# Effect of Fish Oil, Arginine, and Doxorubicin Chemotherapy on Remission and Survival Time for Dogs with Lymphoma

## A Double-Blind, Randomized Placebo-Controlled Study

Gregory K. Ogilvie, D.V.M.[1]
Martin J. Fettman, D.V.M., Ph.D.[2]
Craig H. Mallinckrodt, Ph.D.[3]
Judy A. Walton, A.H.T.[1]
Rodney A. Hansen, M.S.[1]
Deborah J. Davenport, D.V.M.[4]
Kathy L. Gross, Ph.D.[4]
Kristi L. Richardson, M.S.[1]
Quinton Rogers, Ph.D.[5]
Michael S. Hand, D.V.M., Ph.D.[4]

[1] Comparative Oncology Unit, Departments of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado.

[2] Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado.

[3] Department of Statistics, Colorado State University, Fort Collins, Colorado.

[4] Hill's Science and Technology Center, Topeka, Kansas.

[5] University of California, Department of Nutrition, Davis, California.

**BACKGROUND.** Polyunsaturated n-3 fatty acids have been shown to inhibit the growth and metastasis of tumors. This double-blind, randomized study was designed to evaluate the hypothesis that polyunsaturated n-3 fatty acids can improve metabolic parameters, decrease chemical indices of inflammation, enhance quality of life, and extend disease free interval and survival time for dogs treated for lymphoblastic lymphoma with doxorubicin chemotherapy.

**METHODS.** Thirty-two dogs with lymphoma were randomized to receive one of two diets supplemented with menhaden fish oil and arginine (experimental diet) or an otherwise identical diet supplemented with soybean oil (control diet). Diets were fed before and after remission was attained with up to five dosages of doxorubicin. Parameters examined included blood concentrations of glucose, lactic acid, and insulin in response to glucose and diet tolerance tests; alpha-1 acid glycoprotein; tumor necrosis factor; interleukin-6; body weight; amino acid profiles; resting energy expenditure; disease free interval (DFI); survival time (ST); and clinical performance scores.

**RESULTS.** Dogs fed the experimental diet had significantly ($P < 0.05$) higher mean serum levels of the n-3 fatty acids docosahexaenoic acid (C22:6) and eicosapentaenoic acid (C20:5) compared with controls. Higher serum levels of C22:6 and C20:5 were associated with lesser ($P < 0.05$) plasma lactic acid responses to intravenous glucose and diet tolerance testing. Increasing C22:6 levels were significantly ($P < 0.05$) associated with longer DFI and ST for dogs with Stage III lymphoma fed the experimental diet.

**CONCLUSIONS.** Fatty acids of the n-3 series normalize elevated blood lactic acid in a dose-dependent manner, resulting in an increase in DFI and ST for dogs with lymphoma. *Cancer* 2000;88:1916–28. © 2000 American Cancer Society.

**KEYWORDS: lymphoma, fish oil, docosahexaenoic Acid, eicosapentaenoic acid, dogs.**

Cancer cachexia is weight loss in cancer patients despite adequate nutrient intake and is a devastating consequence of malignancy.[1–6] Alterations in resting energy expenditure (REE) have been documented in persons with cancer and are related to derangements in carbohydrate, protein, and lipid metabolism.[2–6] Many of these derangements have been identified for dogs with lymphoma and other malignancies even before overt signs of cachexia were observed.[7–11] Blood concentrations of acute phase proteins, including alpha-1 acid glycoprotein (AG), are also increased for dogs with lymphoma and other malignancies.[11] Tumor necrosis factor (TNF) and interleukin-6

(IL-6) may play a role in the pathogenesis of cancer cachexia.[1–5] One of the most consistent findings for dogs with a wide variety of malignancies includes significant elevations in blood lactic acid and insulin concentrations that increase further in response to the intravenous administration of glucose.[10] These alterations persist after all clinical evidence of cancer is eliminated with chemotherapy or surgery and are associated with cancer cachexia.[7,11]

Studies of polyunsaturated fatty acids (PUFAs) of the n-3 series, especially eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acids, indicate that these fatty acids may prevent the development of carcinogen-induced tumors, the growth of solid tumors, and the occurrence of cachexia and metastatic disease in experimental tumor models.[13–17] The mechanism in which these fatty acids alter tumor growth and metastasis is unknown; however, eicosapentaenoic acid may inhibit cell proliferation and induce its apoptosis.[13,17] Fatty acids of the n-3 series have been shown to normalize elevated blood lactic acid and insulin levels in nonmalignant conditions.[14,15] In contrast, PUFAs of the n-6 series appear to enhance tumor development and metastases.[12,13,16] These data, along with the epidemiologic findings of an inverse relation between dietary n-3 fatty acid intake and incidence of some cancers,[16] are the basis of studies to evaluate the potential benefit of n-3 fatty acids in the prevention of cancer cachexia and therapy of malignancy in cancer patients.

The serum concentration of arginine has been reported to be lower than healthy controls in persons with a wide variety of malignancies.[18] Supplementation with arginine has been reported to improve wound healing and immune responses in elderly persons.[19]

Dogs with spontaneously occurring, high grade lymphoblastic lymphoma are an excellent model to evaluate dietary therapy for the treatment of cancer in humans. The addition of n-3 PUFAs with or without the amino acid arginine enhances the immune system, inhibits tumorigenesis and the spread of cancer in animal models,[20,21] and is the basis of this work in pet dogs with spontaneously occurring neoplasia.

Lymphoma in dogs has some similarities to high grade non-Hodgkin lymphoma in humans. Both respond well to chemotherapy, yet the median duration of remission for dogs rarely exceeds 9 months.[7] Unlike humans, these pets readily adapt to long term feeding with a specific diet and can be repeatedly sampled to document the impact of dietary therapy. In addition, dogs share the human environment, and owners expect the same quality of life during therapy as they would for themselves.

This study was undertaken to determine if an experimental diet supplemented with menhaden fish oil (n-3 fatty acids) and arginine can influence remission time, survival time, and selected aspects of metabolism and the immune system in client-owned dogs treated for lymphoma with doxorubicin. This study was designed to evaluate the hypothesis that the experimental diet would decrease blood concentrations of lactic acid, insulin, acute phase reactant proteins (alpha-1 AG), and inflammatory cytokines (TNF-alpha, IL-6), increase host body weight, energy balance, disease free interval (DFI), and survival time (ST), and improve quality of life for dogs receiving doxorubicin chemotherapy for lymphoma.

## MATERIALS AND METHODS
### Dogs with Lymphoma
Thirty-two client-owned dogs with histologically confirmed, high grade lymphoblastic lymphoma were selected sequentially from the patient population of the Comparative Oncology Unit at Colorado State University. To ensure a more uniform patient population, only dogs with histologically high grade Stage IIIa or IVa lymphoma, according to the World Health Organization classification scheme, were eligible for this study.[22] Dogs were excluded from this study if they were cachectic or if they had received chemotherapy, exogenous steroids, or anesthesia in the 30 days before initial presentation to our study. In addition, client-owned dogs with concurrent diseases such as renal failure, hepatic cirrhosis, endocrine diseases, obesity, or hypercalcemia secondary to lymphoma were excluded. Twenty-eight dogs were classified as Stage IIIa (generalized lymph node involvement, without systemic signs), and four dogs were Stage IVa (clinical evidence of liver and/or spleen involvement plus Stage III, without systemic signs).

