituted a diet change within six months of their dog’s cancer diagnosis, excluding a conventional dietary component (Rajagopaul et al., 2016). These feelings of distrust should be addressed with owners and accounted for when tailoring individual diet recommendations.

### 1.4.1.2 Cooked homemade diets

Pet owners may select homemade diets due to concerns over synthetic additives
and preservatives, difficulty understanding commercial diet labels, misinformation regarding conventional diets, and/or the absence of a commercial product addressing their pet’s medical conditions (Remillard, 2008). In fact 60% of owners stated they believe a homemade diet provides the best nutrition for their pet following a cancer diagnosis (Rajagopaul et al., 2016). The benefits of homemade diets include the ability to control the ingredients and accommodate a combination of medical conditions (Remillard, 2008). This individualized approach to diet allows adjustments for both cancer-associated metabolic changes and concurrent diseases, and often provides increased palatability for patients with reduced appetites. Although this approach may seem ideal, homemade diets for medical conditions require specialized knowledge and expertise to assess and/or formulate the recipe according to patient needs and owner preferences. Without this expertise and ongoing monitoring, homemade diets often provide inadequate nutrient profiles. This and a lack of adherence to or deviations from the recipe, have been associated with nutritional deficiencies in pets (Remillard, 2008; Streiff et al., 2002). Previous analyses of homemade diet recipes demonstrated that the majority are deficient in one or more essential nutrients (Heinze, Gomez, & Freeman, 2012; Stockman, Fascetti, Kass, & Larsen, 2013; Streiff et al., 2002). Moreover, 82% of owners of dogs with cancer feeding homemade diets failed to consult a veterinarian for nutritional advice (Rajagopaul et al., 2016). Information regarding evaluation of homemade diet recipes can be found elsewhere (Parr & Remillard, 2014).

1.4.1.3 Raw-meat based diets

Raw-meat based diets are characterized by ingredients derived from animal tissue that did not undergo heat processing. One study noted that 4% of owners of canine
cancer patients presented to a tertiary referral oncology service fed raw-meat based dietary components and 29% fed raw-meat based treats (e.g. uncooked animal proteins, commercial freeze-dried products, rawhide chews) (Rajagopaul et al., 2016). Similar to homemade cooked diets, raw-meat based diets are often nutritionally inadequate (Dillitzer, Becker, & Kienzle, 2011; L M Freeman & Michel, 2001). Another concern involves bacterial contamination. *E. Coli* was present in nearly 60% of commercial raw-meat based diets (Strohmeyer et al., 2006), *Salmonella* in 21-48% of commercial raw-meat based diets (Finley et al., 2008; Strohmeyer et al., 2006; Weese, Rousseau, & Arroyo, 2005), and *Listeria* in 30% of commercial raw food products and jerky treats (Nemser et al., 2014). Bully sticks have also been found to be contaminated with *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus*, and tetracycline resistant *E. coli* (Lisa M Freeman, Janecko, & Scott Weese, 2013). These pathological concerns pose a risk to canine cancer patients due to cancer and treatment-induced immunosuppression. Similar to recommendations for humans with cancer, it is critical to prevent food-borne infection in dogs by “separating raw meats from other foods, cooking to the right temperature, and refrigerating food appropriately” (Wolfe, Sadeghi, Agrawal, Johnson, & Gupta, 2018). Thus, due to concerns of bacterial contamination and nutritional inadequacy associated with some raw meat-based diets, the authors do not recommend dogs with cancer be fed a raw-meat based diet. These concerns need to be communicated to owners of dogs with cancer.

### 1.4.1.4 Dietary supplements

Around 10% of owners administer supplements to healthy dogs (Lisa M Freeman et al., 2006), while supplement use was reported by 35-40% of owners of canine cancer
Glucosamine was provided most commonly, followed by n-3 PUFA (Rajagopaul et al., 2016). Supplements provided to dogs with cancer may differ from healthy dogs, as one study found that 48% of owners who gave supplements indicated that this was associated with their dog’s cancer diagnosis (Rajagopaul et al., 2016), and another found some significant differences in the types of supplements fed to healthy dogs and dogs with cancer (Bianco et al., 2019). Owners choose to feed their dogs dietary supplements following a cancer diagnosis for various reasons: improving overall well-being, improving immune functionality, symptomatic treatment, and ensuring nutritional adequacy (Lana et al., 2006). The actual effects of dietary supplements may be beneficial, non-existent, or even harmful under various circumstances. Further research regarding cancer-associated efficacy and possible side-effects is required. Dietary supplements should be avoided unless there is clear evidence-based research in veterinary medicine that suggests a benefit to canine cancer patients. Until further research is conducted, the focus should be on a complete and balanced diet (L. Freeman, Becvarova, Cave, Zealand, et al., 2011). The current body of research of dietary supplements conducted under a canine cancer context and the level of evidence for each of these studies is summarized in Table 1.2.

1.5 Key dietary factors for the canine cancer patient

1.5.1 Energy requirement & intake

It is important to compare current caloric intake to calculated energy requirements in all canine cancer patients, no matter if they have an ideal BCS, are underconditioned or overconditioned (Baldwin et al., 2010; Brooks et al., 2014; L. Freeman, Becvarova,
Cave, MacKay, et al., 2011). The equations used to estimate the Maintenance Energy Requirement (MER) of adult dogs are shown in Table 1.3 (Saker & Selting, 2010); however, additional research is needed to determine if and when cancers change individuals' energy requirements (Argilés, Moore-Carrasco, Busquets, & López-Soriano, 2003). These equations provide an initial estimate of energy requirements that can be altered based on current caloric intake, monitoring and reassessment (Baldwin et al., 2010; L. Freeman, Becvarova, Cave, MacKay, et al., 2011). Ideally patients should maintain body weight, BCS, and MCS during cancer treatment. Overweightness and obesity lead to health consequences such as osteoarthritis, hyperlipidemia, immunosuppression, and shorter lifespan (German, 2006). To prevent further weight gain in overweight and obese dogs, calculating MER using the equation for inactive or obese-prone adult dogs (Table 1.3) is a good starting point during cancer treatment (Saker & Selting, 2010). A weight loss plan with further energy restriction (Table 1.3) could be considered to slowly achieve an ideal BCS after successful cancer treatment and evaluation of the pet’s long-term prognosis, or during treatment if obesity is causing other issues affecting quality of life (Mauldin, 2012). Details on designing weight loss plans have been published elsewhere (Brooks et al., 2014).

The prevalence of anorexia among canine cancer patients is unknown. Yet, anorexia was noted as one of the most common clinical signs in dogs with intestinal cancer (Sogame et al., 2018). Inappetance and anorexia can lead to rapid weight loss and underweightness due to a negative energy balance (Saker & Selting, 2010). Weighing below an ideal should also be avoided (K. C. Fearon & Moses, 2002; Mauldin, 2007) as it causes complications such as anemia, protein malnutrition, and other
nutrient deficiencies (Pointer, Reisman, Windham, & Murray, 2013). An underweight BCS suggests a need for nutritional support (Baldwin et al., 2010; L. Freeman, Becvarova, Cave, MacKay, et al., 2011). Patients managed at home should eat enough to meet their estimated MER, while in hospital their estimated Resting Energy Requirement (RER) should be met accounting for reduced activity in a controlled environment (Table 1.3) (Saker & Selting, 2010). A gradual increase, starting with 1/3 RER, then 2/3 RER and finally RER on day three, is recommended for hospitalized anorexic patients (Delaney, 2006). Monitoring and reassessment are critical. Small, frequent meals and a low stress environment are good starting points to encourage voluntary food intake. Gently warming canned food or adding broth can help increase aromas. High protein and high fat foods increase palatability. Handfeeding can be used, but force feeding is strongly discouraged (Delaney, 2006). When dogs have not eaten for 48 hours, or if food intake is lower than RER for 3-5 days, assisted enteral feeding should be considered (Delaney, 2006). Tube feeding is preferred over parenteral feeding as it is less expensive, requires less technical expertise, has fewer complications, and preserves intestinal mucosal architecture, gut permeability and immune function (Saker & Selting, 2010). Pharmacological appetite stimulation is controversial as results can be variable (Delaney, 2006). Mirtazapine is commonly prescribed despite there being little pharmacokinetic information in dogs and cats (Dowling, 2019; Giorgi & Yun, 2012). Alternatively, an appetite stimulant such as capromorelin can be used to increase appetite and food intake by increasing ghrelin levels, an orexigenic hormone, and may improve muscle mass as a result of increased growth hormone and Insulin-like growth factor 1 (IGF-1) production (Rhodes, Zollers, Wofford, & Heinen, 2018). Further
information on these and other drugs has been published elsewhere (Delaney, 2006; Giorgi & Yun, 2012; Rhodes et al., 2018).

1.5.2 Energy sources

Pet foods contain energy as proteins, fats and carbohydrates. Dogs have a dietary requirement for protein and individual essential amino acids, as well as for fat and essential fatty acids, however, they do not require carbohydrates in their diet (Nutrient requirements of dogs and cats, 2006).

Higher protein diets may be supportive for management of canine cancer, especially when weight loss occurs. Moreover, dogs also show a dietary preference for protein, with higher protein diets boosting food intake when appetite is reduced (Delaney, 2006; Hall, Vondran, Vanchina, & Jewell, 2018; Mauldin, 2007). However, protein should be more conservative if co-morbidities that require protein restriction, such as renal disease or liver disease, are present.

Dietary fat is also an important energy source for cancer patients as fat primarily determines the caloric density of the food and is highly palatable (Saker & Selting, 2010). This is especially relevant for inappetant patients, which enables a higher caloric intake while eating a limited amount of food. Conversely, high-fat diets are contraindicated in fat-sensitive diseases such as obesity, pancreatitis, primary hyperlipidemia, and lymphangiectasis.

Diets reduced in digestible carbohydrates (i.e. simple sugars and starches) are commonly suggested for dogs with cancer. The concept that cancer cells preferentially
metabolize glucose and are highly dependent on anaerobic glycolysis, a process referred to as the Warburg effect (Warburg, 1956), is now well-known among the scientific community (Ogilvie, 1998). Glycolysis is upregulated by cancer cells, which, causes excessive production of pyruvate that is rapidly converted into lactate and subsequently converted into glucose through the Cori cycle (J. Wakshlag & Kallfelz, 2006). This cycle explains why dogs with lymphoma showed higher blood insulin and lactate concentrations compared to healthy dogs (McQuown, Burgess, & Heinze, 2018). Rodent and human studies have shown that low carbohydrate or ketogenic diets may slow tumor growth and progression through glucose ‘starvation’ of tumour cells and by reducing the effect of direct insulin-related actions on cell growth (Klement, 2017; Tisdale, Brennan, & Fearon, 1987). Some pet owners are seemingly aware of this link between glucose and cancer, as it was found that 24% of interviewed pet owners believed that carbohydrates should be limited in their pet’s diet following a cancer diagnosis (Rajagopaul et al., 2016). Only one study has investigated low carbohydrate diets for dogs with cancer, however there was no evidence for efficacy (Ogilvie et al., 1993). Clinical trials evaluating the potential benefits of low carbohydrate diets in canine cancer patients are needed to inform nutritional decisions.

1.5.3 Amino acids

Immune function, metabolism as well as tumor growth may be impacted by dietary amino acids, therefore, amino acids provide potential benefits to patients with cancers (Roudebush, Davenport, & Novotny, 2004). Branched chain amino acids (isoleucine, leucine and valine) support muscle mass and diminish protein catabolism (J. J. Wakshlag, Kallfelz, Wakshlag, & Davenport, 2006). In vitro, these branched chain
amino acids have also been found to slow tumor growth by inducing apoptosis in canine OSA cells (J. J. Wakshlag et al., 2006). For management of canine cancer, arginine and glutamine are most often discussed and some research suggests supplementation may reduce tumor growth or development (Lana et al., 2003; Ogilvie et al., 2000; Kaufmann et al., 2003), while another study concludes arginine supplementation in rats was not advantageous (Robinson et al., 1999).

To the authors’ knowledge, only one in vivo study investigated amino acid supplementation in dogs with cancers. Providing a diet containing supplemental arginine increased the disease-free interval and survival time in dogs with LSA (Ogilvie et al., 2000). However, study limitations have to be acknowledged; arginine was not the only dietary intervention (Ogilvie et al., 2000). The dietary macronutrient profile was also altered and n-3 PUFA were supplemented (Ogilvie et al., 2000). Reported benefits may have been due to a combination of diet changes rather than arginine alone (Ogilvie et al., 2000). Another limitation was the methodology used for LSA staging (Ogilvie et al., 2000). This staging was based on subjective assessment of organ size on radiographs, rather than more objective measures on abdominal ultrasound (Ogilvie et al., 2000). This may have decreased accuracy of the study's findings as dogs with stage 4 cancers were removed for statistical analysis (Ogilvie et al., 2000).

Provision of arginine, glutamine, and branched-chain amino acids in the diet or by dietary supplements may potentially provide benefit to canine cancer patients, although further research is required to define appropriate dosing regimens. Until then, it is
recommended to feed a completed and balanced diet that meets essential amino acid requirements for healthy adult dogs.

1.5.4 Omega-3 polyunsaturated fatty acids

Long-chain omega-3 polyunsaturated fatty acids (n-3 PUFA) have been shown to evoke anticancer activity in human cancer cells by induction of apoptotic cell death, inhibition of cancer cell growth and proliferation \textit{in vitro} (Shirota et al., 2005). Moreover, when used in conjunction with cancer treatment, n-3 PUFA improved the response to chemotherapy by enhancing cytotoxicity of anticancer drugs (Menendez, Lupu, & Colomer, 2005). In dogs, only stearidonic acid, a fatty acid derived from plant sources, has been tested \textit{in vitro}. A high dose was cytotoxic to canine LSA cells, and when combined with chemotherapy drugs vincristine and doxorubicin, cell viability was decreased compared to cells treated with chemotherapy alone (Pondugula et al., 2015).

\textit{In vivo}, dogs with nasal carcinoma receiving radiation and menhaden oil supplementation, which contained 9% of metabolisable energy as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), had decreased serum concentrations of prostaglandin E\textsubscript{2} and matrix metalloproteinase-9 (Hansen et al., 2011). This indicated that fatty acid supplementation may be beneficial in alleviating the inflammatory side effects of radiation therapy (Hansen et al., 2011).

It is important to note that EPA and DHA from marine sources would need to be fed rather than the precursor alpha-linolenic acid, due to the limited conversion rate of alpha-linolenic acid into EPA and DHA in dogs (J E Bauer, Dunbar, & Bigley, 1998; John E Bauer, 2007). Potential adverse effects of DHA and/or EPA supplementation include
gastrointestinal upset, decreased platelet function, and impaired short-term wound healing (Lenox & Bauer, 2013). Many conventional foods contain EPA and DHA; however, the appropriate dose of EPA and DHA is currently not known for dogs with cancer. The National Research Council’s recommended allowance for EPA and DHA for healthy adult dogs is $30 \text{ mg} / (\text{kg of body weight})^{0.75}$ and the safe upper limit is $370 \text{ mg} / (\text{kg of body weight})^{0.75}$. N-3 PUFA can be included in the diet as a component of a complete and balanced conventional or homemade diet, or provided as a dietary supplement (Nutrient requirements of dogs and cats, 2006). Currently, there is no research comparing these two methods of dietary n-3 PUFA supplementation (Nutrient requirements of dogs and cats, 2006).

1.5.5 Vitamin D

Research suggests that altered vitamin D metabolism is associated with common canine cancers such as OSA, LSA, and mast cell tumors. However, the mechanisms at work are still unclear and the studies investigating this link have limitations that restrict interpretation of results (Weidner et al., 2017). Dogs with OSA, LSA and mast cell tumors presented with low plasma 25-hydroxyvitamin D concentrations compared to dogs without cancer (J. J. Wakshlag et al., 2011; Weidner et al., 2017). One study indicated the possibility of a protective effect against various abdominal cancers when plasma 25-hydroxyvitamin D reached a concentration of over $100 \text{ ng/mL}$ (Selting, Sharp, Ringold, Thamm, & Backus, 2014). In contrast a separate study found no differences in plasma 25-hydroxyvitamin D between dogs with OSA and healthy controls (Willcox, Hammett-Stabler, & Hauck, 2016).
Further research investigating the link between vitamin D and cancers as well as clinical trials to determine appropriate dosing are needed. Until then, vitamin D should be provided by a complete and balanced diet and, although the NRC has not identified a minimum requirement for vitamin D, intake should at least meet the adequate allowance of 0.76 µg cholecalciferol (vitamin D₃) /kgBW⁰.⁷⁵ or ideally meet the NRC recommended allowance of 0.96 µg cholecalciferol/kgBW⁰.⁷⁵ (Nutrient requirments of dogs and cats, 2006). Additional supplementation beyond the NRC safe upper limit of 5.6 µg cholecalciferol/kgBW⁰.⁷⁵ is not recommended due to risk of hypervitaminosis and toxicity (Nutrient requirments of dogs and cats, 2006).