The following procedures were performed on all dogs for tumor staging purposes: lymph node biopsy, hemogram, serum biochemical profile, urinalysis, bone marrow aspirate, and thoracic and abdominal radiographs. Median and mean body weights were 32 kg and 25 kg, respectively, with a range from 7 to 58 kg. Median and mean ages were 8 and 7.24 years, respectively, with a range from 2 to 16 years. Dogs were categorized as those that were less than 9 years old (n = 20) and those that were more than 9 years old (n = 12). Fifteen of the dogs were spayed females, two were noncycling intact females, nine were neutered males, and four were intact males.

All dogs were entered into the double-blind randomized study design in one of two groups. The diets were isocaloric providing 6.1 kJ metabolizable energy/g (Table 1). Each dog was exclusively fed identical

TABLE 1
Nutrient Content of Diets as g/kg Dry Matter

| Nutrients | Control | Experimental |
|---|---|---|
| Crude protein | 378 | 378 |
| Fat | 329 | 326 |
| CHO | 207 | 216 |
| C20:5 | 0.1 | 29 |
| C22:6 | 0.2 | 24 |
| Linoleic | 123 | 16 |
| α-Linolenic | 16 | 4.2 |
| n-6:n-3 ratio | 7.7:1 | 0.3:1 |
| Total n-6 | 125 | 23 |
| Total n-3 | 16 | 73 |

CHO: carbohydrate.

[a] Ingredients: beef by-products, water, liver, rice, soybean or menhaden fish oil, chicken, cellulose, amino acids, minerals, and vitamins.

amounts (maintenance energy requirement [kcal = $2(70$ weight $kg^{0.75})$] of one of two specialized diets for the duration of their chemotherapy or first remission. The diet was fed twice daily. The experimental diet was supplemented with menhaden fish oil ( 140 g EPA [eicosapentaenoic acid] per kg fish oil, 120 g DHA [docosahexaenoic acid] per kg fish oil; Zapata Protein Corporation, Battle Creek, MI) and arginine (5.54% dry matter basis) whereas the control diet was supplemented with soybean oil without additional arginine (arginine amount 2.5% dry matter basis). Both diets contained identical amounts of vitamin E as alpha-tocopherol at 500 IU per kg dry matter basis. The owner and clinician kept detailed records to document the daily consumption of the experimental and control diets and performance status. Both diets were formulated to be isonitrogenous, equivalent in metabolizable and digestible energy, tested for palatability, stability, and nutritional adequacy. The vitamin and mineral content met or exceeded the requirements set by the National Research Council. Each was labeled in code before the initiation of this study. All dogs that were in remission were sampled and evaluated for all tests and analyses as follows after a 12-hour fast: before the first, second, and fifth evaluation period that coincided with doxorubicin chemotherapy (30 mg/m$^2$ intravenously every 3 weeks).[23]

Tumors were measured before each treatment with doxorubicin. A complete remission was defined as the disappearance of all clinically detectable disease. Progressive disease was defined as an unequivocal increase of at least 50% of the sum of the products of the perpendicular diameters of all measurable tumors, or the appearance of new neoplastic lesions, or uncontrolled hypercalcemia. Dogs in this study did not have any other concurrent treatment for their neoplastic disease. Information on clinical signs of drug-induced toxicoses and complications relating to neoplastic disease included, but was not limited to: history, physical examinations, hemogram, serum biochemical analyses, urinalyses, and radiographs. After dogs with lymphoma failed to go into remission or if progressive disease was documented, additional therapy was initiated with cyclophosphamide, vincristine, and prednisone.[23] Dogs were humanely killed when progressive disease was noted after this second round of therapy or if quality of life was considered unacceptable based on mutual consent of the owner and attending veterinarian.

### Diet Tolerance Test and Concomitant Blood Glucose, Lactic Acid, and Insulin Concentrations
After a 12-hour, overnight fast, venous blood was taken before, immediately after, and at 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, and 360 minutes after dogs were allowed to consume their assigned diets during a 5-minute period. The amount fed was calculated as $1/2[70($body weight$/kg^{0.75})]$. For analysis of glucose and lactic acid, whole blood was anticoagulated with lithium heparin and centrifuged immediately to separate plasma. For analysis of insulin, whole blood was allowed to clot at room temperature and was centrifuged to separate serum. Samples were stored at $-20$ °C immediately after collection and assayed together within 60 days. Glucose and lactic acid concentrations were measured in duplicate on plasma by a semiautomated, membrane-bound, enzyme-based electrochemical method using glucose oxidase and lactic dehydrogenase. Serum insulin concentrations were measured in duplicate by a radioimmunoassay technique with a commercially available kit (catalog number 07-160102; ICN Biomedicals Inc., Carson CA) validated in the dog as previously described.[9–11]

### Intravenous Glucose Tolerance Test and Concomitant Blood Glucose, Lactic Acid, and Insulin Concentrations
The day after the diet tolerance test and after a 12-hour, overnight fast, blood was taken before and 5, 15, 30, 45, and 60 minutes after the intravenous administration, during 30 seconds, of 500 mg/kg of 25% dextrose by volume as previously described.[9–11] Blood samples were collected, processed, stored, and analyzed as described above.

### Body Weight, Diet Consumption, and Performance Status
Body weight was determined using a tared electronic scale. Performance status was assessed by both subjective and clinical criteria using the clinical performance scoring scheme (0 [normal]: fully active, able to perform at predisease level; 1 [restricted]: restricted to

**TABLE 2**
Evaluation Scheme Used to Evaluate Specific Toxicoses Associated with Tumor or Treatment

| | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Leukopenia | | | | | |
| White blood cells $\times 10^3$ | > 5.5 | 3 to < 5.5 | 2 to < 3 | 1 to < 2 | < 1 |
| Neutrophils $\times 10^3$ | > 2.5 | 1.5 to < 2.5 | 1 to < 1.5 | 0.5 to < 1 | < 0.5 |
| Lymphocytes $\times 10^3$ | > 1.5 | 1 to < 1.5 | 0.5 to < 1 | 0 to < 0.5 | 0 |
| Thrombocytopenia | | | | | |
| Platelets $\times 10^3$ | < 130 | 90 to < 130 | 50 to < 90 | 25 to < 50 | < 25 |
| Anemia | | | | | |
| Hematocrit (%) | > 25 | 20 to < 25 | 15 to < 20 | 10 to < 15 | < 10 |
| Clinical | — | — | — | Serious | Transfusion |
| Hemorrhage | — | Minimal | Moderate | Debilitated | Life threatening |
| Infection | — | No therapy | Requires therapy | Debilitated | Life threatening |
| Urinary | | | | | |
| BUN mg/dL | < 20 | 21 to 40 | 41 to 60 | > 60 | Symptomatic |
| Creatinine (mg/dL) | < 2 | 2.1 to 4 | 4.1 to 6 | > 6 | Uremia |
| | — | 1+ | 2+ to 3+ | 4+ | |
| Proteinuria | — | Microscopic | Grossly red | Grossly cloudy | Obstructed |
| Hematuria | | | | | |
| Hepatic ($\times$ normal values) | | | | | |
| Alanine amino | < 1.5 | 1.5 to 2 | 2.1 to 5 | > 5 | |
| Alkaline phosphatase | < 1.5 | 1.5 to 2 | 2.1 to 5 | > 5 | |
| Bilirubin | < 1.5 | 1.5 to 2 | 2.1 to 5 | > 5 | |
| Clinical | | | | Precoma | Coma |
| Nausea and vomiting | None | Nausea | Continuous vomiting | Intractable | |
| Diarrhea | — | Loose | Severe dehydration | Bloody | |
| Pulmonary (clinical) | — | Mild | Moderate | Severe | Requires $O_2$ |
| Cardiac | | HR > 200 | Arrhythmia | Mild heart failure | Severe heart failure |
| Neurologic | | | | | |
| Peripheral nerves | — | Mild | Moderate | Moderate to Severe | Severe Seizures |
| Central nervous system | — | Depress | Moderate +/− tremor | Severe +/− coma | Coma |
| Dermatology | | | | Ulcerogenic | |
| Skin | — | Erythema Pigmented Atrophy | Vesticular Subepithelial | Necrotic | |
| Stomatitis | | Sore | Mild | Severe | |
| Alopecia | | Mild | Ulceration Severe | Ulcerated | |
| Allergy | — | Rash | Urticaria, fever | Requires therapy | Anaphylaxis |
| Fever (°F) | < 102 | 102 to 103 | 103 to 104 | 104 to 105 | > 105 |
| Local toxicity | — | Pain | Phlebitis | Ulcerative | |

BUN: blood urea nitrogen; HR: heart rate.

activity from predisease level but able to function as a normal pet; 2 [compromised]: severely compromised in activity level, ambulatory only to point of eating, sleeping, and consistently urinating and defecating in acceptable areas; 3 [disabled]: completely disabled, must be force-fed, and unable to control urination and defecation to acceptable areas; 4 [dead]: dead). Clinical and toxicity data were scored using the scheme tabulated in Table 2. Food consumption logs were completed on a daily basis and verified by quan-

titating the amount of food dispensed every 3 weeks. Animals were excluded from consideration if absolute compliance to all aspects of the protocol were not confirmed based on evaluation of data from patient history, detailed diet logs, and food consumption profiles, as well as amino acid and fatty acid serum profiles.

## Indirect Calorimetry

Resting energy expenditure (REE, kcal/kg/day) was estimated with a commercially available, open flow, indirect calorimetry system (Oxymax Indirect Calorimetry Unit; Columbus Instruments Corporation, Columbus, OH) using methods similar to those previously described.[24] Briefly, the rate of carbon dioxide production ($VCO_2$, mL/min/kg) and oxygen consumption ($VO_2$, mL/min/kg) were determined. Subsequently, REE was calculated by applying the abbreviated Weir formula.[24,25] The total flow of room air through the system was 10 times the estimated basal oxygen consumption to insure that all expired gases were collected. The percentage of $O_2$ in a dried aliquot of the effluent gas (100 mL/min) was measured continuously by an $O_2$ sensor and $O_2$ analyzer by using electrochemical cell technology. The percentage of $CO_2$ in a similar aliquot also was measured continuously by a $CO_2$ sensor and a $CO_2$ analyzer using infrared absorption technology. Calibration of the system was performed immediately after each measurement period by using medical grade 100% $N_2$ and 10% $CO_2$. The content of each tank of gas was verified by mass spectroscopy before use for calibration. Fedak et al.'s nitrogen dilution technique was used to calibrate $VO_2$.[25] $VCO_2$ was calibrated by infusing a known flow of $CO_2$ into the mask.

## Tumor Necrosis Factor-alpha

The assay for TNF-alpha has been described previously.[26] Briefly, WEHI-164 cells (ATCC#CRL1751) were added to the wells of a 96-well flat bottom microtiter plate at $1 \times 10^5$ cells per well in 100 $\mu$L of fresh Dulbecco's minimal essential medium (DMEM) and allowed to adhere for 4 hours at 39 °C, 5% $CO_2$. After incubation, the medium was aspirated, and 100 $\mu$L (1.0 $\mu$g) of actinomycin D was added to each well to increase sensitivity to toxicity by TNF-alpha and to stop proliferation of cells. One hundred $\mu$L of serum was added to each well in triplicate. In each plate, rhTNF-alpha (0.1, 0.5, and 1 pg) and DMEM were added in triplicate as positive and negative controls, respectively. The plates were incubated for 18 hours at 39 °C, 5% $CO_2$. After incubation, 150 $\mu$L of supernatant was removed from each well, and 40 $\mu$L of 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide (1 mg/mL) was added to each well. The plate then was incubated for 3 hours, and the supernatant was removed. Cell lysis and dye solubilization were achieved with the addition of isopropanol and hydrochloric acid, respectively. The absorbance of the resultant solution was determined in each well and was directly related to the number of viable WEHI-164 cells per well. Optical density of each well was determined by a microtiter plate reader (ICN Biomedicals Inc., Carson, CA) at 750 nm, and results are expressed as percent cytotoxicity. The standard error of the mean was routinely less than 10% of the mean within a given set of replicated wells.

## Interleukin-6

Canine IL-6 activity was measured by bioassay as previously described using the IL-6–dependent murine cell line 7TD1 (American Type Culture Collection, Rockville, MD).[27,28] This cell line has been shown previously to respond to recombinant and native IL-6 and not to other cytokines including interleukin-1, interleukin-2, interleukin-4, and tumor necrosis factor-alpha.[29] Cells ($5 \times 10^3$ 7TD1) were added to triplicate wells in a 96-well plate containing 100 $\mu$L media in which test sera were added. In each plate, 100 $\mu$L DMEM was added in triplicate as a negative control. Serum was treated at 56 °C for 30 minutes to inactivate complement. The microtiter plate was incubated at 39 °C in humidified air with 5% $CO_2$ for 44 hours. After incubation, 50 $\mu$L of 1.0 mg/mL XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt) and phenazine methosulfate (1.53 $\mu$g/mL) in DMEM were added to each well.[30] The plate was incubated for 4–6 hours at 39 °C in humidified air with 5% $CO_2$. During this period, viable cells convert the yellow XTT to water-soluble pink crystals that are released into the supernatant. The absorbance of the supernatant in the well is directly related to the number of viable cells in that well. The optical density of each well was determined with a microtiter plate reader at 450 nm. Differences among wells assayed in triplicate were less than 10%, and results were reported as the average IL-6 for triplicate wells for a given dilution. Data from the samples were compared with a standard curve from the optical density of the wells containing increasing amounts of rhIL-6 (Boehringer-Mannhein Biochemicals, Indianapolis, IN). The assay was sensitive to 1 U/mL IL-6.

## Alpha-1 Acid Glycoprotein (alpha-1 AG)

Serum alpha 1-AG concentrations were determined by use of single radial immunodiffusion (Kaikin Kagaku Institute Co. Ltd., Sendai, Japan) as previously described.[12] Briefly, agarose gel containing anti-canine

alpha 1-AG rabbit sera was prepared in a plastic container, and 2.5-mm diameter wells were punched out. Serum samples were applied to the wells, and the gels were incubated for 24 hours at 21 °C in a humidified chamber. The diameter of the precipitin ring was measured and compared with a standard diameter concentration curve by using samples with known alpha-1 AG concentrations.