1.5.6 Antioxidants

The use of antioxidants in cancer patients is controversial as oxidative stress due to reactive oxygen species can lead to DNA damage, mutations, malignant transformations, and potential tumorogenesis (Mauldin, 2012). Elevated metabolic markers associated with oxidative stress have been demonstrated in dogs with mammary carcinoma and LSA (Szczubial, Kankofer, Lopuszynski, Dabrowski, & Lipko, 2004; Winter et al., 2009). *In vivo* selenium supplementation reduced DNA damage and facilitated apoptosis of DNA damaged non-neoplastic prostate epithelial cells in Beagles, suggesting selenium supplementation may benefit the aging prostate in dogs through preferentially eliminating cells with damaged DNA prior to development of a malignancy (Waters et al., 2003). These findings are promising but need to be confirmed in a cancer context. *In vitro* studies in canine mammary cancer have found variable responses to treatment with selenium, (Fico, Poirier, Watrach, Watrach, & Milner, 1986;
Wallig, Kuchan, & Milner, 1993) however in vivo studies have not been performed. Also, ascorbate, turmeric root extract and rosemary leaf extract have been tested in vitro (Carlson et al., 2018; Levine, Bayle, Biourge, & Wakshlag, 2017; Shin et al., 2018). When tested as a single agent, these antioxidants reduced cellular viability of canine melanoma, OSA, mastocytoma, or mammary carcinoma (Levine et al., 2017; Shin et al., 2018). Combinations of turmeric and rosemary leaf extract were more effective than monotherapies in some cases in activating caspase induced apoptosis (Levine et al., 2017). Furthermore, the combination of resveratrol, an antioxidant found in berries and the skin of grapes, with the drug doxorubicin decreased cellular viability of canine hemangiosarcoma in vitro compared to doxorubicin treatment alone (Carlson et al., 2018). To the authors’ knowledge, only one in vivo study of antioxidant effects in dogs with cancer has been published to date. This study provided evidence that administration of vitamins C and E limited oxidative stresses in a small group of dogs with mammary adenocarcinoma that result from cancer treatments and from the cancer itself, however more robust research is needed to confirm these results (Todorova & Dinev, 2010).

No recommendations regarding antioxidant supplementation can be made at this time, as in vitro efficacy does not necessarily translate into a therapeutic effect. Adequate amounts of vitamins (β-carotene, retinoids, vitamin C and E) and minerals (selenium) are present in complete and balanced conventional and homemade diets, such that feeding above what is required for adult maintenance cannot be recommended until more research and in vivo clinical trials have been conducted.
1.5.7 Pre- and probiotics

Fiber plays a key role in enhancing bowel function and stool quality in chemotherapy-induced diarrhea. Certain types of fibers (e.g. pectins, gums, psyllium) have physical gelling and water-holding properties that are helpful to restore normal fecal consistency (Lecoindre & Gaschen, 2011; Leib, 2000). While others (e.g. cellulose, peanut hulls, bran, pea fiber, psyllium) can assist in the management of diarrhea since these normalize gastrointestinal transit time and increase fecal bulk (Lecoindre & Gaschen, 2011). Select fibers also act as prebiotics, providing substrate for beneficial intestinal microbes. In healthy dogs fiber (soybean husk, lignocellulose, sugar beet pulp, potato fiber, inulin, oligofructose) can help to increase gastrointestinal health by promoting proliferation of beneficial bacteria (e.g. *Lactobacillus spp.*)(Flickinger & Fahey, 2002; Middelbos et al., 2010; Myint, Iwahashi, Koike, & Kobayashi, 2017; Panasevich et al., 2015). This can be of benefit for canine cancer patients as differences were observed between the fecal microbiotas of healthy dogs and dogs with cancer (Gavazza et al., 2018; Herstad, Moen, Gaby, Moe, & Skancke, 2018; Omori et al., 2017). In dogs with multicentric LSA, beneficial bacteria associated with gut health (e.g. *Ruminococcaceae*) and immunomodulation (e.g. *Faecalibacterium spp.*.) were decreased (Gavazza et al., 2018). Dogs with colorectal epithelial tumors presented with a higher presence of potentially pathogenic bacteria (Herstad et al., 2018), and dogs with intestinal LSA had more bacteria involved in tumorigenesis (e.g. *Parabacteroides*) compared to healthy dogs (Omori et al., 2017).

Fiber-enriched diets are commercially available, though they may not be ideal for canine cancer patients that experience decreased appetite or that have increased
energy requirements as fiber decreases digestibility and energy density of a food (de Godoy, Kerr, & Fahey, 2013; Guilford & Matz, 2003). Alternatively, there are many different fiber supplementation options available; each having different water-holding capacity, effects on luminal absorption of nutrients, and fermentation capability. Further information about properties of dietary fibers and their roles in gastrointestinal health has been published elsewhere (de Godoy et al., 2013; Flickinger & Fahey, 2002). Fecal consistency should be monitored throughout cancer treatment, using the fecal scoring chart previously referenced in this review.

Probiotics, or live cultures of perceived beneficial bacteria, might also have a positive impact on gastrointestinal health. Although a few studies have examined probiotic use in the context of gut health (Grześkowiak, Endo, Beasley, & Salminen, 2015; Jugan, Rudinsky, Parker, & Gilor, 2017; Redfern, Suchodolski, & Jergens, 2017), none were performed in dogs with cancer, therefore more research is needed in this area.

1.6 Clinical summary

The purpose of this review was to provide practical information to veterinary health care teams with a focus on the nutritional assessment and dietary considerations of dogs with cancer. When possible, it is important that dogs with cancer maintain (or achieve) a healthy body condition and muscle condition in order to prevent complications related to being overweight or underweight. Practitioners should encourage dog owners to feed dogs a balanced diet, free from food-borne pathogens to ensure adequate nutrition during and after cancer treatments. Lastly, this review
critiqued the existing body of literature surrounding popular dietary supplements in order to further guide practitioners when communicating with clients who are interested in giving dietary supplements to their dogs with cancer. To date, dietary supplementation above nutrient requirements for adult maintenance cannot be recommended as there is insufficient evidence supporting the use of one or more dietary supplements in dogs with cancer. Since cancers present a unique challenge, nutritional support should be individualized for each canine cancer patient for optimal results.
1.7 Acknowledgements

This study declares no sources of funding. Dr. Jacqueline M. Parr is a paid employee of Royal Canin Canada. Dr. Adronie Verbrugghe is the Royal Canin Veterinary Diets Endowed Chair in Canine and Feline Clinical Nutrition at the Ontario Veterinary College.
### 1.8 Tables

Table 1.1: Nutritional risk factors for canine cancer patients adapted from the World Small Animal Veterinary Association (WSAVA) Nutritional Assessment Guidelines (2011) and Parr & Remillard (2014).

<table>
<thead>
<tr>
<th>Nutritional Risk Factors</th>
<th>Present (Yes/No)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient History</strong></td>
<td></td>
</tr>
<tr>
<td>Senior life stage</td>
<td></td>
</tr>
<tr>
<td>Anorexia or hyporexia (i.e. decreased appetite)</td>
<td></td>
</tr>
<tr>
<td>Abnormal gastrointestinal function (e.g., vomiting, nausea, diarrhea, increased frequency of defecation, flatulence, borborygymus, constipation, etc.)</td>
<td></td>
</tr>
<tr>
<td>Previous or ongoing medical conditions or disease other than cancer diagnosis</td>
<td></td>
</tr>
<tr>
<td>Currently receiving medications or medical treatment (including chemotherapy and/or radiation)</td>
<td></td>
</tr>
<tr>
<td><strong>Dietary History</strong></td>
<td></td>
</tr>
<tr>
<td>Currently receiving dietary supplements</td>
<td></td>
</tr>
<tr>
<td>Consuming an unsafe diet (e.g., recalled diet, raw animal proteins or bones)</td>
<td></td>
</tr>
<tr>
<td>Consuming an unfamiliar or unknown to be nutritionally adequate diet (e.g., homemade, raw-meat based diets)</td>
<td></td>
</tr>
<tr>
<td>Treats, snacks, human foods &gt; 10% of total caloric intake</td>
<td></td>
</tr>
<tr>
<td>Free fed or food not measured</td>
<td></td>
</tr>
<tr>
<td>-------------------------------</td>
<td></td>
</tr>
<tr>
<td>Competition for food (e.g. multi-pet household)</td>
<td></td>
</tr>
</tbody>
</table>

**Physical & Clinical Exam**

<table>
<thead>
<tr>
<th>Body condition score: &lt;4 or &gt;5 (on a 9-point scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle condition score: mild, moderate, severe muscle wasting</td>
</tr>
<tr>
<td>Unintended or unexplained weight loss or weight gain</td>
</tr>
<tr>
<td>Dental abnormalities or disease</td>
</tr>
<tr>
<td>Poor skin or coat quality</td>
</tr>
<tr>
<td>Abnormal immune cell counts (e.g., neutropenia)</td>
</tr>
<tr>
<td>Abnormal glucose metabolism (e.g. hyperglycemia, hyperlactemia)</td>
</tr>
<tr>
<td>Protein malnutrition (e.g. hypoalbuminemia)</td>
</tr>
<tr>
<td>New morbidities (may be cancer-associated)</td>
</tr>
</tbody>
</table>
Table 1.2: Summary of in vitro and in vivo nutritional studies in canine cancer.

<table>
<thead>
<tr>
<th>Reference number</th>
<th>Nutritional treatment</th>
<th>Dose</th>
<th>Cancer type</th>
<th>Outcome</th>
<th>Level of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IN VIVO STUDIES</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| 56               | Diet 1: high fat, low carbohydrate | 1. Fat: 36.87 %DM / 62.7 %ME  
NFE: 13.7 %DM / 7.5 %ME | Lymphoma | No effect on energy expenditure | 6 |
|                  | Diet 2: low fat, high carbohydrate | 2. Fat: 8.98 %DM / 22.4 %ME  
NFE: 58.10 %DM / 55.0 % ME |             |         |                  |
| 60               | Arginine + n-3 PUFA | 5.54 %DM  
29g/kg DM EPA, 24g/kg DM DHA | Lymphoma | ↑ Survival time in experimental group | 6 |
<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Dose/Missed Component</th>
<th>Disease Model</th>
<th>Effect Description</th>
<th>Study Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td>Menhaden oil</td>
<td>9% ME</td>
<td>Nasal carcinoma</td>
<td>↓ Inflammatory eicosanoids (11-dehydroTXB2 and prostaglandin E2) in skin biopsies taken from areas exposed to high levels of radiation ↓ Resting energy expenditure</td>
<td>6</td>
</tr>
<tr>
<td>76</td>
<td>Selenium</td>
<td>3-6 ug/kg BW</td>
<td>None</td>
<td>↓ Accumulation of DNA damage in non-neoplastic epithelial cells</td>
<td>6</td>
</tr>
<tr>
<td>82</td>
<td>Vitamin E + Vitamin C</td>
<td>50 mg/kg BW Vitamin C</td>
<td>Mammary adenocarcinoma</td>
<td>↓ plasma malondialdehyde concentration (ie. marker of oxidative stress) combined with chemotherapy</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>IN VITRO STUDIES</td>
<td></td>
<td></td>
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<td>---------------------------------------------------------------------------------</td>
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<td>------------------------------------------------------------------</td>
<td>---</td>
</tr>
<tr>
<td>58</td>
<td>Valine, isoleucine, leucine, arginine</td>
<td>5-100 mM</td>
<td>Osteosarcoma</td>
<td>Anti-prolific effects of arginine and leucine at highest tested</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Broncho-epithelial</td>
<td>concentration.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>Stearidonic acid</td>
<td>50-100 μM</td>
<td>Lymphoma</td>
<td>↑ Anti-tumor activity of chemotherapy drugs</td>
<td>1</td>
</tr>
<tr>
<td>77</td>
<td>Selenium + Cyanohydroxybutene (CHB)</td>
<td>3.2 μM</td>
<td>Mammary</td>
<td>Selenium alone no effect, both doses of CHB alone reduced cell</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 and 20 nM</td>
<td></td>
<td>viability slightly and moderately inhibited cell growth. ↓ tumor</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>cell growth and viability substantially when 3.2 μM selenium</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>was combined with 1mM CHB</td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>Selenium</td>
<td>0.2-1 μg/ml</td>
<td>Mammary</td>
<td>Different cell lines showed variable sensitivity</td>
<td>1</td>
</tr>
<tr>
<td>79</td>
<td>Ascorbate</td>
<td>0-20 mM</td>
<td>Melanoma</td>
<td>↓ Cancer cell viability through ↑ apoptosis</td>
<td>1</td>
</tr>
<tr>
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<td>--------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>80</td>
<td>Turmeric root extract Rosemary leaf extract</td>
<td>Single agent: 6.3 μg/ml / Compound: 3.1 mg/ml each</td>
<td>Mastocytoma, mammary carcinoma and osteosarcoma</td>
<td>Compound ↓ cell viability more than single agent</td>
<td>1</td>
</tr>
<tr>
<td>81</td>
<td>Reversitol</td>
<td>10-100 μM</td>
<td>Hemangiosacoma</td>
<td>Growth-inhibitory effect ↑ Cytotoxic effect of doxorubicin</td>
<td>1</td>
</tr>
</tbody>
</table>

Level of evidence code: In-vitro research=1, single case reports=2, cross-sectional studies=3, case-control studies=4, cohort studies=5, blinded randomized control studies=6, meta-analysis=7, systematic review=8.
Table 1.3: Equations used for the calculation of daily energy expenditure for adult dogs based on ideal body weight (Saker & Selting, 2010)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Maintenance Energy Requirement (MER) (kcal/day)</th>
<th>Body Condition Score (BCS) (9 point scale)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact adult dog</td>
<td>1.8 x RER</td>
<td>Ideal (4 or 5)</td>
</tr>
<tr>
<td>Neutered adult dog</td>
<td>1.6 x RER</td>
<td>Ideal (4 or 5)</td>
</tr>
<tr>
<td>Inactive or obese-prone adult dog</td>
<td>1.4 x RER</td>
<td>Overweight (6 or 7) or obese (≥ 8) cancer patient <strong>undergoing</strong> treatment</td>
</tr>
<tr>
<td>Weight loss plan for an adult dog</td>
<td>1.0 x RER</td>
<td>Overweight (6 or 7) or obese (≥ 8) patient that has <strong>completed</strong> cancer treatment</td>
</tr>
<tr>
<td><strong>NOTE:</strong> Must feed a veterinary therapeutic diet labelled for weight loss to avoid deficiencies in essential amino acids, vitamins and/or minerals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Critical care/hospitalization</td>
<td>1.0 x RER</td>
<td>All body condition scores</td>
</tr>
<tr>
<td>Weight gain required for an adult dog</td>
<td>Increase caloric intake by 5-10% based on patient’s current caloric intake and continue adjusting to achieve ideal body condition score of 4 or 5 out of 9</td>
<td>Underweight (&lt;4)</td>
</tr>
</tbody>
</table>
Using Resting Energy Requirement (RER) (kcal/day):

\[ \text{RER} = 70(BW_{kg})^{0.75} \text{ or } \text{RER} = 30(BW_{kg}) + 70 \text{ (if body weight between 2-45 kg)} \]

Calculated based on ideal body weight

(exception: underweight anorectic patients in hospital; calculate based on current body weight, then once they go home calculate with ideal body weight)
2 Omega-3 fatty acids and cancer: A review of the literature

2.1 Introduction

Modern day researchers have been investigating the potential role of various fatty acids in cancer prevention and treatment since at least 1924, when it was found that injecting the fatty acids linoleic acid and α-linolenic acid into mice with transplantable tumors aided in increased resistance to subsequent cancer grafts by slowing the growth of cancer cells (Nakahara, 1924). Since then, many in vitro, and in vivo studies in a variety of species have been published to further investigate the connection between fatty acids and cancer. Despite the findings of these studies, no one mechanism of action has been elucidated which may imply fatty acids work via multiple mechanisms of action. It is the purpose of this review to describe the unique structure and classification of fatty acids, in particular n-3 PUFAs, their potential roles in treating cancers, and how they interact with chemotherapy drugs.

2.2 Structure and classification of fatty acids

Fatty acids are a diverse group of molecules that are characterized by long chains of carbon atoms, typically 12-24 carbon atoms in length, as well as hydrogen atoms bonded to a carboxyl group (COOH)(Gyamfi, Awuah, & Owusu, 2019). They are used to build larger lipids such as triacylglycerols and phospholipids(Gyamfi et al., 2019). In addition, fatty acids can be broken down in the liver into acetyl CoA or glucose and incorporated into various metabolic processes(Gyamfi et al., 2019; Valenzuela & Videla, 2011).
Depending on the nature of the carbon bonds within the fatty acid molecule, fatty acids can be classified as either saturated or unsaturated, which directly affects the availability of carbon to bind to hydrogen (Gyamfi et al., 2019). Fatty acids that contain double bonds between the carbon atoms are less available to bind to hydrogen and are, therefore, classified as unsaturated fatty acids; alternatively whereas fatty acids that do not contain double bonds are classified as saturated (Gyamfi et al., 2019). Unsaturated fatty acids are further characterized by the degree of double bonding present; an unsaturated fatty acid with only one carbon-carbon double bond is a monounsaturated fatty acid while fatty acids with two or more double bonds are classified as polyunsaturated fatty acids (PUFA) (Gyamfi et al., 2019). This is illustrated in Figure 2.1.