## Amino Acid Profile Analysis

The plasma was assayed by high performance liquid chromatography methodology as previously described.[31,32] Blood was collected in heparin and within 15 minutes was centrifuged at $5000 \times g$, and we removed the plasma taking care not to collect any of the buffy coat. Within 15 minutes, 200 $\mu$L of 275 mmol/L of sulfosalicylic acid was added to 200 $\mu$L of plasma, and the mixture was centrifuged at $5000 \times g$, and the supernatant was removed and stored at −70 °C until the samples were assayed for amino acids. At the time of amino acid analysis, the acid supernatant was thawed and centrifuged at 4 °C at $14{,}000 \times g$ for 20 minutes; the supernatant was filtered through a 0.45-$\mu$m filter, and to 100 $\mu$L of this supernatant we added 12.5 $\mu$L of 0.8 moles/L of lithium hydroxide and 12.5 $\mu$L of a solution containing 2 mmols/L of the internal standard, L-alpha-amino beta-guanidino propionic acid, and after mixing, 50 $\mu$L of this solution was placed on the column, by using an automated amino acid analyzer (7300 Beckman Amino Acid analyzer; Beckman Instruments, Palo Alto, CA). The results are reported in nmols/mL plasma.

## Fatty Acid Analysis

Fatty acids were analyzed as previously described.[33,34] Briefly, blood was obtained from the lateral saphenous vein and aliquoted into each of two evacuated glass tubes with ethylenediamine tetraacetic acid and centrifuged for 5 minutes at $500 \times g$. Butylated hydroxytoluene was added (0.1 mg/mL) as a stabilizer, and 1-mL serum aliquots were placed in cryovials, capped in nitrogen gas, and frozen at −70 °C until analysis. Sera were batched, allowed to thaw, thoroughly vortexed, and run in duplicate. Two hundred microliters of serum was placed in 15-mL glass vials with Teflon lined caps. Two hundred $\mu$L of heptadecanoic methyl ester (200 mg/dL) was added to each sample as an internal standard. A 2:1 chloroform-methanol solution was added to each sample and vortexed for 1 minute. One microliter of isotonic saline was added, and the sample was vortexed again for 1 minute before centrifugation at $1000 \times g$ for 10 minutes. The resulting solution was composed of an aqueous layer on top, a proteinaceous wafer, and a bottom chloroform layer.

The aqueous layer was drawn off and discarded. The chloroform then was drawn off and evaporated until dry with a slow nitrogen stream. One milliliter of hexane reagent was added to each sample, followed by three milliliters boron trichloride/methanol, as an esterification reagent. After incubation at 60 °C for 2 hours, the vials were cooled to room temperature whereupon 1 mL of water was added to each vial followed by vortexing to quench the reaction. The samples then were placed in a 4 °C refrigerator for 15 minutes. After cooling, the hexane layer was aspirated and placed in a chromatograph vial and capped with nitrogen gas. Samples were analyzed on a Hewlett Packard 5890 gas chromatograph using an HP Innowax capillary column (Hewlett Packard, Bedford, MA). Injector temperature was set at 255 °C, and detector temperature was set at 255 °C. Quantification was accomplished by first determining the area ratio of each sample (ratio of internal standard to peak of interest) and then comparing with an external set of standards. The ratios then were matched to their corresponding molar amounts by simple linear regression.

## Statistical Analysis

### Effect of diet on serum fatty acid and arginine levels and metabolic health parameters

The significance and magnitude of factors potentially influencing serum levels of fatty acids, arginine, and metabolic health parameters (glucose, insulin, lactate, TNF-alpha, IL-6, alpha 1-AG, REE, and weight of the patient) were analyzed with least squares analysis of variance for repeated measures by using the Mixed Model procedure in SAS.[35] A first-order autoregressive error structure was included in each analysis to account for the correlation of errors between the repeated measurements.

Effects tested (independent variables) in each analysis were the fixed effects of each dietary treatment (DIET), time of measurement (TIME), diet by time interaction (DIET*TIME), age of the patient, stage category (Stage III or IV), gender (male, female, neutered, non-neutered), and the random effect of subject nested within diet (SUBJECT[DIET]). Fisher unprotected least significant difference test for preplanned comparisons was used to test the significance of the differences between treatment groups at each time period.

DIET was not hypothesized to have an effect on fatty acid levels until Week 3 for dogs fed the experimental diet. DIET included data from all time periods—including those times when diets had not had time to take effect. Overall diet effects could have been masked by similarity of groups at baseline. Therefore,

DIET*TIME was used in addition to the main effect of diet to assess treatment differences.

Normality of errors was confirmed in each analysis to verify the validity of analysis of variance (ANOVA) for these data.

### Comparing results from intravenous glucose tolerance test and diet tolerance test

Baseline, total area under the curve (AUC), and change in blood values for insulin, glucose, and lactic acid were assessed for both the intravenous glucose tolerance test (IVGTT) and the diet tolerance test (DTT). The similarity of measurements taken via both tests was assessed with parametric and nonparametric procedures.

The parametric analyses were multivariate analyses of variance (MANOVA)[36] in which corresponding values from IVGTT and DTT were included as dependent variables. Independent variables included in the MANOVA were TIME, DIET, and DIET*TIME. Partial correlations between the corresponding IVGTT and DTT measures were calculated from the error sum of squares and cross-products matrix.[36]

The nonparametric analyses were Spearman rank correlations[37] in which corresponding values from IVGTT and DTT were correlated to ascertain the strength of the relation between the rankings.

### Effect of diet on survival time and disease free interval

Metabolic health parameters were evaluated at several time points. The repeated measures of metabolic health parameters were averaged for each subject. The averages were included as the independent variables in all ST and DFI analyses.

The bivariate and independent associations of demographic factors and health parameters with Kaplan–Meyer product limit estimates ST and DFI were assessed using the Lifetest procedure in SAS.[37,38] Strata were defined by diet. Significance of independent variables were assessed using the log rank test. Independent associations were tested using a forward stepwise algorithm. Differences between strata (DIET) also were assessed using log rank tests. A second complete replicate of these analyses were performed in which serum levels of free fatty acids and arginine were included in place of diet.

Cox proportional hazards regression model[38] also was used to test the bivariate and independent associations of demographic factors and metabolic health parameters with ST and DFI. A second complete replicate of proportional hazards analyses were conducted in which serum levels of free fatty acids and arginine were included in place of diet.

Least squares analysis of variance, as previously described, was used to investigate potential interactions of diet and stage category that could not be readily evaluated with tests from either the Kaplan–Meyer or the Cox proportional hazards analyses.[38] Least squares analyses did not account for the censoring in the data, and normality assumptions were not always valid. Therefore, least squares analyses of ST and DFI are considered only exploratory and suggestive.

### Effect of diet on quality of life and toxicity parameters

The significance and magnitude of factors potentially influencing quality of life and toxicity parameters (Table 2) were analyzed with least squares analysis of variance as previously described for fatty acid, arginine, and metabolic health parameters.

Quality of life variables were categorical. Categorical data are typically analyzed with logistic regression. However, variation in scores at any time period was minimal, which limited the utility and validity of logistic regression. Therefore, quality of life and toxicity scores for each subject were averaged during all time periods. These averages were approximately normally distributed. Factors tested included diet, stage category, age, and gender.

A second complete replicate of the least squares analyses of quality of life parameters was performed in which serum levels of free fatty acids and arginine were included in place of diet. Bivariate associations of serum free fatty acid and arginine levels with average quality of life and toxicity scores (Tables 2 and 3) also were tested using Spearman rank correlations. These nonparametric analyses were performed due to concerns about potential violations of normality assumptions in the ANOVAs.

## RESULTS
### Effect of Diet on Serum Fatty Acid and Arginine Concentrations

There were no significant ($P > 0.10$) differences between treatment and control groups in the serum concentrations of fatty acids or of arginine before initiation of experimental diet feeding. Dietary supplementation with arginine and fish oil resulted in significant ($P < 0.05$) increases in serum arginine (Fig. 1) and n-3 fatty acid (Fig. 2) concentrations. Concentrations of C20:5, C22:6, and arginine increased ($P < 0.05$) after 3 weeks of feeding the experimental diet and remained so for the duration of the trial. An increase in these parameters was not seen in the controls. Serum concentrations of linoleic acid and arachidonic acid were significantly ($P < 0.05$) depressed by feeding the n-3 supple-
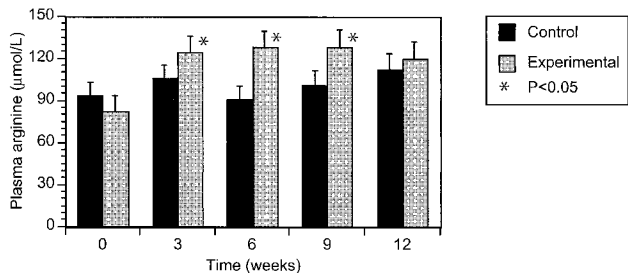
**FIGURE 1.** Mean ± standard deviation concentrations of arginine in the plasma of dogs with lymphoma fed the diet supplemented with n-3 fatty acids and arginine or a control diet are shown. Note significant elevations (*) beginning 3 weeks after initiation of feeding in the dogs fed the experimental diet.



**FIGURE 2.** Mean ± standard deviation concentrations of n-6 fatty acids linoleic acid (first graph, 18:2), arachidonic acid (second graph, 20:4) and n-3 fatty acids eicosapentaenoic acid (third graph, 20:5), and docosahexaenoic acid (fourth graph, 22:6) in the serum of dogs with lymphoma fed the diet supplemented with n-3 fatty acids and arginine or a control diet are shown. Note significant decreases (*, $\Psi$) in n-6 fatty acids and elevations in n-3 fatty acid concentrations beginning 3 weeks after initiation of feeding in the dogs fed the experimental diet.

mented diet from Weeks 3 to 12. Serum concentrations of $\alpha$-linolenic acid were not affected ($P > 0.10$) by the experimental diet and likewise were unchanged in control animals.

### Effect of Diet on Serum Insulin and Plasma Glucose and Lactic Acid Concentrations

There were no significant ($P > 0.10$) differences between treatment and control groups in the serum concentrations of insulin or the plasma concentrations of glucose and lactic acid before the initiation of experimental diet feeding (data available upon request). Results of the IVGTT and DTT challenges were similar ($P > 0.10$) between treatment groups before the initiation of feeding the experimental diet (Fig. 3). Baseline plasma glucose and lactic acid concentrations and baseline serum insulin concentrations were not affected ($P > 0.10$) by dietary treatment. Values of AUC for plasma lactic acid ($P = 0.043$) and serum insulin ($P = 0.039$) decreased significantly for dogs fed the experimental diet for 3 weeks or more (Fig. 3). Values of AUC for plasma glucose were not affected ($P > 0.10$) by dietary treatment (Fig. 3). However, differences over time between groups in baseline values for lactic acid and insulin were predictive of changes in AUC and peak response values during the IVGTT and DTT and appeared to account for much of the treatment effect observed in these parameters. Baseline and AUC measures were moderately to strongly correlated for insulin during the IVGTT (r = 0.66, $P = 0.0001$) and DTT (r = 0.78, $P = 0.0001$). Baseline and peak response measures were weakly correlated for insulin during the IVGTT (r = 0.33, $P = 0.01$) and DTT (r = 0.49, $P = 0.0001$). Baseline and AUC measures were moderately to strongly correlated for lactic acid during the IVGTT (r = 0.60, $P = 0.0001$) and DTT (r = 0.76, $P = 0.0001$). Baseline and peak response measures
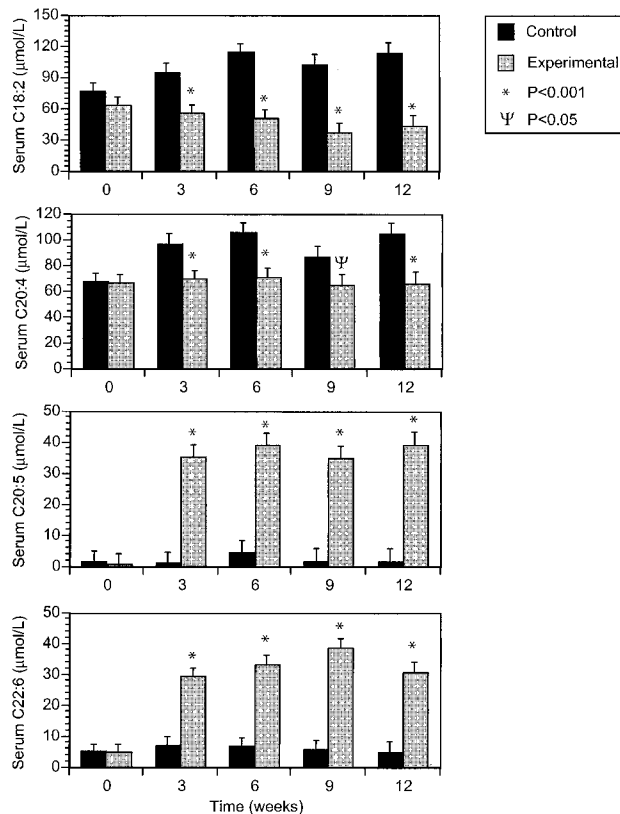
were weakly correlated for lactic acid during the IVGTT (r = 0.43, $P = 0.0002$) and DTT (r = 0.36, $P = 0.0001$).

### Effect of Diet on Serum Inflammatory Cytokine and Alpha 1-AG Concentrations

There were no significant ($P > 0.10$) differences between treatment and control groups in the serum concentrations of IL-6, TNF, or alpha 1-AG before the initiation of experimental diet feeding (Table 3). Serum IL-6 and alpha 1-AG values decreased significantly ($P < 0.001$) with time in both groups, but there was a further significant ($P = 0.05$) decrease for dogs fed the experimental diet owing to particularly high baseline values in this group before initiation of treatment (Table 3).
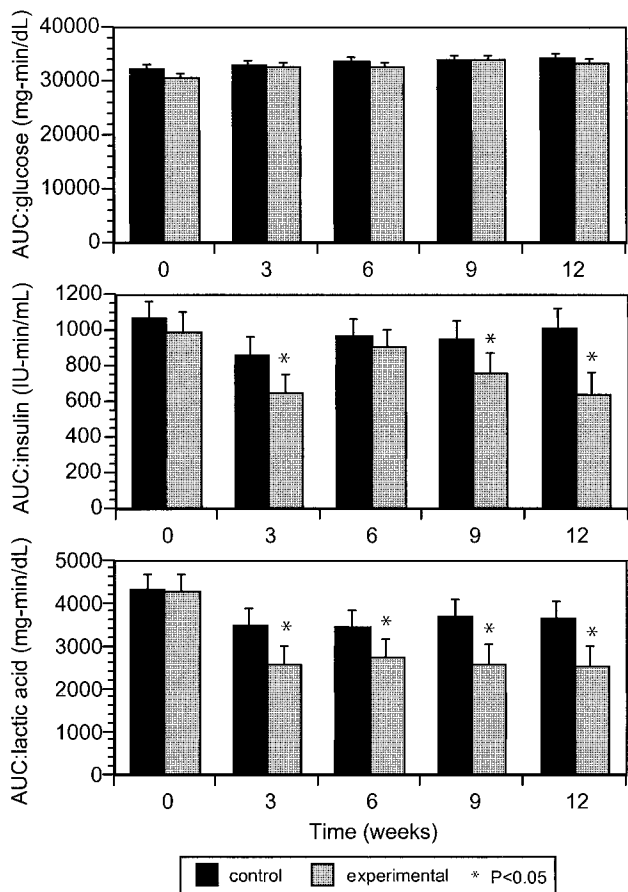
**FIGURE 3.** Mean area under the curve $\pm$ standard deviation concentrations of glucose, lactate, and insulin in response to an intravenous glucose tolerance test in the plasma of dogs with lymphoma fed the diet supplemented with n-3 fatty acids and arginine or a control diet are shown. Note significant decreases (*) 3 weeks beginning after initiation of feeding in the dogs fed the experimental diet. AUC: area under the curve.