Fatty acids that cannot be synthesized and must be acquired via dietary sources are considered to be essential (Gyamfi et al., 2019). Most mammals, including dogs, have the ability to synthesize nonessential fatty acids by producing a double bond at carbon 9, but are unable to produce double bonds any closer to the functional COOH group (Gyamfi et al., 2019). Therefore, fatty acids such as linoleic acid (LA) and α-linolenic acid (ALA) are considered essential due to the presence of their first double bond at carbon 6 and carbon 3 respectively (Gyamfi et al., 2019); these fatty acids are often referred to as omega-6 and omega-3 fatty acids (Gyamfi et al., 2019). Other omega-6 fatty acids, such as arachidonic acid and other omega-3 fatty acids, such as, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are considered to be “conditionally essential” fatty acids, as they play important roles in nervous tissue development (Heinemann & Bauer, 2006) as well as in both inflammatory and immune responses (Hall, Wander, Gradin, Du, & Jewell, 1999). Arachidonic acid can be
synthesized by dogs through metabolism of the precursor molecules linoleic acid and linolenic acid via Δ6 desaturase activity (Gyamfi et al., 2019; Nutrient requirments of dogs and cats, 2006). It is not possible for dogs and other mammals to convert between omega-3, 6, 7, and 9 fatty acids, due to an inability to place double bonds in position 12 and 15 (Nutrient requirments of dogs and cats, 2006). In dogs fed a diet devoid of PUFA, monounsaturated fatty acids (such as oleic acid) can also provide a substrate for Δ6 desaturase. When essential fatty acids are present, the Δ6 desaturase reaction is the rate-limiting step in the cascade of reactions that result in the production of long-chain PUFAs such as EPA and DHA (Nutrient requirments of dogs and cats, 2006). Figure 2.2 illustrates these pathways. It has been found that EPA rapidly accumulates in plasma lipids of dogs fed a diet with moderate ALA content (J E Bauer et al., 1998), however there appears to be limited conversion of ALA to DHA, even when docosapentaenoic acid (DPA) is produced (J E Bauer et al., 1998; Purushothaman, Yvonne Brown, Wu, & Vanselow, 2011). This finding is consistent in humans (Burdge & Calder, 2005) and cats (Pawlosky, Barnes, & Salem, 1994), as they have limited Δ6 desaturase activity (Nutrient requirments of dogs and cats, 2006).

### 2.2.1 Eicosapentaenoic and docosahexaenoic acid

The fatty acid DHA is an n-3 PUFA that contains 22 carbon atoms with six double bonds (22:6). EPA is also an n-3 PUFA but with 20 carbon atoms and five double bonds (20:5). Sources of DHA and EPA are marine animals such as krill and fatty fish like mackerel, salmon, trout, herring, tuna, and sardines (Calder, 2014). Supplements such as cod liver oil and krill oil can also be used as a source of dietary DHA and
EPA (Calder, 2014). Alternatively, dietary DHA and EPA can be provided by marine plants such as algae, and microorganisms such as phytoplankton (Nutrient requirements of dogs and cats, 2006). Although DHA and EPA are only considered to be conditionally essential in healthy adult dogs, the NRC (2006) recommendations state that the adequate intake and recommended allowance of DHA + EPA for healthy adult dogs is 0.44 g/kg DM, or 0.03 g/kg of BW$^{0.75}$ and the safe upper limit is 11 g/kg DM, or 0.37 g/kg of BW$^{0.75}$. Recommended allowance and adequate intake of DHA + EPA in growing puppies after weaning, is 0.5 g/kg DM, or 0.036 g/kg per kg of BW$^{0.75}$ and the safe upper limit has been determined to be 11 g/kg DM or 0.77 g/kg per kg of BW$^{0.75}$.

Both DHA and EPA interactions with cancerous cells have been under investigation since the early 1980’s, when it was found that in vitro supplementation of MG63 human osteogenic sarcoma cells with DHA or EPA resulted in suppression of cancer cell proliferation (Booyens et al., 1984). Since then many studies have attempted to elucidate the potential mechanisms of action for the observed anti-cancer effects of DHA and EPA (Abdi, Garssen, Faber, & Redegeld, 2014; Ding et al., 2018; Fukui, Kang, Okada, & Zhu, 2013; Kang et al., 2010; Shirota et al., 2005; Ogo et al., 2018), however, there is no consensus on the scope of mechanisms of action. A variety of different mechanisms of action have been described, such as induction of apoptosis or reduction of tumor growth (Abdi et al., 2014; Ding et al., 2018; Fukui et al., 2013; Kang et al., 2010; Shirota et al., 2005). In addition, synergistic activity has been observed when n-3 PUFAs are used in combination with chemotherapy drugs (Ogo et al., 2018). These proposed mechanisms will be described in the next section.
2.3 Proposed mechanisms of action of eicosapentaenoic acid and docosahexaenoic acid in human cancers

2.3.1 Induction of apoptosis

One of the most commonly studied mechanisms of action for anticancer activity of DHA and EPA is through their ability to induce apoptosis. Apoptosis, also known as programmed cell death, is mediated by a group of protease enzymes called caspases (Cruickshanks, Booth, Tang, & Dent, 2013). Activation of caspases requires energy in the form of adenosine triphosphate (ATP) and results in degradation of eukaryotic DNA and encapsulation of the DNA fragments (Cruickshanks et al., 2013). There are two well documented pathways that lead to apoptosis; the extrinsic pathway and the intrinsic pathway (Malladi, Challa-Malladi, & Bratton, 2010). The extrinsic pathway is mediated by Tumor necrosis Factors (TNFs), a common superfamily of cell signaling proteins (Cruickshanks et al., 2013; Malladi et al., 2010). When TNF binds to its receptor on the cell it is targeting, a structural change occurs that eventually recruits and activates caspase-8. Activated caspase-8 then can induce apoptosis through two different mechanisms (Malladi et al., 2010). The first is by directly cleaving effector caspases which then causes caspase-3 to initiate apoptosis (Cruickshanks et al., 2013). The alternate mechanism is to induce apoptosis via the intrinsic pathway; this involves cleaving the BH3-interacting domain death agonist (BID) protein into its active form, tBID, which inserts itself into the mitochondrial membrane (Malladi et al., 2010). Following this, tBID binds to Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK), which are two proteins that aide in increasing mitochondrial membrane permeability (Malladi et al., 2010). Next, tBID forms pores in the
mitochondrial membrane, a condition called mitochondrial outer membrane permeability (MOMP), through which caspase activator cytochrome c can leak into the cytoplasm and eventually activate caspase-9 in the apopotosome (Malladi et al., 2010). Activated caspase-9 will then trigger caspase-3 and subsequently resulting in apoptosis (Cruickshanks et al., 2013). The intrinsic pathway can also be activated by stress or damage to the cell. Both pathways are illustrated in Figure 2.3. In either case, apoptosis can be detected in the laboratory through methods such as staining with Annexin V and flow cytometry (Cruickshanks et al., 2013).

The literature describes a diversity of roles that DHA and EPA play in caspase-dependent apoptosis in various cancer types. In vitro treatment of human gastric cancer cells, breast cancer cells, multiple myeloma cells and pancreatic cancer cells with DHA or EPA as single agents has been shown to increase expression and activity of caspase-3, caspase-8 and/or caspase-9, resulting in apoptosis of cancer cells (Abdi et al., 2014; Ding et al., 2018; Fukui et al., 2013; Kang et al., 2010; Shirota et al., 2005). Furthermore, inhibition of caspase-8 expression in human gastric and human colon cancer cells, as well as knockdown of caspase-8 in human breast cancer cell lines, effectively blocked DHA-mediated cell death; these findings confirmed DHA’s critical role in mediating caspase-8 dependent apoptosis (Kang et al., 2010; S. J. Lim et al., 2012). Evidence of increased cleaved caspase-9 concentrations has also been found in human breast cancer cells treated with DHA-dopamine and EPA-dopamine conjugates (Rovito et al., 2015). When used in combination, DHA- and EPA-treated human colorectal cancer stem-like cells showed increased caspase-3 activity and subsequent apoptosis which was not observed when used as monotherapies (Sam,
Ahangar, Nejati, & Habibian, 2016).

DHA and EPA have also been proposed to induce apoptosis by suppression of cyclo-oxygenase–2 (COX-2), a protein that, when inhibited, is thought to modulate apoptosis through subsequent activation of caspase-3 (Yu et al., 2004). Inhibition of COX-2, and subsequent cancer cell apoptosis, has been observed in human hepatocellular carcinoma cells, human pancreatic cancer cells and human colon adenocarcinoma cells when treated with either DHA or EPA (Calviello et al., 2004; K. Lim, Han, Dai, Shen, & Wu, 2009; Shirota et al., 2005).

2.3.2 Reduction of tumor growth

An alternative mechanism by which DHA and EPA have been found to impact cancer cells is by reducing tumor growth, and in some cases causing a reduction of tumor size (Hicklin & Ellis, 2005). This is possible by decreasing expression of vascular endothelial growth factor (VEGF) proteins, as they play a pivotal role in angiogenesis (Hicklin & Ellis, 2005). There are six glycoproteins in the VEGF family: VEGF-A (often referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placenta Growth Factor (PIGF) 1 and 2 (Hicklin & Ellis, 2005). In addition, there are three tyrosine kinase receptors that have specific binding affinity for VEGF proteins: VEGFR1, VEGFR2 and VEGFR3 (Hicklin & Ellis, 2005). In vivo study of human cancers have found VEGF expression to be associated with poor prognosis in colon cancer (Hui & Meng, 2015), poor efficacy of treatment in breast cancer (Berns et al., 2003) and reduced survival time in prostate cancer (Humphrey et al., 2006).

In addition to activating caspase-3 in the induction of apoptosis, COX-2 inhibition
can also regulate reduced expression of VEGF *in vitro* in human colon cancer cell lines and human colorectal cancer cell lines treated with DHA or EPA (Calviello et al., 2004; Zou et al., 2015), though DHA has been found to reduce VEGF expression with greater efficacy than EPA (Moradi Sarabi et al., 2018). DHA has also been found to downregulate VEGF expression *in vitro* in lung cancer cell lines (Yin, Sui, Meng, Ma, & Jiang, 2017) and in breast cancer cell lines (Chen et al., 2014). These *in vitro* results may explain the reductions of tumor size observed in *in vivo* studies, where rodents fed a diet enriched in n-3 FA had significant decreases in tumor volume over time in a murine colon cancer model (Zou et al., 2015), decreases in tumor weight in a murine breast cancer model (Chen et al., 2014) and lower tumor weight and volume in rats injected with methylcholanthrene sarcoma cells (Ramos et al., 2004). It is possible that a reduction of VEGF expression plays a role in reducing tumor vascularity in humans with colorectal cancer who had never received fish oil supplements before, when given moderate doses of fish oil (Cockbain et al., 2014).

### 2.3.3 Combinations of eicosapentaenoic and docosahexaenoic acid with chemotherapy

Several studies have been published that suggest n-3 PUFAs may have additive and/or synergistic effects on chemotherapy drugs. In particular, DHA has been found to enhance cytotoxicity of the chemotherapy drugs doxorubicin and taxane in human breast cancer cells *in vitro* (Germain, Chajès, Cognault, Lhcollery, & Bougnoux, 1998; Menendez et al., 2005), however, this finding may be cell line specific (Mahéo et al., 2005). Synergy has been reported *in vitro* between DHA and the chemotherapy drug 5-fluorouracil in human colon adenocarcinoma cell lines (Calviello et al., 2005), and
between EPA and chemotherapy drugs paclitaxel, docetaxel and cis-
diamminedichloridoplatinum[II] in human esophageal cancer cells (Ogo et al., 2018).

Though the result of these in vitro studies seemed promising, in vivo results have been inconsistent. DHA has been found to enhance effects of epothilone, while EPA was found to enhance effects of 5-fluorouracil and cyclophosphamide and neither DHA nor EPA had any effect on gemcitabine in mice bearing colon adenocarcinoma (Wynter, Russell, & Tisdale, 2004). EPA has been further studied in mice with triple-negative breast cancer tumor xenographs, where it was found to produce modest reductions in tumor growth when used as a single agent. However, when used in combination with dasatinib significant reduction of tumor growth was observed, coupled with an increase in survival (Torres-Adorno et al., 2019). In humans with metastatic breast cancer, it was found that supplementation with DHA while receiving anthracycline-based chemotherapy treatment may improve disease outcome by increasing both survival time and time to progression, while also finding no adverse side-effects of DHA supplementation (Bougnoux et al., 2009). Similar results have been found in humans with lung cancer receiving fish oil supplements in addition to platinum-based chemotherapy (Murphy et al., 2011).

Many potential mechanisms of action have been proposed to explain the synergistic effects observed in treating various cancers with a combination of n-3 PUFAs and chemotherapy. In particular, it has been found that DHA increases sensitivity of human gastric cancer cells to doxorubicin by inhibiting the NF-κβ pathway (Xin-xin et al., 2011). Furthermore, this pathway was inhibited in human breast
cancer cells treated with DHA (Hwang et al., 2017) and in human esophageal cancer cells treated with EPA (Kubota et al., 2013). Similar to single agent treatments, combination therapy may also induce apoptosis through cleavage of caspase-3 (Torres-Adorno et al., 2019). However, the full scope of mechanisms at work are still unclear, due to limitations such as sample size, compliance issues, types of supplements and contents of supplements (Morland, Martins, & Mazurak, 2016). Therefore, more research is needed before clinical application of these findings can be established.

### 2.4 Proposed mechanisms of action of fatty acids in canine cancers

Compared to other species (humans and rodents), few studies have evaluated the utility of DHA and EPA against canine cancers, and fewer still have succeeded in elucidating potential mechanisms of action. A recent *in vitro* study of canine lymphoid tumor cells found that the plant-based fatty acid stearidonic acid was able to reduce viability of tumor cells both as a single agent and when combined with chemotherapy drugs vincristine and doxorubicin at non-toxic doses (Pondugula et al., 2015). In addition, stearidonic acid had no cytotoxic effects on healthy dog mononuclear cells when used at chemosensitizing concentrations (Pondugula et al., 2015). Although more research needs to be done to determine specific dosing, these findings suggest that stearidonic acid is likely safe for dogs. Further analysis indicates that stearidonic acid may be mediating chemosensitization through inhibition of P-gp efflux activity (Pondugula et al., 2015).

Menhaden oil is another fatty acid that may have direct effects on cancer cells (Ogilvie et al., 2000), and has been found to play a role in reducing inflammatory...
eicosanoids in dogs with nasal carcinoma (Hansen et al., 2011). An *in vivo* study of dogs with lymphoma found that dogs receiving a diet supplemented with menhaden oil had improved the disease-free interval and survival compared to controls; however, it was unclear what mechanisms resulted in these observations (Ogilvie et al., 2000). In that study, menhaden oil was not the only nutritional intervention, as dogs fed the control diet were also receiving supplemental arginine (Ogilvie et al., 2000). The authors found no differences in quality of life score or toxicity scores at any time during the study, which suggests that feeding a diet containing up to 73 g/kg Dry Matter of total n-3 PUFAs may be safe for dogs with lymphoma (Ogilvie et al., 2000). This would be expected since this amount of n-3 PUFAs does just fall below the safe upper limit of total n-3 PUFA identified by the NRC for healthy adult dogs (*Nutrient requirments of dogs and cats*, 2006).

Another application for n-3 PUFA in canine cancer is its potential to reduce inflammation, which can occur as a result of malignant disease (Mantovani, Allavena, Sica, & Balkwill, 2008). Following the extrinsic and intrinsic pathways, tumor cells produce chemokines, cytokines and prostaglandins, which then recruit inflammatory cells that, in turn, produce even more inflammatory mediators through activation of transcription factors in inflammatory cells and tumor cells (Mantovani et al., 2008). This cascade effect ultimately results in cancer-related inflammation, which creates a microenvironment that further promotes tumor growth (Mantovani et al., 2008). *In vitro*, it has been found that murine fibrosarcoma cells treated with menhaden oil had a significant decrease in inflammatory cytokine TNF-α compared to controls (Ogilvie et al.,
Furthermore, a canine *in vivo* study found menhaden oil supplementation of dogs with nasal carcinoma resulted in a decrease of PGE$_2$ in skin exposed to high levels of radiation (Hansen et al., 2011). These studies are promising; however, more research is needed to confirm therapeutic applications of n-3 PUFA in different canine cancers.

### 2.5 Conclusions and future directions

Current research of n-3 PUFA in various human cancers shows promise for its potential to directly impact cancer cells, and their ability to reduce side-effects of cancer and cancer treatments. Unfortunately, extensive research is currently lacking in dogs. Preliminary findings are promising and there are some similar findings in human/murine cancer cells and canine cancer cells, such as the ability for n-3 PUFAs to reduce cellular viability of cancer cells, and the potential for n-3 PUFAs to reduce inflammatory markers. However, more research is needed in dogs before clinicians can confidently advise owners of dogs with cancer interested in feeding n-3 PUFA supplements to their dogs. In particular, more studies are needed to compare the effects of various types of n-3 PUFA, such as EPA and DHA, on the most common types of canine cancer such as OSA, or LSA.
2.6 Thesis objectives and hypotheses

The research objectives of this thesis are as follows:

Objective 1 (Chapter 2):

a. To investigate and compare diet type and supplement use between dogs with cancer and healthy dogs.

b. To assess the sources of information dog owners consult when researching pet health topics, pet nutrition, and nutritional supplements.

Objective 2 (Chapter 3):

a. To investigate the impact of EPA and DHA on the viability of canine OSA cell lines.

b. Determine if combination treatments of EPA or DHA with the chemotherapy drug doxorubicin are additive, synergistic, or antagonistic.

It was hypothesized that:

1. Dogs with cancer would be less likely to receive a commercial diet type compared to healthy dogs.

2. Owners of dogs with cancer would feed their dog a greater number of nutritional supplements per dog compared to owners of healthy dogs.