## Effect of Diet on Body Weight and Resting Energy Expenditure

There were no significant ($P > 0.10$) differences between treatment and control groups in body weight or REE before the initiation of experimental diet feeding. Neither parameter was significantly altered by the experimental diet, but resting energy expenditure standardized to metabolic body size did decrease with time ($P < 0.001$) in Stage III dogs fed either the experimental or the control diets.

## Effect of Diet on Quality of Life Parameters, Disease Free Interval, and Survival Time

No factors were found to have significant effects ($P > 0.10$) on quality of life or toxicity scores (Tables 2 and 3) at any time point, with either treatment, by both parametric and nonparametric tests. Dogs fed the ex-

TABLE 3
**Serum Inflammatory Cytokine and Alpha 1-AG Concentrations**

| | IL-6 | | TNF-alpha | | Alpha 1-AG | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE |
| Week 0 | | | | | | |
| Control | 0.221 | 0.073 | 62.114 | 26.233 | 740.25 | 77.494 |
| Experimental | 0.586 | 0.083 | 27.354 | 30.210 | 576.40 | 91.589 |
| Week 3 | | | | | | |
| Control | 0.151 | 0.073 | 39.305 | 26.288 | 528.06 | 78.592 |
| Experimental | 0.222 | 0.083 | 48.825 | 30.527 | 409.05 | 92.216 |
| Week 6 | | | | | | |
| Control | 0.128 | 0.073 | 31.444 | 26.288 | 449.31 | 78.592 |
| Experimental | 0.195 | 0.083 | 51.175 | 32.449 | 395.05 | 94.919 |
| Week 9 | | | | | | |
| Control | 0.139 | 0.079 | 26.761 | 27.926 | 405.75 | 81.780 |
| Experimental | 0.214 | 0.085 | 41.850 | 33.051 | 348.67 | 95.903 |
| Week 12 | | | | | | |
| Control | 0.140 | 0.079 | 23.702 | 29.090 | 410.51 | 84.008 |
| Experimental | 0.196 | 0.085 | 39.187 | 33.051 | 350.10 | 97.352 |

AG: acid glycoprotein; TNF: tumor necrosis factor; SE: standard error.

TABLE 4
**Summary Statistics for DFI and ST by Stage of Lymphoma**

| | | 95% Confidence interval | |
|---|---|---|---|
| Quantile | Point estimate | Lower | Upper |
| DFI | | | |
| Stage III | | | |
| 75% | 268.0 | 200.0 | 332.0 |
| 50% | 157.5 | 107.0 | 224.0 |
| 25% | 94.5 | 63.0 | 144.0 |
| Mean | 181.8 | 131.0 | 231.0 |
| Stage IV | | | |
| 75% | 71.0 | 36.0 | 87.0 |
| 50% | 45.5 | 0.0 | 87.0 |
| 25% | 18.0 | 0.0 | 55.0 |
| Mean | 44.5 | 7.0 | 81.0 |
| ST | | | |
| Stage III | | | |
| 75% | 413.0 | 324.0 | 569.0 |
| 50% | 319.0 | 202.0 | 390.0 |
| 25% | 149.0 | 85.0 | 312.0 |
| Mean | 308.7 | 268.0 | 348.0 |
| Stage IV | | | |
| 75% | 112.0 | 57.0 | 130.0 |
| 50% | 75.5 | 36.0 | 130.0 |
| 25% | 46.5 | 36.0 | 94.0 |
| Mean | 79.3 | 39.0 | 119.0 |

DFI: disease free interval; ST: survival time.

perimental diet had numerically ($P = 0.29$) greater mean $\pm$ standard error (SE) ($181.5 \pm 26.8$ days), median (144 days), 75th quantile DFI (270 days), and 25th quantile DFI (84 days) compared with the dogs fed the control diet (mean plusmn; SE: $136.7 \pm 34.5$ days;

**FIGURE 5.** Plasma concentrations of docosahexaenoic acid (22:6) compared with survival time for dogs fed the experimental diet are shown. ST: survival time.
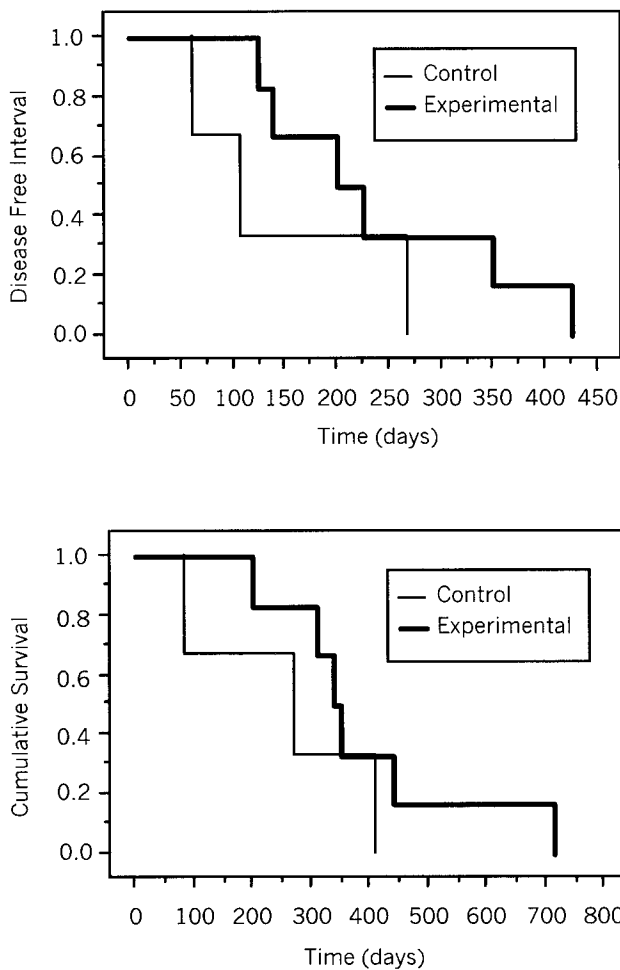
**FIGURE 4.** Kaplan–Meyer curves for disease free interval (top) and survival time (bottom) for all dogs with Stage III lymphoma are shown. Dogs fed the n-3 and arginine supplemented diet had a significantly longer disease free interval and survival time.

median: 107 days; 75th quantile: 209 days; 25th quantile: 63 days). Dogs fed the experimental diet had numerically ($P = 0.23$) greater mean ± SE (318.6 plusmn; 57.0 days), median (319 days), 75th quantile ST (440 days), and 25th quantile ST (124 days) compared with the dogs fed the control diet (mean ± SE: 227.6 ± 42.1 days; median: 232 days; 75th quantile: 388 days; 25th quantile: 94 days) (Table 4). Stratification for age older than or younger than 9 years did not improve the statistical analysis. Stratification for stage of lymphoma influenced DFI and ST outcomes, because dogs with Stage IV lymphoma had markedly shorter ($P < 0.001$) DFI and ST (Table 4).