3. Owners of dogs with cancer would spend more time researching pet health, pet nutrition and nutritional supplements while also favoring different sources of information compared to owners of healthy dogs.
4. Single agent treatment of EPA and DHA would reduce viability of canine OSA cells in a dose-dependent manner

5. Combinations of EPA or DHA with doxorubicin would be synergistic or additive.
2.7 Figures

Figure 2.1: Classification of Fatty Acids
Figure 2.2: Steps in fatty acid metabolism in dogs, and enzymes required to complete each reaction.
Figure 2.3: Intrinsic and Extrinsic Apoptosis Pathways.

TNF, Tumor necrosis factor; BID, BH3 interacting-domain death agonist; tBID, active form of BID; BAX, Bcl-2-associated X protein; BAK, Bcl-2 homologous antagonist killer.
CHAPTER 2:

3 Unconventional diets and nutritional supplements are more common in dogs with cancer compared to healthy dogs: An online global survey of 345 dog owners

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Submitted to the Journal of Comparative and Veterinary Oncology: November 29th, 2019
3.1 Abstract

**Objective:** Investigate and compare diet type and supplement use between dogs diagnosed with cancer and a population of owner-reported healthy dogs and to assess the sources of information that dog owners consult.

**Sample:** Respondents were mainly from English-speaking countries. Dogs were considered healthy (N=213) if owners reported them to be in overall good health. Dogs were included in the cancer group (N=132) if the owner reported that their dog had been diagnosed with cancer.

**Procedures:** An online survey was distributed to clients presenting to a tertiary oncology service, clients presenting to a local primary care veterinary practice, and through social media.

**Results:** Owners of dogs with cancer spent more time researching pet health topics, pet nutrition topics, and nutritional supplements than owners of healthy dogs. While a veterinarian was most commonly reported to be an information source for both groups, owners of healthy dogs more likely consulted pet stores, while owners of dogs with cancer tended more to consult social media groups and blogs. Healthy dogs were more likely to be fed commercial dry food, whereas homemade cooked and raw diets were more prevalent among dogs with cancer. Supplement use, especially cannabidiol products, mushroom extracts or turmeric/curcumin, was also more common for this group.
Conclusions and Clinical Relevance: Alternative diets and supplements were more popular among owners of dogs with cancer compared to owners of a population of healthy dogs. These findings highlight the need for nutritional counselling and education of pet owners regarding nutrition-related topics, especially when their dog is diagnosed with cancer.
3.2 Introduction

Cancers are the reported cause of death in about 30% of dogs both in the United Kingdom and in North America, making cancers the most commonly reported cause of death among adult dogs (Adams et al., 2010; Fleming, Creevy, & Promislow, 2011). Advances in cancer research have resulted in the availability of a variety of treatment and palliative care options for dogs with cancer that can improve overall health and quality of life, including nutritional management (Mauldin, 2007; J. Wakshlag & Kallfelz, 2006).

It has been previously reported that owners of dogs with cancer share concerns about their pet's diet and nutrition following a cancer diagnosis (Rajagopaul et al., 2016). Certain components of the diet and the diet type itself may be called into question for its nutritional adequacy, leading about 80% of owners to believe a diet change for their dog may be needed post-diagnosis; such as inclusion of homemade cooked or raw dietary components, and exclusion of conventional dry or wet dietary components (Rajagopaul et al., 2016). This is a concern, as homemade diets are often deficient in some essential nutrients (Heinze et al., 2012; Stockman et al., 2013; Streiff et al., 2002), and raw diets possess increased risk of bacterial contamination (Finley et al., 2008; Nemser et al., 2014; Strohmeyer et al., 2006; Weese & Rousseau, 2006). Therefore, it is important to monitor dietary decisions made by owners of dogs with cancer to target nutrition education and how clinicians can best advise clients following a cancer diagnosis.

Inclusion of nutritional supplements into the daily feeding routine is a dietary change commonly reported in diseased animals (Lisa M Freeman et al., 2006).
Supplement use has been reported at even higher proportions in dogs with cancer (Lana et al., 2006; Rajagopaul et al., 2016). Despite this observed difference in supplement use between healthy dogs and dogs with cancer, no study has yet directly compared this relationship. In addition, no study has evaluated or compared the sources of information favored by owners of dogs with cancer and owners of healthy dogs when researching pet nutrition topics. This information is important as it is a potential force driving dietary decisions owners make for their dogs.

The objectives of this study were to investigate and compare diet type and supplement use between dogs with cancer and healthy dogs, and to assess the sources of information dog owners consult when researching pet health topics, pet nutrition, and nutritional supplements. Based on results from previous studies (Lana et al., 2006; Rajagopaul et al., 2016). It was hypothesized that dogs with cancer would be less likely to receive a commercial diet type compared to healthy dogs, owners of dogs with cancer would feed a greater number of nutritional supplements per dog and would spend more time researching pet health, pet nutrition and nutritional supplements while also favoring different sources of information compared to owners of healthy dogs.

### 3.3 Materials and methods

This study was approved by the University of Guelph’s Research Ethics Board (REB No. 18-09-026) and involved a survey of dog owners’ attitudes and practices regarding their dog’s diet and use of nutritional supplements in dogs with a cancer diagnosis and in healthy dogs.
3.3.1 Survey

An online survey was administered using Qualtrics© Research Suite (Provo, Utah). The survey contained a total of 51 questions, 28 multiple-choice style, nine one-word fill-in-the-blank style questions and 14 open-ended fill-in-the-blank style questions. The researchers developed the survey by consulting survey questions used in previous studies and adapting some, while also developing several original questions. The survey began with a consent form that participants completed. The next section of multiple-choice and fill-in-the-blank questions collected information about time spent and resources consulted when researching pet health topics, pet nutrition, and nutritional supplements. Following that, dog owners were asked about their dog’s age, sex, breed, and health status. Emphasis was placed on inputting information about only one dog in the home. Participants were then asked if their dog had cancer. If “yes”, they were directed to questions asking for more information about the cancer such as type, date of diagnosis, and treatments the dog had or would receive. Participants who selected “no” were asked if and/or how their behavior would change with respect to feeding practices if their dog were to receive a cancer diagnosis. All participants were then asked what diet type they fed to their dog and if they currently (or ever had) fed nutritional supplements to their dogs. Participants administering nutritional supplements were prompted to answer questions designed to gather more information about individual supplements and behaviors surrounding each supplement, such as the dose and how often the supplement is provided. The supplements reported by participants were sorted into categories by the researchers following data collection. Categories were developed by consulting categories reported in a previous study(Rajagopaul et al.,
and by developing original categories to sort supplements that did not otherwise fit in categories previously reported. Participants who indicated they were not feeding their dog nutritional supplements were asked why they made this choice and if they would consider feeding nutritional supplements and under what conditions.

The final section of the survey collected demographic data about each participant, such as gender, age, country of residence, level of education and the name of their family veterinary clinic or oncology practice. Depending on how a participant responded they may have answered a minimum of 25 questions in total (i.e. such as a respondent with a healthy dog who had never been fed a nutritional supplement), or a maximum of 177 questions (i.e. respondents who had a dog with cancer and received as many as 20 supplements, which was the maximum number of supplements reported to be fed per dog). The survey questions and consent form can be found in Appendix 1.

3.3.2 Recruitment

This survey was distributed among a population of dog owners aged 18 or older presenting to a tertiary oncology service from October 2018 until February 2019. Recruitment was done purposively in the waiting room where a researcher was present with information about the survey during clinic hours. Clients were informed about the option to participate in the study through signage hung in the waiting room and verbally by reception staff. Clients interested in participating had the choice to voluntarily approach a researcher and could complete the survey on a tablet provided by a researcher. In addition, post cards with a link to the survey were available and handed out to clients by the researchers or by reception staff to provide an option for clients who
preferred to complete the survey from their home computer. Owners of healthy dogs were recruited from a population of clients at a local university-based primary care veterinary practice from October 2018 until February 2019. Post cards with a link to the survey were distributed with client invoices, and posters with a link to the survey were hung in the waiting room and in the exam rooms. For four weeks, January 2019 until February 2019, the survey was distributed through social media. To stimulate recruitment of both owners of healthy dogs and owners of dogs with cancer, the link to the survey was shared by the researcher through social media groups that were identified as pertaining to canine health and nutrition as well as canine cancer support groups based on the names of the groups. The survey was available internationally; however, the questions were only available in English. Surveys were excluded from the analysis if they contained incomplete information, if the respondent chose to provide information about a deceased animal, more than one animal, an animal less than one year of age, or if the respondent indicated their dog was not healthy but did not have cancer. Incomplete surveys included those that were opened but none of the questions were completed, and surveys in which the participant dropped out before indicating if their dog was healthy, had cancer, was in remission or if they were unsure of their dog’s cancer status.

3.3.3 Statistical analysis

Data was analysed using IBM© SPSS Statistics for Macintosh, Version 25. Descriptive statistics including frequency, distributions, mean and median responses were calculated. Frequencies were reported for owner and dog demographic data.
Categorical data included comparisons of sources of information, diet type and nutritional supplement types in healthy dogs and dogs with cancer. These were analysed using Chi-squares and odds ratios. Continuous data, such as number of supplements fed and length of time spent researching pet health, pet nutrition and nutritional supplements, were assessed for normality using a Shapiro-Wilk test. All were found to be non-normal and were analyzed using the Mann-Whitney U test. Statistical significance was set at P<0.05 because, despite conducting multiple tests comparing variables, each test contained different variables; therefore, the P value did not require adjusting. Figures and tables were created using Microsoft® Excel for Mac, Version 16.

3.4 Results

A total of 640 surveys were submitted when the survey was closed, 54% (345/640) were included in statistical analysis. The largest group of excluded surveys (n=256) were incomplete. Five surveys containing information about a deceased dog and five surveys containing information pertaining to more than one animal were excluded; 15 surveys containing information about a dog less than one year of age were excluded as well as five surveys where respondents indicated their dog was not healthy but did not have cancer. In total, only eight participants (2.3%) indicated being clients from the local primary care veterinary practice and four (1.2%) from the tertiary oncology service. 306 (88.7%) participants were not clients at either practice involved in clinic recruitment whereas 71 (20.6%) chose not to disclose this information.

Respondents were included in the healthy group (N=213) if they self-reported that their dog was in overall good health, and dogs were included in the cancer group
(N=132) if the owner self-reported that their dog had cancer. The study population consisted of owners from around the world; most respondents were from the United States of America (44.9% 155/345) and Canada (29.6% 102/345) while 7.8% (27/345) came from other countries and the remaining 17.7% (61/345) participants chose not to disclose their location. Owner demographic data are summarized in Table 3.1 and dog characteristics are in Table 3.2.

The sources of information consulted by owners of dogs with cancer and owners of healthy dogs are shown in Figure 3.1. A veterinarian was the most commonly reported source of information when researching pet health topics, pet nutrition, and nutritional supplements in both groups. However, owners of dogs with cancer have greater odds of consulting social media groups than owners of healthy dogs when researching pet health, pet nutrition and nutritional supplements (Table 3.3). They also spend more time researching pet health topics (p<0.001), pet nutrition topics (p<0.01), and nutritional supplements (p<0.001) than owners of healthy dogs (Table 3.5). Owners of healthy dogs have greater odds of consulting pet stores for information when researching pet health, pet nutrition and nutritional supplements compared to owners of dogs with cancer (Table 3.3).

As shown in Figure 3.2, commercial dry food was the most commonly fed diet type in both healthy dogs and dogs with cancer. The odds of owners of healthy dogs feeding a commercial dry food was 4.4 times that of owners of dogs with cancer (p<0.001); and the odds of owners of dogs with cancer feeding a homemade raw diet were 2.2 times the odds of owners of healthy dogs (p<0.05), while the odds of owners of
dogs with cancer feeding a homemade cooked diet were 4.3 times the odds of owners of healthy dogs (p<0.001) (Table 3.4).

Owners of dogs with cancer were more likely to feed nutritional supplements to their dogs compared to owners of healthy dogs (p<0.01). Owners of dogs with cancer also reported feeding significantly more nutritional supplements per dog (p<0.001). The median number of supplements fed to healthy dogs was three and the interquartile range (IQR) for this same group ranged from two to six supplements. Healthy dogs received a median of two supplements with an IQR ranging from one to three. The maximum number of supplements reportedly fed to one animal in healthy dogs and dogs with cancer was 15 and 20, respectively (Table 3.5).

Overall, there were 279 individual supplements reported among owners of dogs with cancer, and 226 reported by owners of healthy dogs. Supplement groups are coded in Table 3.6 and are presented in Figure 3.3 in alphabetical order with the number and proportion of supplements fed to dogs with cancer and healthy dogs.

The most common supplements fed to dogs with cancer, in order from most common to least common, were: marine-derived n-3 FA supplements (9%, 25/279 of total supplements reported among dogs with cancer), mushroom supplements (8%, 21/279), multivitamins/minerals (8%, 21/279), cannabidiol (CBD)/tetrahydrocannabinol (THC) products (8%, 23/279), glucosamine/chondroitin (6%, 18/279), multi/mixed supplements supporting immune function (5%, 15/279), turmeric/curcumin (5%, 15/279), probiotics (5%, 14/279) and plant-derived n-3 FA supplements (4%, 12/279).
The same supplements were reported in healthy dogs with higher, lower or similar frequency. At a higher frequency: marine-derived n-3 FA supplements (14%, 32/226 of total supplements reported among healthy dogs), glucosamine/chondroitin (21%, 48/226), probiotics (9%, 20/226); at a lower frequency: plant-derived n-3 FA (1%, 3/226), mushroom supplements (1%, 2/226), CBD and THC products (4% 8/226), multi/mixed supplements supporting immune function (1%, 2/226), turmeric/curcumin (2%, 4/226); and at a similar frequency: multivitamins and minerals (7%,16/226). Odds ratios comparing significant differences in use of CBD/THC products, turmeric/curcumin, mushroom supplements and multi/mixed supplements for supporting immune function between healthy dogs and dogs with cancer can be found in Table 3.7. Use of probiotics, glucosamine/chondroitin, multivitamin/minerals, and marine or plant-derived n-3 FA supplements were not significantly different between healthy dogs and dogs with cancer (p>0.05).

3.5 Discussion

The findings of this study suggest that, as hypothesized, owners of dogs with cancer are more likely to feed a non-conventional diet. Particularly, the odds of owners of dogs with cancer feeding homemade cooked or homemade raw diets were higher compared to owners of healthy dogs. This finding is consistent with previously reported attitudes of owners of dogs with cancer(Rajagopaul et al., 2016), and may be based in part on have been feelings of distrust toward conventional dry and canned foods, worry that commercial foods contribute to cancer, beliefs that ingredients used in commercial products are of poor quality, a desire to avoid dietary carbohydrates following a cancer
diagnosis, and/or the belief that homemade cooked or raw diets could provide superior nutrition (Rajagopaul et al., 2016). Use of homemade cooked or raw diets may be of concern for dogs with cancer because previous analysis of homemade diet recipes found the majority to be unbalanced in one or more essential nutrients (Heinze et al., 2012; Stockman et al., 2013; Streiff et al., 2002), which puts dogs with cancer- or treatment-induced appetite-loss at higher risk for nutrient deficiencies. In addition, diets containing raw meat are at greater risk of containing bacterial contamination (Finley et al., 2008; Nemser et al., 2014; Strohmeyer et al., 2006; Weese & Rousseau, 2006), which is concerning for dogs affected by immunocompromising side-effects from cancer or cancer treatment. Unconventional diets have become more popular, but commercial dry food was still the most common diet type fed to both healthy dogs and dogs with cancer. Previous research in dogs with cancer reported that a conventional dry or wet diet was fed by 71% of owners (Rajagopaul et al., 2016), whereas the present study noted about 61% of owners of dogs with cancer were feeding a commercial dry or wet food as the primary diet type. This decrease in conventional diet feeding coincides with the observed increase in homemade diets fed to dogs with cancer. Previously, 7% of dogs with cancer received a homemade cooked diet and 4% received a homemade raw diet (Rajagopaul et al., 2016). In the present study, 39% of dogs with cancer were fed a homemade cooked diet and 18% of dogs received a homemade raw diet. One difference between how these studies were conducted that may impact results is the option to select more than one diet type; the present study allowed participants to select multiple answers, whereas the earlier study only reported on primary diet type. Our results suggest that owners are choosing to feed a combination of diet types, which may
be an area for future research. It is also important to note differences in these study populations since the previous study population consisted exclusively of owners presenting their pet to a referral oncology clinic, whereas the majority of participants in the present study were recruited via social media. More research is warranted to further investigate how feelings of distrust may impact the dietary decisions owners make for their dogs. Overall, following a cancer diagnosis, the present study suggests that owners may consider switching from a conventional diet to a homemade diet. Clinicians should be prepared to advise on species-appropriate homemade diet recipes to guide clients through this process to ensure dogs receive a complete and balanced diet (Parr & Remillard, 2014).

More than a decade ago, 10% of owners recruited through a telephone survey from the United States of America and Australia reported administering nutritional supplements to their healthy dogs and cats (Lisa M Freeman et al., 2006). The present study asked a similar question and found this number to be higher, with about half of owners of healthy dogs indicating nutritional supplement use. Chondroprotective agents and N-3 FA are still the most popular, similar to previous reports (Lisa M Freeman et al., 2006). In dogs with cancer, the majority of participants in the present study reported feeding their dog nutritional supplements, and the median number of supplements fed per dog was greater in dogs with cancer compared to healthy dogs. The most commonly reported products fed to dogs with cancer consisted of supplements made from herbs, botanicals and spices, fungal extracts, and fats or oils. Observed differences in supplement use, such as greater odds of feeding CBD/THC products,
mushroom supplements, turmeric or curcumin, and multi/mixed supplements for immune support in dogs with cancer may be a result of favoring different sources of information, since many of the supplements reported to be fed to dogs with cancer are products that are widely marketed in human health.