Bivariate analysis for the effect of diet on dogs with Stage III lymphoma revealed a significant effect of dietary n-3 and arginine supplementation on increased DFI ($P = 0.0038$) and ST ($P = 0.0015$) (Fig. 4).
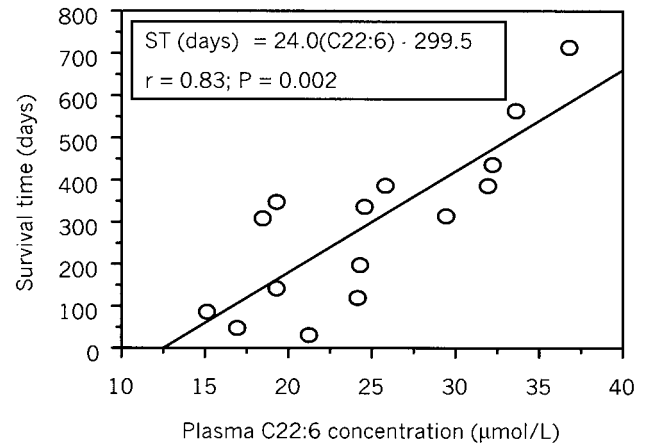
Stage IV patients were not affected by diet.

## Bivariate Associations with DFI and ST

Further tests for the bivariate association of metabolic parameters and indices of inflammation with DFI and ST revealed some relations independent of diet, gender, or age, after stratification for stage. Higher plasma concentrations of lactic acid and IL-6 were associated with shorter DFI and ST. The DFI was significantly lower for dogs with higher baseline plasma lactic acid concentrations during the IVGTT ($P = 0.011$) and DTT ($P = 0.008$), as well as higher AUC for lactic acid during the IVGTT ($P = 0.015$) and DTT ($P = 0.007$). The ST was significantly lower for dogs with higher baseline plasma lactic acid concentrations during the IVGTT ($P = 0.004$) and DTT ($P = 0.009$), as well as higher AUC for lactic acid during the IVGTT ($P = 0.006$) and DTT ($P = 0.042$). Higher plasma IL-6 was associated with significantly lower DFI ($P = 0.0008$) and ST ($P = 0.031$), but these associations may have been confounded by extremely high baseline values for dogs fed the experimental diet. Higher REE standardized to metabolic body size also was associated with significantly lower DFI ($P = 0.029$) and ST ($P = 0.028$).

Plasma concentrations of arginine, which were increased by the experimental diet, were significantly associated with longer DFI ($P = 0.065$) and longer ST ($P = 0.0007$) for dogs with Stage III but not Stage IV lymphoma. Serum concentrations of n-3 fatty acids, which were increased by the experimental diet, also were associated with longer ST (C22:6; $P = 0.037$ [Fig. 5]; C20:5; $P = 0.086$) for dogs with Stage III but not Stage IV lymphoma. Least squares regression analysis

suggested that for each 1-mmol/L increase in serum C22:6, the probability of dying by any given time point decreased by approximately 3.7% in Stage III patients fed the experimental diet. For an increase in serum C22:6 concentrations of 1 standard deviation (approximately 11.0 mmol/L), the probability of dying at any given time point would decrease by nearly 40%. Dogs with Stage IV lymphoma had DFI of less than 70 days and ST of less than 100 days regardless of serum C22:6 or C20:5 concentrations. Serum n-3 fatty acid values did not influence DFI or ST for dogs fed the control diet, regardless of stage of lymphoma.

## DISCUSSION

These results document for the first time as far as we know in an outbred species with spontaneously occurring malignancy that higher serum levels of C22:6 and C20:5 were associated with a longer DFI and ST. These results are consistent with previous in vitro studies, which have shown that PUFAs have direct antitumor activity. The mechanisms by which PUFAs kill cancer cells is unknown, but there is evidence to suggest that cancer cell cytotoxicity may be related to lipid peroxidation and production of superoxide radicals.[39] In addition, the n-3 fatty acids C20:5 and C22:6 can inhibit the synthesis of arachidonic acid from linoleic acid and compete with arachidonate for the 2-acyl position in membrane phospholipids. These fatty acids are preferentially metabolized by cyclooxygenase and lipoxygenase to eicosanoids and leukotrienes with differing biologic activities from those of the n-6 series.[40] For example, one recent study demonstrated that n-6 PUFAs, primarily linoleic acid, promote the proliferation of breast carcinoma cells directly and indirectly through increased synthesis of cyclooxygenase- and lipoxygenase-catalyzed products.[41,42] In contrast, the n-3 PUFAs C20:5 and C22:6 suppress breast tumor cell proliferation. In addition, PUFAs such as C20:5 have been shown to sensitize cancer cells to doxorubicin cytotoxicity.[43]

Another important observation in this study was that the area under the curve for plasma lactic acid in response to glucose and diet tolerance testing decreased significantly for dogs fed the experimental diet for 3 weeks or more. In addition, lower plasma concentrations of lactic acid were associated with a more favorable DFI and ST. This is logical because lactic acidosis is a marker of unfavorable metabolic conditions associated with many forms of cancer. However, it is unclear whether lactic acidosis is a cause or effect of changes in DFI or ST. The mechanisms for elevated blood lactic acid levels for dogs and humans with a wide variety of malignancies remain uncertain, but increased rates of anaerobic glycolysis have been documented in persons with cancer and lactic acidosis.[44,45] Tumor cells also have been hypothesized to be the source of the increased lactic acid. Neoplastic cells may use embryonic forms of key enzymes of anaerobic metabolism, such as hexokinase, 6-phosphofructokinase, and pyruvate kinase, which are less subject to host feedback mechanisms.[46] It is likely, although still conjectural, that inflammatory cytokine-mediated increases in host tissue glycolysis are responsible for increased production of lactic acid in many, if not all tissues in the body. Futile cycles resulting from elevated Cori cycle activity of lactic acid interconversion with glucose may increase the daily energy requirements of human patients with cancer cachexia by approximately 20%.[44–46] The reason why decreased lactic acid levels were not correlated with an improved quality of life in this study remains unknown but may be the limited number of animals studied for these subjective parameters.

We also report herein that the area under the curve for serum insulin decreased significantly for dogs fed the experimental diet for 3 weeks or more. Elevated blood insulin concentrations have been reported for dogs, laboratory animals, and persons with cancer.[8-10,44,45] Results of studies in human beings and laboratory animals with cancer cachexia provide ample evidence of decreased postreceptor insulin responsiveness resulting in part from increased serum concentrations of free fatty acids and lipoproteins, as well as other insulin-antagonistic metabolic defects.[44–47] Normalizing insulin levels, and presumably normalizing carbohydrate kinetics, would have been of theoretical benefit to the dogs in this study. Additional studies are essential to examine the effect of changes in insulin values on quality of life, DFI, and ST.