On October 17, 2018 the Government of Canada legalized the possession and recreational use of cannabis. Since the Cannabis Act was passed during the distribution of this survey it is possible that it influenced off-label use of cannabis products, such as CBD oil, by Canadian owners for their pets. Use of CBD oil in dogs should proceed cautiously due to the potential for toxicity (Brutlag & Hommerding, 2018) and due to the limited in vitro and in vivo research that has been conducted to establish safety and efficacy of these products. CBD oil products have been found to be both bioavailable and effective in increasing comfort and activity in a study of 16 client-owned dogs presenting to the Cornell University Hospital for Animals for evaluation or treatment of osteoarthritis (Gamble et al., 2018). CBD oil was also found to reduce frequency of seizures in a sample of 26 dogs living in Colorado, USA with idiopathic epilepsy (McGrath, Bartner, Rao, Packer, & Gustafson, 2019). However, more research is needed before CBD oil can be applied as a treatment for dogs with cancers.

Fatty acid supplements were not significantly different in healthy dogs or dogs with cancer, but they were highly reported among dogs with cancer, and this was not surprising because research has found that popular supplements among owners of dogs with cancer include n-3 FA supplements (Rajagopaul et al., 2016). The body of literature showing positive effects of fatty acids on cancers is larger than that of CBD oil.
Many \textit{in vitro} studies have been conducted and provide evidence that fatty acids like DHA and EPA are effective in reducing cancer cell viability through a variety of mechanisms in human cell lines (Abdi et al., 2014; Ding et al., 2018; Fukui et al., 2013; Kang et al., 2010; Shirota et al., 2005); however, only one \textit{in vitro} study has been conducted in canine cell lines (Pondugula et al., 2015). One \textit{in vivo} study investigated supplementation of menhaden oil in dogs with nasal carcinoma and found it may alleviate inflammatory side-effects of radiation therapy (Hansen et al., 2011) while another found n-3 PUFA supplementation increased the disease-free interval and survival time in dogs with lymphoma (Ogilvie et al., 2000). However, arginine was also supplemented in this group and lymphoma staging was based on subjective methodology, both of which greatly limits interpretation of the results (Ogilvie et al., 2000). Since N-3 FA supplements have also been previously reported at a high frequency among healthy animals, it is possible that other conditions, such as those impacted by aging, may be confounding reasons to administer these and other supplements that were highly reported among the dogs with cancer. It was previously reported that in dogs with cancer, half of owners who chose to feed a nutritional supplement did so because of their dog’s cancer diagnosis (Rajagopaul et al., 2016) and 17% of owners of dogs with cancer indicated choosing to pursue alternative and complementary therapies to attempt to cure cancer and/or reduce treatment-related toxicities (Lana et al., 2006). The present study did not evaluate owners’ rationale for choosing to feed specific types of supplements; therefore, this could be an area of investigation for future studies.
The use of herbal and fungal-derived products, such as turmeric/curcumin and various mushroom supplements have not been previously reported at high frequencies and may represent growing trends among dogs with cancer. There is limited research indicating that these products are safe or effective. Furthermore, it is not well known how any nutritional supplement may interact with conventional cancer therapy. So far, only one in vitro study of canine cancer cell lines has found turmeric extract to have some efficacy in inducing apoptosis of mastocytoma, mammary carcinoma, and osteosarcoma cells (Levine et al., 2017). Research investigating the use of mushroom supplements in cancer treatment is more extensive; however, the results are conflicting. In vitro and in vivo studies of human and murine cancers provide evidence that mushroom supplements may be a useful complementary cancer treatment (Alonso et al., 2017; Louie, Rajamahanty, Won, Choudhury, & Konno, 2010; Phil Kim, Hyun Nam, & Friedman, 2013; Xu, Huang, & Cheung, 2012), while a prospective non-controlled in vivo study of 15 dogs with lymphoma found no objective response to a nutritional supplement that included Maitake mushroom extract (Griessmayr, Gauthier, Barber, & Cotter, 2007). Clinicians should be aware of these growing trends and be equipped with evidence-based knowledge when providing nutritional advice, especially following a cancer diagnosis.

An interesting trend detected among owners of dogs with cancer was the influence of internet sources. As hypothesized, owners of dogs with cancer favored different sources of information when researching pet health, pet nutrition, and nutritional supplements. In particular, they were more likely to consult social media
groups for topics related to pet health, pet nutrition, and nutritional supplements than owners of healthy dogs. Owners of dogs with cancer were also more likely to consult blogs for information on nutritional supplements. This is in contrast to a 2006 study, which found only a minority of owners of dogs with cancer consulted internet sources when researching alternative therapies such as nutritional supplements (Lana et al., 2006). More recently, however, half of owners of dogs with cancer participating in qualitative interviews at an oncology referral centre in Ontario, Canada reported consulting internet sources in search of more information about cancer and treatment options for their dogs (Stoewen, Coe, MacMartin, Stone, & Dewey, 2019). The increase of internet use for information-seeking may be related to the growth in popularity of social media in recent years, as an estimated 2.7 billion people use social media websites each month (Facebook Reports Fourth Quarter and Full Year 2018 Results, 2019). The current study found owners of dogs with cancer consistently reported consulting internet sources, blogs, and social media at higher frequencies than owners of healthy dogs when researching pet health, pet nutrition, and nutritional supplements. This may explain why owners of dogs with cancer spent more time researching pet health, pet nutrition, and nutritional supplements than owners of healthy dogs, as internet sources tend to be easily accessible from a variety of locations and devices. It is also possible to consult internet sources at any time of the day, which may provide owners relief of uncertainty when it is not possible to consult a member of their pet's health care team. Provision of 24-hour information support is regarded as integral in managing feelings of uncertainty (Stoewen et al., 2019), therefore, clinicians should be aware of this and refer clients to reputable sources of information that are accessible at
any time for use in emergency, as well as in benign situations when advice or guidance is needed.

The use of different sources of information by owners of healthy dogs and owners of dogs with cancer may play a role in the observed differences in diet and supplement use between these groups, as supplements available online for purchase or research may vary from supplements available for purchase in pet stores. The source of information usually reported at the highest frequency among both owners of dogs with cancer and owners of healthy dogs is the veterinarian (Lana et al., 2006; Stoewen et al., 2019). Previous studies have reported that clients highly value information delivered by someone they have an established a relationship with, such as a regular oncology care provider or their dog’s primary care practitioner (Stoewen, Coe, MacMartin, Stone, & Dewey, 2014a; Stoewen et al., 2019). However, in situations when primary practitioners seemed to have inadequate knowledge of cancer or showed hesitancy to refer to a specialist, dog owners expressed concerns regarding the quality of information provided to them by their dog’s primary veterinarian (Stoewen et al., 2019). Clinicians should be aware that clients want to be informed about the details of their dog’s cancer as well as treatment options, especially during the early stages of treatment (Stoewen, Coe, MacMartin, Stone, & Dewey, 2014b). In addition, most clients arrive at an oncology service with little to no background information (Stoewen et al., 2014b). This offers clinicians with opportunities to provide clients with information and reputable resources to consult as a means to build trust and confidence with the oncology service (Stoewen et al., 2014b). Establishing a trusting relationship and equipping clients with timely, clear
and appropriate information allows clients to confidently make educated and informed decisions regarding treatment options for their dog (Stoewen et al., 2014b). This has yet to be studied in a nutrition context explicitly and more research should be conducted to determine if establishing a trusting client-clinician partnership and equipping clients with the information necessary to make decisions for their dog leads to changes in feeding practices in dogs with cancer. Until then, clinicians should equip clients with reliable resources, online and otherwise, that are available 24-hours a day and focus on establishing a partnership founded on trust with clients, that allows for open and judgement-free communication throughout the duration of their dog’s cancer treatment.

There are some limitations present in this study that may impact interpretation of the results. As the survey was only available in English, the sample population was representative of pet owners from some English-speaking countries (United States of America, Canada, United Kingdom, and Australia). The sample of pet owners responding to the survey was based on self-selection, which risks the possibility of sampling bias, and likely accounts for the gender bias detected. This was not unexpected considering previous studies in pet nutrition, and survey studies in general, that tend to show strong bias toward female pet owners (Dodd, Cave, Adolphe, Shoveller, & Verbrugghe, 2019; Lana et al., 2006; Rajagopaul et al., 2016). Furthermore, recruitment bias likely occurred due to the two types of recruitment strategies utilized. Participants recruited through social media may be different than participants recruited via a referral oncology centre or a primary care centre, which may have accounted for some of the differences in the results found in this study compared
to previous studies that sampled exclusively from oncology referral centres. It has been found that, however, that internet samples are more representative of the population at large with respect to gender, socioeconomic status, geographic location and age when compared to traditional sampling methods (Gosling, Vazire, Srivastava, & John, 2004).

Furthermore, current evidence indicates that internet-based findings are consistent with results based on traditional methods (Gosling et al., 2004), thereby justifying use of an online survey for this study. To minimize bias during recruitment, no reference to nutritional supplements was made in the distribution of the survey, however, references to feeding practices were made. Therefore, it is possible that owners who chose to include nutritional supplements in their dog’s daily feeding routine were more likely to participate. Owners self-reported the disease prevalence and health status of their dogs; therefore, it is possible that dogs included in the healthy group may have undiagnosed diseases that would have disqualified them from inclusion. In a separate study, some owners of dogs with cancer participating in a qualitative interview demonstrated having a limited understanding of their dog’s cancer diagnosis (Stoewen et al., 2014b). It is possible that some participants in the present study may demonstrate a similar limited understanding, which could have impacted the true prevalence of dogs with cancer and prevalence of types of cancer reported. Dogs with cancer that also had other disease or disease-associated signs were not excluded from analysis, which may bias the findings from the present study.

Another limitation is that owners were asked to report accurate information about their pet’s diet, which allows for recall bias if owners cannot accurately remember
details about their pet’s food or the supplements fed to their pets. Due to the cross-sectional nature of the study, researchers were not able to determine if supplements fed to dogs with cancer came before or after the cancer diagnosis was made. This means it is not possible to rule out the interpretation that some nutritional supplements may have been introduced due to conditions other than cancer, or that nutritional supplements played a role in causing the onset of cancer. Finally, though the sample population exceeds a priori sample size calculation requirements, conducting a similar study with a larger sample size (particularly a larger sample of dogs with cancer), could give rise to further analysis of the connections between diet, nutritional supplements, and cancer status. Further studies should compare attitudes and dietary decisions made by owners of dogs with cancer that are clients at an oncology referral centre to those who are not and should also compare attitudes and decisions between owners of dogs with different cancer types and prognoses. In addition, qualitative interviews could help uncover if the differences and similarities observed in nutritional supplements fed to healthy dogs and dogs with cancer are due to the presence of cancer or confounding disease.

3.6 Conclusion

This study represents one of the few studies investigating diet and nutritional supplements in dogs with cancer; it is the first to compare diet type, supplement use, and sources of information used in researching dietary decisions in healthy dogs and dogs with cancer. It was found, as hypothesized, that owners of dogs with cancer favored different sources of information when researching pet health, pet nutrition, and nutritional supplements as compared to owners of healthy dogs. In particular, owners of
dogs with cancer were more likely to consult social media when researching these topics than owners of healthy dogs. Diet differences were also found in dogs with cancer compared to healthy dogs, such as increased odds that owners of dogs with cancer would feed a homemade cooked diet or homemade raw diet compared to owners of healthy dogs, and that dogs with cancer were more likely to be fed nutritional supplements containing CBD/THC, mushrooms, turmeric/curcumin, or compounds for immune support. Alternative diets and supplements come with concerns regarding both safety and efficacy; therefore, it is important that clinicians partner with their clients when making decisions in order to be in a position to raise concerns when needed.
3.7 Acknowledgements

The authors declare no conflicts of interest. Dr. Verbrugghe is the Royal Canin Veterinary Diets endowed Chair in Canine and Feline Clinical Nutrition at the Ontario Veterinary College, serves on industry-related scientific advisory boards and has received honoraria and research grants in association with various pet food companies. Dr. Abood is the owner of Sit Stay Speak Nutrition, consults with various manufacturers within the pet food industry and is associated with Pet Recipe Designers. Dr. Coe regularly receives honoraria and research funding from various commercial pet food companies.

The authors thank Nada Hafez, Data Analyst, Research and Scholarship, University of Guelph for assistance with statistical analysis.

Presented as an oral presentation at the Institute for Comparative Cancer Investigation Symposium, Guelph, ON, Canada, May 2019 and as a poster presentation at the 23rd Congress of the European Society of Veterinary and Comparative Nutrition, Turin, Italy, September 2019.
3.8 Figures

**Figure 3.1:** Number of survey respondents (N) and proportion (%) of owners of dogs with cancer and owners of healthy dogs consulting various sources of information when researching pet health (A), pet nutrition (B) and nutritional supplements (C). Other includes animal
behaviorists, books, conferences, dog shows, dog trainers, health food stores, holistic veterinarians, magazines, nutritionists, other dog owners, pet food companies, pharmacists, and universities.* Indicates significant (Chi-square, P < 0.05) differences detected between sources consulted by owners of healthy dogs and owners of dogs with cancer.
Figure 3.2: Number (N) and proportion (%) of diet types reported among owners of dogs with cancer (N=132) and owners of healthy dogs (N=213) responding to an online survey regarding diet and supplement use.

“Other” includes diets that did not fit into the categories given, or responses that did not provide adequate information for the researchers to categorize manually. These include: grain free diet, commercial hand cooked food, commercial homemade wet food, dehydrated food, freeze dried food, food toppers, green beans, base for a low-carb diet, and veterinary therapeutic diets.

* Indicates significant (Chi-square, P < 0.05) differences detected between diet types fed to healthy dogs and dogs with cancer.
Figure 3.3: Number (N) and proportion (%) of supplements fed to dogs with cancer and healthy dogs as reported by dog owners responding to an online survey regarding diet and supplement use.

“Other” includes apple cider vinegar, bone broth, greens powder, dehydrated lamb tripe, fermented veggies and nuts, flax seeds, raw egg, artemisinin and when the participant indicated they were unsure what the supplement they provided was.
* Indicates categories where significant (Chi-square, P < 0.05) differences were detected in types of supplements fed to healthy dogs and dogs with cancer.

### 3.9 Tables

Table 3.1: Demographic characteristics of owners of dogs with cancer and owners of healthy dogs responding to an online survey regarding diet and supplement use.

<table>
<thead>
<tr>
<th></th>
<th>Owners of dogs with cancer (N = 132)</th>
<th>Owners of healthy dogs (N = 213)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>6</td>
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</tr>
<tr>
<td>Female</td>
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<tr>
<td><strong>Age</strong></td>
<td></td>
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</tr>
<tr>
<td>18-29</td>
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<td>30-39</td>
<td>21</td>
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<td>40-49</td>
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<td>50-59</td>
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<tr>
<td>60-69</td>
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</tr>
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<tr>
<td>No response</td>
<td>27</td>
<td>20.5</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td><strong>Country of residence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>78</td>
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</tr>
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<td>Canada</td>
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<td>United Kingdom</td>
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</tr>
<tr>
<td>Australia</td>
<td>7</td>
<td>5.3</td>
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<tr>
<td>Other</td>
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<tr>
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<tr>
<td><strong>Level of education</strong></td>
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<td>Secondary school diploma</td>
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### Table 3.2: Characteristics of dogs with cancer and healthy dogs that were the subject of an online dog owner survey regarding diet and supplement use. (N = 345)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Dogs with cancer (N=132)</th>
<th>Healthy dogs (N=213)</th>
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<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Neutered male</td>
<td>59</td>
<td>44.7</td>
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<td>Intact male</td>
<td>4</td>
<td>3.0</td>
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<tr>
<td>Spayed female</td>
<td>66</td>
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<tr>
<td>Intact female</td>
<td>3</td>
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<tr>
<td>Age (Mean ± SD)</td>
<td></td>
<td></td>
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<tr>
<td>Breed</td>
<td>Dogs with cancer (N=132)</td>
<td>Healthy dogs (N=213)</td>
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<tr>
<td>Mixed breed</td>
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<td>30.3</td>
</tr>
<tr>
<td>Pure breed</td>
<td>90</td>
<td>68.2</td>
</tr>
<tr>
<td>No response</td>
<td>2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

N, number of respondents; %, proportion.
Countries of residence reported under “Other” included Austria, Brazil, Denmark, Germany, Greece, Hong Kong, New Zealand, and South Korea.
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<th>Frequency of wellness exam</th>
<th>Less than annually</th>
<th>Annually</th>
<th>Biannually</th>
<th>Triannually</th>
<th>Quarterly</th>
<th>Bimonthly</th>
<th>Monthly</th>
<th>Biweekly</th>
<th>Weekly</th>
<th>Daily</th>
<th>Never</th>
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<td>66</td>
<td>31</td>
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<td>6</td>
<td>4</td>
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<td>2.3</td>
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<td>21.6</td>
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<table>
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<th>Adenocarcinoma</th>
<th>Transitional cell carcinoma</th>
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<td></td>
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<tr>
<td>----------------------------------</td>
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<table>
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<tr>
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<td>2016</td>
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<td>8.3</td>
</tr>
<tr>
<td>2015</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>2013 or earlier</td>
<td>6</td>
<td>4.5</td>
</tr>
<tr>
<td>No response</td>
<td>4</td>
<td>3.0</td>
</tr>
</tbody>
</table>

N, number of respondents; %, proportion; SD, standard deviation. Other cancer types included hepatocellular carcinoma, intestinal, kidney, leukemia, lung, mammary, multiple myeloma, nasal, oral melanoma, soft tissue sarcoma, spleen, squamous cell carcinoma, stromal sarcoma, and thyroid.
Table 3.3: Odds of owners of dogs with cancer and healthy dogs responding to an online survey regarding diet and supplement use consulting pet stores, social media groups and blogs as an information source about pet health, pet nutrition and supplements

<table>
<thead>
<tr>
<th>Sources of information</th>
<th>Research topics</th>
<th>Dogs with cancer (N=132)</th>
<th>Healthy dogs (N=213)</th>
<th>OR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Pet stores</td>
<td>Pet health topics</td>
<td>12</td>
<td>9.1</td>
<td>50</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>Pet nutrition topics</td>
<td>23</td>
<td>17.4</td>
<td>64</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>Nutritional supplements</td>
<td>16</td>
<td>12.1</td>
<td>51</td>
<td>24.4</td>
</tr>
<tr>
<td>Social media groups</td>
<td>Pet health topics</td>
<td>103</td>
<td>78.0</td>
<td>101</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>Pet nutrition topics</td>
<td>86</td>
<td>65.1</td>
<td>84</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td>Nutritional supplements</td>
<td>84</td>
<td>63.6</td>
<td>70</td>
<td>34.8</td>
</tr>
</tbody>
</table>
N, number of respondents; %, proportion; OR, odds ratio Significant (chi-square test, P < 0.05) differences between dogs with cancer and healthy dogs are indicated by p-values.

Table 3.4: Odds of owners of dogs with cancer and healthy dogs responding to an online survey regarding diet and supplement use feeding their dogs commercial dry, homemade cooked and homemade raw diets.

<table>
<thead>
<tr>
<th>Diet type</th>
<th>Dogs with cancer (N=132)</th>
<th>Healthy dogs (N=213)</th>
<th>OR</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Commercial dry food</td>
<td>61</td>
<td>46.2</td>
<td>168</td>
<td>78.9</td>
</tr>
<tr>
<td>Homemade cooked diet</td>
<td>54</td>
<td>40.9</td>
<td>27</td>
<td>12.7</td>
</tr>
<tr>
<td>Homemade raw diet</td>
<td>25</td>
<td>18.9</td>
<td>20</td>
<td>9.4</td>
</tr>
</tbody>
</table>
Table 3.5: Time spent researching pet health topics, pet nutrition and nutritional supplements, proportion of pet owners feeding nutritional supplements and number of supplements fed per dog in dogs with cancer and healthy dogs from an online dog owner survey

<table>
<thead>
<tr>
<th>Time spent researching (min)</th>
<th>Dogs with cancer (N=132)</th>
<th>Healthy dogs (N=213)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pet health topics – median (IQR)</td>
<td>120 (60-405)</td>
<td>60 (30-180)</td>
<td>1.1x10^-5</td>
</tr>
<tr>
<td>Pet nutrition topics – median (IQR)</td>
<td>60 (30-240)</td>
<td>60 (10-120)</td>
<td>1.9x10^-3</td>
</tr>
<tr>
<td>Nutritional supplements – median (IQR)</td>
<td>60 (15-180)</td>
<td>15 (0-60)</td>
<td>5.1x10^-9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supplement use</th>
<th>Respondents - N</th>
<th>88</th>
<th>105</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respondents - %</td>
<td>66</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Number of supplements – median (IQR)</td>
<td>3 (2-6)</td>
<td>2 (1-3)</td>
<td>9.8x10^-5</td>
</tr>
</tbody>
</table>

| Number of supplements – range (min, max) | 1, 20 | 1,15 |

N, number of respondents; %, proportion; IQR, Inter-Quartile Range reported as the range from Q1-Q3; min, minimum; max, maximum.
Significant (Mann Whitney U, P < 0.05) differences between dogs with cancer and healthy dogs are indicated by p-values.

Table 3.6: Supplement category code book to categorise supplements reported by owners of dogs with cancer and healthy dogs responding to an online survey regarding diet and supplement use.

<table>
<thead>
<tr>
<th>Category</th>
<th>Supplements</th>
</tr>
</thead>
</table>
| Amino acids                     | L-glutamine
S-adenosyl methionine
Taurine |
| Bee products                    | Bee propolis
Honey |
| Chondroprotective agents        | Glucosamine/chondroitin
Green-lipped mussel powders
Methysulfonylethanolamine |
| Herbs, botanicals spices       | Adaptogenic supplements
Boswellia
Broccoli extract
Chaparral
Chinese herbs (various)
Chlorella/spirulina
Cleavers tincture
Copaiba
Cranberry supplements
Diatomaceous earth
Dorwest tree bark powder
Essiac tea
Great sassurea coptis
Herbal flea and tick preventative
Milk thistle
Moringa oleifera
Red clover extract
Si miao san
Slippery elm
Stasis breaker
Turmeric/curcumin/golden paste
Yunaan baiyao |
| Enzymes and co-enzymes          | Coenzyme Q10
Digestive enzymes
Systematic enzymes |
| Fats and oils                   | Butyric acid
CBD and THC products
Coconut oil
Essential oils
Lethicin |
<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine and plant-derived n-3 FA supplements (various)</td>
<td>Olive oil</td>
</tr>
<tr>
<td>Fiber, carbohydrates, and sugars</td>
<td>D-mannose</td>
</tr>
<tr>
<td></td>
<td>Fibre supplements</td>
</tr>
<tr>
<td></td>
<td>Psyllium husk</td>
</tr>
<tr>
<td></td>
<td>Pumpkin</td>
</tr>
<tr>
<td>Fungi and fungal extracts</td>
<td>Mushroom supplements (various)</td>
</tr>
<tr>
<td></td>
<td>Nutritional yeast</td>
</tr>
<tr>
<td>Multi/mixed supplements for organ support</td>
<td>Variety of active compounds that were designed to support organ systems including:</td>
</tr>
<tr>
<td></td>
<td>Anal gland supplements</td>
</tr>
<tr>
<td></td>
<td>Calming supplements</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular system support</td>
</tr>
<tr>
<td></td>
<td>Dental health supplements</td>
</tr>
<tr>
<td></td>
<td>Supplements supporting eye health</td>
</tr>
<tr>
<td></td>
<td>Immune system support</td>
</tr>
<tr>
<td></td>
<td>Liver support supplements</td>
</tr>
<tr>
<td></td>
<td>Skin and coat supplements</td>
</tr>
<tr>
<td></td>
<td>Thyroid supplements</td>
</tr>
<tr>
<td></td>
<td>Urinary system supports</td>
</tr>
<tr>
<td>Multivitamins/minerals</td>
<td>Various brands and types of supplements containing a combination of vitamins and minerals to support overall health</td>
</tr>
<tr>
<td>Probiotics</td>
<td>Variety of brands and types of live cultures of bacteria for gut health</td>
</tr>
<tr>
<td>Minerals</td>
<td>Calcium</td>
</tr>
<tr>
<td></td>
<td>Himalayan salt</td>
</tr>
<tr>
<td></td>
<td>Kelp</td>
</tr>
<tr>
<td></td>
<td>Magnesium</td>
</tr>
<tr>
<td></td>
<td>Manganese</td>
</tr>
<tr>
<td></td>
<td>Phosphorous</td>
</tr>
<tr>
<td></td>
<td>Potassium</td>
</tr>
<tr>
<td></td>
<td>Selenium</td>
</tr>
<tr>
<td></td>
<td>Silver colloidal</td>
</tr>
<tr>
<td></td>
<td>Zinc</td>
</tr>
<tr>
<td>Vitamins and vitamin-like substances</td>
<td>Astaxanthin</td>
</tr>
<tr>
<td></td>
<td>B-vitamins</td>
</tr>
<tr>
<td></td>
<td>Folate</td>
</tr>
<tr>
<td></td>
<td>Inositol</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
</tr>
<tr>
<td></td>
<td>Vitamin C</td>
</tr>
<tr>
<td></td>
<td>Vitamin D</td>
</tr>
<tr>
<td></td>
<td>Vitamin E</td>
</tr>
<tr>
<td>Other</td>
<td>Apple cider vinegar</td>
</tr>
<tr>
<td></td>
<td>Artemisinin</td>
</tr>
<tr>
<td>Supplement</td>
<td>Dogs with cancer (N=132)</td>
</tr>
<tr>
<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>CBD/THC</td>
<td>23</td>
</tr>
<tr>
<td>Tumeric/curcumin</td>
<td>15</td>
</tr>
<tr>
<td>Mushroom supplements (various)</td>
<td>19</td>
</tr>
<tr>
<td>Multi/mixed supplements supporting immune function</td>
<td>13</td>
</tr>
</tbody>
</table>

N, number of respondents; %, proportion; OR, odds ratio

Significant (chi-square test, P < 0.05) differences between dogs with cancer and healthy dogs are indicated by p-values
CHAPTER 3

4 *In vitro* study of eicosapentaenoic and docosahexaenoic acid in combination with doxorubicin in canine osteosarcoma cell lines
4.1 Abstract

Objective: Determine if EPA and DHA reduce viability of canine OSA cell lines and determine if combination treatments of EPA or DHA with the chemotherapy drug doxorubicin are additive or synergistic.

Samples: Cultures of canine osteosarcoma cell lines D17 and Abrams were used.

Procedures: Cells were treated with increasing doses of doxorubicin, EPA and DHA as single agents for 72 hours. Following incubation, cells were stained with crystal violet and absorbance was read to estimate cell viability. Half-maximal inhibitory concentration (IC50) values were estimated based on the crystal violet assay data, and combination drug treatments were performed for doxorubicin with EPA or DHA. Synergism and efficacy of the drug combinations were assessed by computing combination index (CI) values using the Chou-Talalay method.

Results: EPA and DHA did not consistently impact cell viability when used as single agents. Doxorubicin treatment yielded IC50 values in the nanomolar range in both cell lines, whereas EPA and DHA both yielded IC50 values in the micromolar range, when values could be calculated. When CI values could be calculated, pharmacological antagonism (CI>1) was observed in all doxorubicin plus EPA or DHA combinations.

Conclusions and Clinical Relevance: Although the results of this study have limited interpretation, it was found that DHA and EPA had no consistent significant effects on canine OSA cells alone or in combination with chemotherapy. These findings support a need for more in vitro research evaluating the effects of EPA and DHA in canine OSA.
4.2 Introduction

OSA is the most common primary bone malignancy in dogs, with an estimated annual incidence of at least 139 cases per million dogs in the United States of America (Rowell, McCarthy, & Alvarez, 2011). The disease typically manifests as a tumor in the appendicular skeleton of older dogs and large breed dogs. Tumors then eventually metastasize, especially in dogs with tumors of the trunk (Diessner et al., 2019). Standard of care treatment of canine OSA includes surgical excision of the primary tumor, either through amputation of the affected limb or by limb salvage surgery (MacEwen & Kurzman, 1996). In addition to surgery, adjuvant chemotherapy is used to delay metastasis and increase overall survival time (MacEwen & Kurzman, 1996). In the past, OSA has been shown to respond well to treatment with chemotherapy drugs, in particular carboplatin and doxorubicin (MacEwen & Kurzman, 1996). However, current research has found a variety of mechanisms resulting in chemoresistance, presenting a need for research of novel treatment strategies (He, Ni, & Huang, 2014). Additionally, clients may choose to supplement chemotherapy treatment with alternative medicine, or forego chemotherapy treatment entirely based on fears of toxicity (MacEwen & Kurzman, 1996), and instead choose to provide their dog with alternative therapies such as nutritional supplements, as described in previous surveys of clients visiting tertiary oncology centres (Lana et al., 2006; Rajagopaul et al., 2016) and in Chapter 2 of this thesis.

In recent years, approximately 40% of surveyed clients chose to feed nutritional supplements to their dogs with cancer (Lana et al., 2006; Rajagopaul et al., 2016),
though in 2019 this figure has increased to 66%, as described in chapter 2. In particular, supplements containing n-3 fatty acids such as DHA and EPA have been found to be popular among owners of dogs with cancer (Rajagopaul et al., 2016) despite a lack of compelling evidence for the safety and efficacy of these products. To date, there has been one in vitro study of fatty acid use in canine lymphoma cells where it was found that the plant-based n-3 fatty acid stearidonic acid successfully increased sensitivity of two chemoresistant cell lines to the chemotherapy drugs doxorubicin and vincristine (Pondugula et al., 2015). However, previous in vitro and in vivo studies investigating fatty acid treatment in human and murine cancers have found conflicting results, with some studies citing enhanced toxicity of chemotherapy drugs when used in combination with n-3 fatty acids, while others uncovered inconsistent results and declared a need for more research (Calviello et al., 2005; Germain et al., 1998; Mahéo et al., 2005; Menendez et al., 2005; Wynter et al., 2004). These inconsistencies in the literature, coupled with the fact that dogs with cancer are already receiving fatty acid supplementation drives a need for more research pertaining to dogs in order to aid oncology clinicians in providing advice for owners who show an interest in feeding these supplements.

The purpose of this study was to investigate the impact of EPA and DHA on the viability of canine OSA cell lines and determine if combination treatments of EPA or DHA with the chemotherapy drug doxorubicin are additive, synergistic, or antagonistic. The hypotheses were that single agent treatment of EPA and DHA would reduce viability of canine OSA cell in a dose dependent manner, and that combinations of EPA or DHA with doxorubicin would be synergistic or additive.
4.3 Materials and methods

4.3.1 Cell lines and culture conditions

Canine osteosarcoma cell lines D17 and Abrams were used. D17 cells were obtained from the Sigma-Aldrich/European Collection of Cell Cultures (ECACC). Abrams cells were provided by Dr. Michael K. Huelsmeyer at the University of Wisconsin. Both D17 and Abrams cell lines have been characterized and published as canine OSA cells based on morphology and xenograft analysis (Legare, Bush, Ashley, Kato, & Hanneman, 2011). All cells were cultured in high glucose Dulbecco's Modified Eagle Medium\(^1\) with 10% fetal bovine serum (FBS)\(^1\) and 100 U/mL penicillin/streptomycin\(^1\). All cell cultures were maintained at 37°C and 5% CO\(_2\) in a humidified incubator.

4.3.2 Fatty acids and chemotherapy drugs

The fatty acids DHA and EPA were purchased from a commercial manufacturer\(^2\). Stock solution was stored at -80°C and flushed with nitrogen for long-term storage. Pure DHA and EPA was suspended in 100% ethanol at 1 mg/mL. This diluted solution was stored at -20°C. Fatty acids were stored in glass and handled with glass instruments whenever possible to prevent loss of fatty acids due to adhesion to plastic surfaces. Fatty acids were prepared for use in experiments by combining DHA or EPA with FBS in a glass culture tube, which was then incubated in a water bath set to 37°C for 30 minutes. The glass culture tube was vortexed every 10 minutes to ensure a

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\(^1\) Thermo Fisher Scientific, Waltham, Massachusetts.
\(^2\) NuCheck Prep Inc., Elysian, Minnesota.
homogenous mixture. Fatty acid and FBS mixture was then added to conditioned media that had not been previously supplemented with FBS, such that the total amount of FBS was 10%. Doxorubicin was obtained from the Ontario Veterinary College pharmacy and was maintained at its stock concentration of 2 mg/mL in isotonic solution.

4.3.3 Crystal violet cytotoxicity assay

OSA cell lines were seeded at 2,000 cells per well in a 96-well plate and incubated for 24 hours. This cell density was previously optimized to reach confluency following a 72-hour incubation period. For dose-response curves, each cell line was treated with increasing doses of doxorubicin (1, 3, 10, 30, 100, 300, 1000 nM), EPA (6.25, 12.5, 25, 50, 100, 150, 200 μM), and DHA (6.25, 12.5, 25, 50, 100, 150, 200 μM) for 72 hours. Doses were selected based on previously conducted experiments. The cells were then fixed with 0.5% crystal violet in 20% methanol, and the dye was eluted with 10% acetic acid after drying overnight. The absorbance at 590 nm (A₅₉₀) was read in by a Synergy 2 Multi-detection Microplate Reader. Each sample was tested in three replicate wells, therefore absorbance was read in triplicate and normalized to a vehicle control. Half-maximal inhibitory concentration (IC50) curves were fit by nonlinear regression and averaged by experiments conducted in triplicate.

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3 Accord Healthcare, Kirkland, Quebec.
4 Thermo Fisher Scientific, Waltham, Massachusetts.
5 BioTek Instruments Inc., Winooski, Vermont.
4.3.4 Combination index experiments

Combination drug treatments were performed for doxorubicin with EPA or DHA compared to single agent treatment. Five drug concentration pairings were chosen near the estimated IC50 values for each drug. Drugs were given simultaneously, and incubation time was 72 hours. Synergism and efficacy of the drug combinations were assessed by computing CI values using the Chou-Talalay method for drug combination, which is based on the median-effect equation (Chou, 2010). This method defines additive drug effects as CI=1, synergism as CI<1 and antagonism as CI>1 (Chou, 2010).

4.3.5 Statistical analysis

Statistical testing was performed and figures were made using GraphPad Prism\(^6\). Software\(^7\) based on the Chou-Talalay method was used for the computation of CI. Means were reported with SE for descriptive statistics.

4.4 Results

Doxorubicin treatment yielded IC50 values in the nanomolar range in both cell lines, whereas EPA and DHA both yielded IC50 values in the micromolar range (Figure 4.1). In one trial with EPA and doxorubicin, no CI values could be calculated due to a lack of reduction in cellular viability. In all other trials, pharmacological antagonism (CI>1) was observed in all doxorubicin plus EPA or DHA combinations (Table 4.1-4).

\(^6\) GraphPad Software Inc, La Jolla, California.

\(^7\) ComboSyn Inc, Paramus, New Jersey.
4.5 Discussion

To the authors' knowledge, this study is the first to investigate the effects of n-3 FA, in particular DHA and EPA, on canine OSA cells as single agents and to evaluate the effects of DHA and EPA in combination with chemotherapy drugs. The results of this study indicate that in these cell lines, and under the specified conditions, both EPA and DHA decreased cell viability when applied as a single agent, however, these results were inconsistent both within technical replicates and between experiments. This inconsistency may have occurred for a number of reasons. It is possible that limitations of the viability assay may have influenced the results. Crystal violet staining (CVS) was chosen to estimate cellular viability because compared to other options, it is a simple, non-enzymatic assay (Saotome, Morita, & Umeda, 1989). CVS lacks some of the limitations observed by enzymatic reactions (for example MTT assays) such as influence from compounds that modify cell metabolism by increasing the NADPH level or the activity of lactate dehydrogenase (Bruggisser, Von Daeniken, Jundt, Schaffner, & Tullberg-Reinert, 2002; Shoemaker, Cohen, & Campbell, 2004; Vistica, Monks, Pittman, & Boyd, 1991; Wang, Henning, & Heber, 2010; Zhang, Li, Xu, & Zeng, 2005). Furthermore, the MTT tetrazolium salt used in the MTT reduction can be reduced in areas other than the mitochondria, such as the cytoplasm, cell surface, endosome or lysosome membranes or even in the extracellular environment (Berridge, Herst, & Tan, 2005). The reduction process can be further influenced by factors such as phase of cell growth, cell cycle phase and reaction conditions such as pH and D-glucose concentration (Berridge et al., 2005; Chiba, Kawakami, & Tohyama, 1998; Vistica et al., 1991; Wang et al., 2010).
By contrast, CVS can quickly quantify viable cells adhering to the plate by taking advantage of the affinity between the dye and the external surface of the DNA double helix (Śliwka et al., 2016). The amount of DNA in the cell culture determines how much of the dye is absorbed and provides an estimate of viable cells (Śliwka et al., 2016).

Despite these advantages, there are also some limitations to CVS assays. If cell culture grows beyond confluence, the absorbance of stained cells does not correspond well to viable cell numbers (Chiba et al., 1998). In addition, non-uniform cell growth on the bottom of wells can also cause variable results (Chiba et al., 1998). This could be confirmed by using a microscope in future studies as a quality control step. Since the estimate of viability relies on cells adhering to the well, it is also possible for dead cells to be counted among the living cells if these cells remain adherent, due to non-specific nature of the CVS binding (Chiba et al., 1998). To mitigate these limitations, studies should ensure cells only just reach confluence after a 3-day incubation, as cell size tends to become smaller and cell size will not correspond to the absorbance of the stained cells if the culture is continued beyond confluence (Saotome et al., 1989). To ensure uniform cell growth along the bottom of each well, plates should be left undisturbed for 20 minutes before they are carried to the incubator (Saotome et al., 1989). Other possible confounding factors that may have impacted results include pipetting errors, and lot-lot variability of reagents. When possible, a multi-channel pipettor was used to dispense an equal volume to each well, however channels were not checked for accuracy, and future studies should incorporate an objective check for accuracy following pipetting to prevent pipetting errors. Future studies should also
prioritize using reagents within the same lot to ensure as little variability in reagent quality as possible.

Fatty acids must be handled and prepared carefully as they are sensitive to light and temperature. As with any reagent, precise instruments and careful technique should be used when preparing the fatty acids for use in cell culture and when preparing doses of fatty acids. Preparation methods of fatty acids in this study were similar to those previously reported, however, use of dark-coloured glass vials for storage of stock solution should be considered in future studies to better preserve stored fatty acid (Weng, Leung, Pang, Kuo, & Hsu, 2018). In addition, a positive control was not included in this study to prove the bioactivity of the fatty acids, therefore future studies could include a positive control to confirm fatty acids are bioactive and rule out the possibility that negative results are due to the fatty acids themselves.

When EPA and DHA were tested in combination with the chemotherapy drug doxorubicin antagonistic effects were observed, however, CI values were inconsistent both within and between experiments. These inconsistencies were first observed when working with the cell line D17 and the reproducibility issues persisted in all experiments with Abrams cell lines. These inconsistencies within experiments and between experiments limit interpretation of the results. Reagents were not tested as monotherapies and as combination therapies in parallel, which means it is not possible to determine if the results observed in combination and the previously determined IC50 values could be recapitulated. Therefore, future experimental designs should incorporate parallel testing of monotherapies and combination therapies.
Doxorubicin was chosen as the chemotherapy agent in these studies because it has been commonly used in the treatment of dogs with OSA (Kochevar DT, Barber LG, & Burgess KE, 2012). The mechanisms of action for doxorubicin include disrupting helical DNA structure, targeting topoisomerase II (Nitiss, 2009) and RNA polymerases, and generating free radicals (Kochevar DT et al., 2012). These multiple mechanisms of action make doxorubicin more broadly toxic to cancer cells, especially compared to other chemotherapy drugs commonly used in treating OSA, such as carboplatin (Kochevar DT et al., 2012).

Previously, only one study to-date determined that the combination of n-3 FA and chemotherapy drugs is effective in reducing cellular viability in canine cancer cells (Pondugula et al., 2015). The present study shared similarities in methodology to the prior study, in that doxorubicin was used as a chemotherapy drug, canine cancer cells were grown and cultured under similar conditions, and the n-3 FA used were reconstituted in ethanol. Despite these similarities, there were four main differences in methodology that may explain the observed differences in results. The first is the source of n-3 FA, which was stearidonic acid in the prior report and fish oil in the present study. It is possible that the use of different sources and types of n-3 FA produced the different results. Several in vitro studies in various human cancer cell lines (Abdi et al., 2014; Calviello et al., 2004; Ding et al., 2018; Fukui et al., 2013; Kang et al., 2010; K. Lim et al., 2009; Shirota et al., 2005; Zou et al., 2015), as well as various in vivo murine cancer models (Chen et al., 2014; Ramos et al., 2004; Zou et al., 2015) have found DHA and EPA to have potent anticancer effects. However, it is possible that differences exist between canine and human/murine cell lines that may account for the observed
differences in DHA and EPA activity. There may also be a sarcoma-related lack of effect, as there is little research investigating the role of n-3 PUFAs in osteosarcoma. To the authors' knowledge, one study has been conducted to investigate the role of DHA derivatives, EDP-EAs in human osteosarcoma cell lines (Roy, Watson, Hong, Fan, & Das, 2018). It was found that EDP-EAs exhibited antiangiogenic, antitumorigenic, and antimigratory properties, however, these compounds are structurally different from DHA and any observed effects may be due to these differences (Roy et al., 2018).

In addition to using different n-3 FA types and sources, the doses tested in the current study were different from doses used in a study in canine cancer cells by Pondugula et al. (2015). The Pondugula et al. (2015) study used a wider range of doses, starting at 10 µM and ending at 400 µM, while the present study used a narrower range of n-3 FA doses, 6.25 to 200 µM. These differences in dose may be warranted in a dose-response study based on differences in cell density. Though both studies seeded cells in 96-well plates, the previous study seeded cells at 10,000 cells per well and the present study seeded cells at 2,000 cells per well. The reduction in cellular viability observed by Pondugula et al. (2015) may be due to overgrowth of cells, which would induce cell death, therefore future studies should prioritize optimizing seeding density prior to testing therapeutic agents to ensure there is no overgrowth.

Differences in cell type may have also played a role in the different observed results. While the current study used canine OSA cells, the previous study investigated n-3 FA activity in a culture of chemoresistant canine LSA cell lines, defined by cells showing little to no response to increasing doses of chemotherapy drugs vistacrine and
doxorubicin. This may indicate that n-3 FA action in canine cancer is cell line- or even cancer-specific and may have contributed to the differing results. Cell lines used in the present study were not chemoresistant, which may also have impacted the results; it is possible that n-3 FA are most effective in chemoresistant canine cancer cells. Different methodology was used to assess cellular viability. CVS was not used in the previous study, rather a CellTiter-Glo luminescent cell viability assay was used. This assay estimates viable cells by quantifying the presence of ATP as a measure of metabolically active cells.

Finally, the method used to compare cell viability between treatments was different. The previous study did not calculate CI values when combining chemotherapy drugs and n-3 FA; rather cellular viability was simply compared between combination and single agent treatment of chemotherapy and n-3 FA. This makes sense, since chemoresistant cell lines were studied; it was, therefore, not possible to determine an IC50 value for the chemotherapy drugs in these cells, which is an important step in calculating CI values. The present study suffered the opposite problem in that IC50 values could be estimated for chemotherapy drugs in both OSA cell lines tested, but it was difficult to determine consistent dose response curves for DHA and EPA, and the IC50 value used for CI calculations was roughly estimated based on dose response curves that could be generated.

Future studies should prioritize refining delivery of DHA and EPA to ensure consistent dose-response curves and IC50 values. Other aspects of cancer biology should also be evaluated, through the use of apoptosis assays, interrogation of the
effects on growth signaling pathways, and assays to detect potential changes in metabolic activity of cancer cells following treatment with n-3 FA. Chemoresistant cell lines may also prove to be an interesting future area of application for fatty acids, therefore, future studies could investigate the effects of fatty acids on different types of chemoresistant cancers.

4.6 Conclusion

This study represents the first investigating effects of EPA and DHA in canine OSA cells, both as single agents and in combination with the chemotherapy drug doxorubicin. The results of this study have limited interpretation due to reproducibility issues observed within and between experiments. Therefore, more research is needed to evaluate the effects of EPA and DHA in canine OSA.
4.7 Acknowledgments

Funding for the project was provided through a Pet Trust grant.

Dr. Verbrugghe is the Royal Canin Veterinary Diets endowed Chair in Canine and Feline Clinical Nutrition at the Ontario Veterinary College, serves on industry-related scientific advisory boards and has received research grants in association with various pet food companies.
4.8 Figures

Figure 4.1: Crystal violet assays show that high doses of DHA and EPA reduce viability of canine OSA cells, based on IC50 curves of cells treated with increasing concentrations of doxorubicin, DHA and EPA for 72 hours and the corresponding cell viability measured by CVS.
Graphs of Abrams cells represent mean SE of three technical replicates and one biological replicate, graphs of D17 cells represent mean SE of three technical replicates and three biological replicates.

IC50, half-maximal inhibitory concentration; OSA, osteosarcoma; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; CVS, Crystal Violet Staining; Relative A590 is defined as; Log[doxorubicin] nM, the log of each tested dose of doxorubicin in nM; Log[EPA] µM, the log of each tested dose of EPA in µM; Log[DHA] µM, the log of each tested dose of DHA in µ
### 4.9 Tables

**Table 4.1: CI values of combinations of doxorubicin and EPA in D17 cell lines**

<table>
<thead>
<tr>
<th>Doxorubicin dose (nM)</th>
<th>EPA dose (µM)</th>
<th>CI values – Trial 1</th>
<th>CI values – Trial 2</th>
</tr>
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<tr>
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NaN, CI values could not be calculated

**Table 4.2: CI values of combinations of doxorubicin and DHA in D17 cell lines**

<table>
<thead>
<tr>
<th>Doxorubicin dose (nM)</th>
<th>DHA dose (µM)</th>
<th>CI values – Trial 1</th>
<th>CI values – Trial 2</th>
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Table 4.3: CI values of combinations of doxorubicin and EPA in Abrams cell lines

<table>
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<tr>
<th>Doxorubicin dose (nM)</th>
<th>EPA dose (µM)</th>
<th>CI values – Trial 1</th>
<th>CI values – Trial 2</th>
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Table 4.4: CI values of combinations of doxorubicin and DHA in Abrams cell lines

<table>
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<th>DHA dose (µM)</th>
<th>CI values – Trial 1</th>
<th>CI values – Trial 2</th>
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CHAPTER 4

5 General discussion
5.1 Introduction

The purpose of this thesis was to describe supplement use in dogs with cancer and compare this to supplement use in healthy dogs. In particular it aimed to determine and compare sources of information consulted by owners of dogs with cancer and owners of healthy dogs when researching pet health, pet nutrition and nutritional supplement topics, as well as compare diet types and nutritional supplements fed to dogs with cancer and healthy dogs. This drove the authors to further investigate popular supplements reported among dogs with cancer, which fueled the final purpose of this thesis; investigating \textit{in vitro} anti-cancer properties of n-3 FA DHA and EPA both as single agents and in combination with doxorubicin in canine OSA cells. Determining differences in diet between dogs with cancer and healthy dogs, and how pet owners inform their choices regarding their pet’s nutrition, serves to guide veterinarians when advising their clients, especially following a cancer diagnosis. Few studies have investigated anti-cancer properties of n-3 PUFA in canine cells; therefore, \textit{in vitro} study of DHA and EPA is warranted to determine if products containing these fatty acids have an effect on cancer cells. This research can then be used to justify further \textit{in vitro} and \textit{in vivo} research in dogs to expand the limited body of literature investigating the effects of n-3 PUFA supplementation in companion animals.

The findings of this research have improved knowledge of differences in pet feeding practices in dogs with cancer and healthy dogs. Previous research studying feeding practices in dogs with cancer did not compare the responses obtained from owners of dogs with cancer to a similar population of owners of healthy dogs. In
addition, the *in vitro* study described in Chapter 3 is the first to investigate DHA and EPA in canine cancer cell lines. The findings from these studies reinforce some of the results from prior work, such as high reported frequencies of alternative diets and nutritional supplements among dogs with cancer. In addition, the results described in Chapter 2 add novel information to the current body of literature, such as differences in sources of information consulted by owners of healthy dogs and dogs with cancer. Chapter 3 also describes novel information that builds on the small body of literature investigating the effects of n-3 PUFAs in canine OSA.

5.2 Sources of information

Based on the data collected from an online survey, Chapter 2 reports that owners of dogs with cancer are more reliant on online sources of information when researching pet health, pet nutrition and nutritional supplements compared to owners of healthy dogs. In contrast, owners of healthy dogs were more reliant on accessible in-person sources of information, such as pet stores, compared to owners of dogs with cancer.

Sources consulted when pet owners conduct research is an area that has not been closely examined in dogs with cancer; and no reports of a direct comparison had been made of sources of information consulted by owners of dogs with cancer and owners of healthy dogs. In 2006, it was found that the most commonly reported source of information for owners of dogs with cancer when researching complementary and alternative therapies was the veterinarian (Lana et al., 2006). This is consistent with the findings from the current study; often the most frequently reported source of information in owners of dogs with cancer and owners of healthy dogs was the veterinarian.
However, social media groups were also a commonly reported source of information in dogs with cancer and were reported at similar frequencies to the veterinarian. This is in contrast to the 2006 survey, where internet sources were reported as a source of information by only 10% of survey respondents (Lana et al., 2006).

The common use of biased, commercially-based, or uninformed sources of information by owners of dogs with cancer may be potentially problematic as these pet owners may be exposed to incorrect information, leading them to incorporate unsafe or understudied dietary components into their pet’s daily feeding routine. This study thus highlights the need for communication among clients and clinicians following a cancer diagnosis to ensure clients’ concerns and questions about diet and nutritional supplements can be answered. Clinicians can also become adequately informed when guiding the nutritional decisions of their clients.

5.3 Dietary differences in dogs with cancer and healthy dogs

In Chapter 2, as hypothesized, owners of dogs with cancer made different dietary decisions for their dogs than owners of healthy dogs. In particular, alternative diets and nutritional supplements were more commonly fed to dogs with cancer.

This thesis is the first to compare the dietary decisions owners of dogs with cancer and owners of healthy dogs make for their pets. Owners of dogs with cancer were found to be more likely to feed their dog a homemade raw diet or a homemade cooked diet compared to owners of healthy dogs; whereas owners of healthy dogs were found to be more likely to feed their dog a commercial dry food. These trends are
consistent with those reported in previous surveys conducted with owners of healthy dogs and owners of dogs with cancer (Lisa M Freeman et al., 2008; Rajagopaul et al., 2016). Interestingly, the proportion of owners of dogs with cancer choosing to include homemade cooked or raw food as a component of their pet’s diet appears to be on the rise when compared to a 2016 survey (Rajagopaul et al., 2016). This necessitates continued communication between clinicians and clients following a cancer diagnosis to ensure owners who choose to feed their pet a homemade diet are equipped with the tools and knowledge needed to offer a diet that is complete and balanced, since many homemade diets recipes have been found to be lacking in one or more essential nutrients (Heinze et al., 2012; Stockman et al., 2013; Streiff et al., 2002). Furthermore, owners choosing to include a raw component to their pet’s diet should be aware of heightened concerns of potential bacterial contamination, especially in dogs with cancer that may have reduced immune function (Finley et al., 2008; Nemser et al., 2014; Strohmeyer et al., 2006; Weese & Rousseau, 2006).

5.4 Differences in supplement use in dogs with cancer and healthy dogs

Another significant finding from this study was the increased use of nutritional supplements among dogs with cancer. A significant portion of owners of dogs with cancer were more likely to feed their dogs nutritional supplements, in particular CBD products, mushroom supplements, turmeric/curcumin, and multi/mixed supplements marketed to support immune function. This is concerning as many of these products are not regulated and have not been clinically tested to ensure they are effective or even
safe for use in dogs with cancer. In Chapter 3 it was found that EPA and DHA had limited efficacy as single agents, and these fatty acids had an antagonist effect on cellular viability when used in combination with chemotherapy. Collectively, these results combined with the fact that owners of dogs with cancer are already turning to nutritional supplements dictates a need for further research investigating the safety, efficacy and potential for possible interaction of these compounds with chemotherapy drugs.

5.5 Future directions

A specific limitation of the study conducted in Chapter 2 involved recruitment methodology. Although the survey was available both to clients visiting an oncology clinic and a local primary practice, as well as through social media, the majority of respondents were recruited through social media. This means the results of this study only represent a small sub-set of pet owners visiting clinics and a larger sub-set of pet-owners active on social media during the data collection period who were members of pet-related groups. Future studies should aim to include a greater diversity of pet owners by practicing a broad variety of recruitment strategies. In addition, sample bias was also introduced by providing pet owners the option to self-recruit. Although no mention of nutritional supplements or diet type was made in the title of the survey, it is possible that individuals who chose to participate had a keen interest in pet nutrition and were practicing vastly different feeding routines than those who chose not to participate. If owners following alternative or unconventional feeding routines were more likely to participate, this could account for the observed higher prevalence. Use of a random
sampling methodology in future studies may be valuable in reducing the presence of sampling bias.

There are also opportunities to expand on the data collected in Chapter 2 in future studies. In particular, collecting temporal information from pet owners about nutritional supplement use could help differentiate between supplements introduced after a cancer diagnosis and those provided prior to a cancer diagnosis. It would also be useful to collect detailed information about why pet owners choose to feed certain diet or supplement types, in order to further understand the motivations behind pet owners' feeding practices.

In Chapter 3, two OSA cell lines were tested to determine the effects of n-3 PUFAs EPA and DHA. Future studies investigating the effects of EPA and DHA on OSA cell lines could aim to choose different or more cell lines to determine if the results found in Chapter 3 are consistent across cancer types and across multiple lines of OSA. In addition, since only one chemotherapy drug was tested when conducting CI experiments in Chapter 3, future studies could aim to include other chemotherapy drugs commonly used in treating canine cancer (such as carboplatin), to determine if EPA and DHA can be combined with other chemotherapy drugs to produce additive or even synergistic effects.
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Nutrition Survey of Healthy Dogs and Dogs with Cancer

Q52
Thank you for considering being a participant in this study.

On the next page you will find the consent form.

Please read it thoroughly and indicate whether you would like to consent to participate by clicking Yes or No.

Once you have reached the end of the survey you will have the option to be entered into a draw to win one of eight $25 gift cards to a pet supply retailer of your choice.
eligible to participate in this study as a client of the OVC Health Sciences Center (HSC)-Animal Cancer Center, OVC Primary Health Care Centre, or a private small animal practice. **NO IDENTIFYING INFORMATION** will be collected at any time during the survey, unless you wish to be entered into a draw for a 25$ gift certificate to a pet food retailer, in which case you will have the option to provide an email address that the researchers will use to contact you if you are selected as the winner for the draw. After the draw all email addresses will be deleted. As the survey is completely anonymous (researchers will NOT connect the information you supply for the draw to the survey data), data provided by you may NOT be withdrawn from the research project once your completed survey is submitted. **You may withdraw during the survey by closing your browser.** Please note that confidentiality cannot be guaranteed while data are in transit over the Internet. Non-identifying information may be used in published materials and presentations. Please remember your **PARTICIPATION IS VOLUNTARY**, and at any time you may decide to skip a question or not participate. None of the questions asked in this interview are in any way meant to suggest a dietary change is needed/required for your pet, nor is it meant to imply your pet’s diet had something to do with a cancer diagnosis. We have no doubt that you had your pet’s best interest in mind when choosing a diet. If you do have questions regarding your pet’s diet, please consult your oncology clinician or technician or your family veterinarian. With your cooperation, we hope to use the information provided towards future research in companion animal cancers, to better understand nutritional supplement use and validate future clinical trials on the safety and efficacy of nutritional supplements in pets with cancer. **Also, your decision to participate OR not to participate in this research project will have absolutely NO consequences.** Please feel free to print this page for your records. You are invited to visit the Ontario Veterinary College’s website once the survey is complete [http://bulletin.ovc.uoguelph.ca/](http://bulletin.ovc.uoguelph.ca/) to learn about the results of the study.

**Researcher Information:**
- Dr. Adronie Verbrugghe, DVM, PhD, Dip. ECVCN, Associate Professor
- Dr. Sarah Abood, DVM, PhD, Assistant Professor
- Dr. J. Paul Woods, DVM, MS, DACVIM (Internal Medicine Oncology), Professor
- Dr. Jason Coe, DVM, PhD, Associate Professor
- Adriana Bianco, BScH, MSc Student

**Student Contact Information:** csnutri@uoguelph.ca  This research project is supported by the Department of Clinical Studies at the Ontario Veterinary College. You are not waiving any legal claims, rights or remedies because of your participation in this research study. This study has been reviewed and received ethics clearance through the University of Guelph Research Ethics Board, REB# 18-09-026. If you have questions regarding your rights as a research participant, please contact: Director, Research Ethics (519) 824-4120, ext. 56606 sauld@uoguelph.ca.

Click the button below if you would like to participate in this survey.
I agree to participate  (1)

I do not agree to participate  (2)

Q1 Where do you go to find information about pet health topics? Check all that apply.

- Pet store  (1)
- Breeder  (2)
- Friends and Family  (3)
- Social media groups  (4)
- Blogs  (5)
- Other  (6)  
- Veterinarian  (7)
Q2 How much time per week do you spend researching pet health related topics? Please answer in the following format: X hours, Y minutes

Q3 Where do you go to find information about pet nutrition? Check all that apply.

- Pet store (1)
- Breeder (2)
- Friends and Family (3)
- Social media groups (4)
- Blogs (5)
- Other (6) _________________________________
- Veterinarian (7)
Q4 How much time per week do you spend researching pet nutrition? Please answer in the following format:  $X$ hours, $Y$ minutes

Q5 Where do you go to find information about nutritional supplements? Check all that apply.

- [ ] Pet store (1)
- [ ] Breeder (2)
- [ ] Friends and Family (3)
- [ ] Social Media groups (4)
- [ ] Blogs (5)
- [ ] Other (6) ________________________________
- [ ] Veterinarian (7)
Q6 How much time per week do you spend researching nutritional supplements? Please answer in the following format: X hours, Y minutes

End of Block: Block 13

Start of Block: Default Question Block

Q7 How many dogs do you have? (if more than one please choose one dog for which you will be providing responses for in this questionnaire)

Q8 How old is your dog in years?
Q9 What is the sex of your dog?

- Neutered male (1)
- Intact male (2)
- Spayed female (3)
- Intact female (4)
- Unsure (5)

Q10 What is your dog's breed? (If unsure, indicate "unsure")

________________________________________________________________

________________________________________________________________

________________________________________________________________

________________________________________________________________

Page Break
Q13 How frequently does your dog visit a veterinarian for a wellness exam?

Q14 Would you consider your dog to be generally in good health?

- Yes (1)
- No (2)
- Unsure (3)

End of Block: Default Question Block

Start of Block: Block 23

Q15 Does your dog have cancer?

- Yes (1)
- No (2)
- My dog is currently in remission (3)
- Unsure at this time (4)

End of Block: Block 23
<table>
<thead>
<tr>
<th>Display This Question</th>
<th>If Does your dog have cancer? = Yes</th>
</tr>
</thead>
</table>

**Q15a** What type(s) of cancer(s) does your dog have?

________________________________________________________________

**Q15b** In what year was your dog first diagnosed with cancer?

________________________________________________________________
Q15c Of the cancer treatment options listed below, which ones has your dog received up until today? Check all that apply

☐ No treatment (1)
☐ Surgery (2)
☐ Chemotherapy (3)
☐ Radiation (4)
☐ Other (5) ________________________________________________

Display This Question:
If Does your dog have cancer? = Yes
Q15d Of the cancer treatment options listed below, which ones will your dog be receiving in the future? Check all that apply.

- No treatment (1)
- Surgery (2)
- Chemotherapy (3)
- Radiation (4)
- Other (5) ________________________________________________

End of Block: Cancer yes questions

Start of Block: Cancer NO

Display This Question:
If Does your dog have cancer? = No

Q15a How would your current behaviours, with respect to pet nutrition, change if your dog were to receive a cancer diagnosis?
Check all that apply:

☐ My behaviour would not change, I would continue to feed my dog the same as I do now  (1)

☐ I do not know how/if my behaviour would change  (2)

☐ I would consider changing my dog’s primary diet type  (3)

☐ I would consider adding nutritional supplements to my dog’s diet  (4)

☐ I would consult my veterinarian for advice about making changes to my dog’s diet  (5)

☐ I would conduct my own nutrition research using internet sources, books, magazine articles etc.  (6)

☐ Other  (7) __________________________________________

Display This Question:

If How would your current behaviours, with respect to pet nutrition, change if your dog were to rece... = I would consider changing my dog’s primary diet type
Q15a1 What changes would you consider making to your dog's primary diet? Check all that apply:

☐ Change pet food brand (1)

☐ Switch to veterinary therapeutic diet (2)

☐ Switch to a homecooked diet (3)

☐ Switch to a raw meat diet (4)

☐ Switch to an organic, holistic and/or natural diet (5)

☐ Switch to a vegetarian diet (6)

☐ other (7) ........................................................................................................
Q16 Does your dog have a history of any medical condition that requires periodic veterinary attention (other than cancer)?

- Yes (1)
- No (2)
- Not sure (3)

Display This Question:
If Does your dog have a history of any medical condition that requires periodic veterinary attention... = Yes

Q16a Please list those medical conditions requiring periodic veterinary attention.

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

End of Block: Periodic vet attention
Start of Block: Prescription meds
Q17 Does your dog have any current health issues requiring prescription medication (other than cancer)?

- Yes (1)
- No (2)
- Not sure (3)

Display This Question:
If Does your dog have any current health issues requiring prescription medication (other than cancer)? = Yes

Q17a Please list all current health issues requiring prescription medication

________________________________________________________________________________
________________________________________________________________________________
________________________________________________________________________________
________________________________________________________________________________
________________________________________________________________________________

End of Block: Prescription meds
Start of Block: Health issues no prescription meds
Q18 Does your dog have any current health issues that do not require prescription medication?

- Yes (1)
- No (2)
- Not sure (3)

Display This Question:
If Does your dog have any current health issues that do not require prescription medication? = Yes

Q18a Please list all current health issues that do not require prescription medication (other than cancer)?

___________________________________________________________________________
___________________________________________________________________________
___________________________________________________________________________
___________________________________________________________________________

End of Block: Heath issues no prescription meds

Start of Block: Block 3
Q19 Currently what is the primary type of pet food you put in your dog's food bowl? Check all that apply:

☐ Commercial dry food (ie. kibble) (1)

☐ Commercial wet food (ie. cans, pouches) (2)

☐ Commercial raw food (3)

☐ Homemade raw food (4)

☐ Homemade cooked food (5)

☐ Other (6) ________________________________________________

Q20 Do you, or have you ever provided a nutritional supplement to your dog?

☐ Yes, I have and continue to administer nutritional supplements to my dog (1)

☐ Yes, I have previously administered nutritional supplements to my dog but do not any more (2)

☐ No, I have never administered nutritional supplements to my dog (3)
Q21 How many different types of nutritional supplements have you previously fed to your dog?

Please enter a numerical value (eg. " 1 ")

________________________________________________________________

End of Block: Block 29

Start of Block: Block 7

Q22 Please list one nutritional supplement you have fed to your dog using the following format:  
Brand name (optional)  Type of supplement (eg. Fish oil/omega-3 fatty acids)  
Dosage (eg. 1 capsule/day)  Approximate start date month/year (eg. since May 2014)  
Approximate end date month/year (eg. Until present day, or until January 2017)  
Please note this question will repeat if you previously entered that you feed more than 1 nutritional supplement

________________________________________________________________

________________________________________________________________

________________________________________________________________
Q22a Why did you stop administering this nutritional supplement to your dog?  Check all that apply

☐ Dog’s health condition was not improving with supplement (1)

☐ Dog’s health condition changed, so supplement was no longer needed (2)

☐ Veterinarian recommended stopping (3)

☐ Dog did not cooperate/did not enjoy consuming the nutritional supplement (4)

☐ Financial reasons (5)

☐ I read/heard conflicting information about this nutritional supplement (6)

☐ Other (7) ___________________________
Q23 Would you consider feeding a nutritional supplement to your dog? Check all that apply

- Yes, if recommended by my veterinarian (1)
- Yes, if recommended by friends or family (2)
- Yes, if recommended on the internet, blogs, books or magazines (3)
- Yes, if one became available at the pet store (4)
- Yes, if more information about the safety and efficacy of nutritional supplements becomes available (5)
- No (6)
- Yes, if it were more affordable (7)

End of Block: Block 4

Q23a If yes, which supplement(s) would you consider feeding? If unsure, indicate so.

________________________________________________________________

End of Block: Block 5

Start of Block: Block 6

166
Q23b You indicated you would not feed supplements to your dog. Why not?

________________________________________________________________________

End of Block: Block 6

Start of Block: Block 31

Q21 How many nutritional supplements do you feed to your dog?

Please enter a numerical value (eg. " 1 ")

________________________________________________________________________

End of Block: Block 31

Start of Block: Block 8

Q22 Please list one nutritional supplement you have fed to your dog using the following format: Brand name (optional) Type of supplement (eg. Fish oil/omega-3 fatty acids) Dosage (eg. 1 capsule/day) Approximate start date month/year (eg. since May 2014) Approximate end date month/year (eg. Until present day, or until January 2017) Please note this question will repeat if you previously entered that you feed more than 1 nutritional supplement

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________
Q23 What is your goal in administering this nutritional supplement to your dog? Check all that apply:

☐ Improve overall health (1)

☐ Increase or enhance my dog’s immune function (2)

☐ Ensure nutritional adequacy of my dog’s diet (3)

☐ Decrease pain associated with my dog’s condition (4)

☐ Decrease side effects of my dog’s treatments (5)

☐ Other (6) ________________________________
Q24 How did you decide on the dose you are currently feeding your dog?

☐ Veterinarian recommended (1)

☐ Internet/blog recommended (2)

☐ Followed human recommendations on the label (3)

☐ Followed canine recommendations on the label (4)

☐ Book/magazine recommended (5)

☐ Other (6) ________________________________________________

Q25 Have you noticed a difference in your dog’s health or behaviour while on this nutritional supplement?

☐ Yes (1)

☐ No (2)

☐ Unsure at this time (3)
Q25a What differences in your dog’s health or behaviour have you noticed while feeding this supplement?

_________________________________________________________________________

_________________________________________________________________________

_________________________________________________________________________

_________________________________________________________________________

_________________________________________________________________________
Q26 What or who influenced your decision to feed this nutritional supplement, please check all that apply:

- [ ] My veterinarian (1)
- [ ] A website/Blog (2)
- [ ] Social media (3)
- [ ] I have fed this nutritional supplement to previous pets of mine (4)
- [ ] Friends/family (5)
- [ ] Book/magazine (6)
- [ ] Breeder (7)
- [ ] Pet store employee (8)
- [ ] Other (9) _______________________________
Q27 Does your veterinarian support the use of this supplement? Check all that apply:

- Yes, my veterinarian supports the use of the supplement (1)
- No, my veterinarian has advised against feeding this nutritional supplement (2)
- My veterinarian does not know I am administering this nutritional supplement (3)
- I am not sure what my veterinarian thinks about the use of this supplement (4)
- Other (5) ________________________________________________

End of Block: Block 8

Start of Block: Block 20

Q53 The following questions will be used to collect dog owner demographic data

End of Block: Block 20

Start of Block: Block 9

Q D1 What is your gender?

- Male (1)
- Female (2)
- Other (3)
Q D2 Please indicate your age category:

- 18 to 29 (1)
- 30 to 39 (2)
- 40 to 49 (3)
- 50 to 59 (4)
- 60 to 69 (5)
- 70 or older (6)

Q54 What is your country of residence?

- Canada (1)
- United States of America (2)
- Australia (3)
- United Kingdom (4)
- Other (5) ____________________________________________
Q D3 What level of education have you achieved?

- Some secondary school (1)
- Secondary school diploma (2)
- Some College (3)
- Achieved college diploma (4)
- Some university (5)
- Achieved university Bachelor’s degree (6)
- Advanced degree (Master’s, PhD, doctorate) (7)

Q55 Are you a client at the Ontario Veterinary College?

- Yes, I am a client at the Mona Campbell Centre for Animal Cancer (1)
- Yes, I am a client at the Smith Lane Animal Hospital (2)
- No, I am not a client at the Ontario Veterinary College (3)

Display This Question:
If Are you a client at the Ontario Veterinary College? = No, I am not a client at the Ontario Veterinary College
Q56 At what veterinary clinic(s) are you a client?

________________________________________________

Q D5 Would you like to be entered into a draw for a $25 pet store gift card?

If yes is selected, you will be re-directed to a different survey where your contact information will be collected.

☐ Yes (1)

☐ No (2)