Whereas this appears to be first time as far as we know that a diet supplemented with n-3 fatty acids and arginine has been shown to improve disease free interval and survival in an outbred species with cancer, others have shown that n-3 fatty acids, arginine, and nucleotides can have significant impact on hospitalized human patients. One study of patients undergoing major abdominal surgery showed that this dietary therapy resulted in improved immune competence.[48] Another study showed that patients in a critical care unit fed the n-3 fatty acid, arginine, and nucleotide supplemented diet had a substantial reduction in length of hospital stay and a reduction in the number of nosocomially acquired infections.[49] Finally, Daly et al. documented that diet significantly improved immunologic, metabolic, and clinical outcome in persons who under-

went surgery for upper gastrointestinal malignancies.[50]

One obvious question not answered in this study is if the beneficial results noted are a result of the absolute dietary amount of n-3 fatty acids or the ratio of n-3 to n-6 fatty acids in the diet. Dose-responsive suppression of eicosanoid synthesis from arachidonic acid by dietary n-3 fatty acids is lost when the ratio of n-3 to n-6 fatty acids is maintained at a constant level by simultaneous dietary safflower oil addition.[51] Thus, it appears that mechanisms for n-3 effects relying on membrane fatty acid replacement depend on relative, rather than absolute, dietary amounts of the various essential fatty acid classes. Studies in piglets and neonatal rats have shown that increasing brain and retina levels of arachidonic acid and docosahexaenoic acid result with increasing dietary intakes of linoleic and $\alpha$-linolenic acid, respectively. This continues until a plateau is reached at approximately 2.4% of dietary energy for linoleic acid and 0.7% of dietary energy for $\alpha$-linolenic.[52] Further increases in the C18 precursors do not affect plasma or tissue levels of their elongated counterparts. However, increased consumption of the long chain metabolites appear to increase circulating plasma phospholipid levels without dose limitations.[52] Thus, effects of n-3 fatty acid dose versus n-3:n-6 fatty acid ratio may depend on independent factors including dietary energy density, fatty acid chain length, and biologic availability of supplements. For dogs supplemented for 12 weeks with increasing ratios of n-6:n-3 fatty acids manipulated by altering dietary menhaden, flaxseed, and safflower oil content, ratios of 5:1 and 10:1 significantly altered isolated neutrophil synthesis of leukotriene B4 (30–33% less) and B5 (370–500% more), whereas ratios of 25:1, 50:1, and 100:1 were without effect.[53] Fatty acid dose and ratio of these diets were without clinically important effects on platelet function and blood coagulation pathways.

## REFERENCES

1. Vail DM, Ogilvie GK, Wheeler SL. Metabolic alterations in patients with cancer cachexia. *Comp Cont Ed Pract Vet* 1990;12:381–7.
2. Brennan MF. Uncomplicated starvation versus cancer cachexia. *Cancer Res* 1977;37:2359–64.
3. Chlebowski RT, Heber D. Metabolic abnormalities in cancer patients: carbohydrate metabolism. *Surg Clin N Am* 1986;66:957–68.
4. Heber D, Byerly LO, Chlebowski RT. Metabolic abnormalities in the cancer patient. *Cancer* 1985;55:225–9.
5. Dewys WD. Pathophysiology of cancer cachexia: current understanding and areas of future research. *Cancer Res* 1982;2(Suppl 5):721–6.
6. Kern KA, Norton JA. Cancer cachexia. *J Parenteral Enteral Nutr* 1988;12:286–98.
7. Ogilvie GK, Vail DM, Wheeler SL, Fettman MJ, Salman MD, Cooper MF, et al. Effect of chemotherapy and remission on carbohydrate metabolism in dogs with lymphoma. *Cancer* 1992;69:233–8.
8. Ogilvie GK, Ford RD, Vail DM, Walters LM, Babineau C, Fettman MJ. Alterations in lipoprotein profiles in dogs with lymphoma. *J Vet Intern Med* 1994;8:62–6.
9. Vail DM, Ogilvie GK, Wheeler SL, Fettman MJ, Johnston SD, Hegstad RL, et al. Alterations in carbohydrate metabolism in canine lymphoma. *J Vet Intern Med* 1990;4:8-14.
10. Ogilvie GK, Walters L, Salman MD, Fettman MJ, Johnston SD, Hegstad RL. Alterations in carbohydrate metabolism in dogs with nonhematopoietic malignancies. *Am J Vet Res* 1997;58:277–81.
11. Ogilvie GK, Walters LM, Greeley SG. Concentration of alpha 1-glycoprotein in dogs with malignant neoplasia. *J Am Vet Med Assoc* 1993;203:1144–6.
12. Karmall RA. Historical perspective and potential use of n-3 fatty acids in therapy of cancer cachexia. *Nutrition* 1996;12:S2–4.
13. Kinoshita K, Noguchi M, Tanaka M. Effects of linoleic acid, eicosapentaenoic acid and docosahexaenoic acid on the growth and metastases of MM48 mammary tumor transplants in mice. *Int J Oncol* 1996;8:575–81.
14. Chicco A, D'Alessandro ME, Karabatas L, Gutman R, Lombardo YB. Effect of moderate levels of dietary fish oil on insulin secretion and sensitivity, and pancreatic insulin content in normal rats. *Ann Nutr Metab* 1996;40:61–70.
15. Pomposelli JJ, Flores EA, Blackburn GL, Zeisel SH, Bistrian BR. Diets enriched with n-3 fatty acids ameliorate lactic acidosis by improving endotoxin-induced tissue hypoperfusion in guinea pigs. *Ann Surg* 1991;213:166–77.
16. Kaiser L, Boyd NF, Kriukow V, Trichler D. Fish consumption and breast cancer risk: an ecological study. *Nutr Cancer* 1989;12:61–8.
17. Yamamoto D, Kiyozuka Y, Adachi Y, Takada H, Hioki K, Tsubura A. Synergistic action of apoptosis induced by eicosapentaenoic acid and TNP-470 on human breast cancer cells. *Breast Cancer Res Treat* 1999;55:149–60.
18. Norton JA, Gorschboth CM, Wesley RA, Burt ME, Brennan MF. Fasting plasma amino acid levels in cancer patients. *Cancer* 1985;56:1181–6.
19. Kirk SJ, Hurson M, Regan MC, Holt DR, Wasserkrug HL, Barbul A. Arginine stimulates wound healing and immune function in elderly human beings. *Surgery* 1983;114:155–60.
20. Lowell JA, Parnes HL, Blackburn GL. Dietary immunomodulation: beneficial effect on oncogenesis and tumor growth. *Crit Care Med* 1990;18:S145–65.
21. Daly JM, Reynolds J, Thom A, Kinsley L, Dietrick-Gallagher M, Shou J, et al. Immune and metabolic effects of arginine in the surgical patient. *Ann Surg* 1988;208:512–23.
22. Owen LN. TNM classification of tumors of domestic animals. Geneva: World Health Organization, 1981.
23. Valerius KD, Ogilvie GK, Mallinckrodt CH, Goetze DM. Doxorubicin alone or in combination with asparaginase, followed by cyclophosphamide, vincristine, and prednisone for treatment of multicentric lymphoma in dogs: 121 cases (1987–1995). *J Am Vet Med Assoc* 1997;210:512–6.
24. Ogilvie GK, Walters LM, Fettman MJ, Hand MS, Salman MD, Wheeler SL. Energy expenditure in dogs with lymphoma fed two specialized diets. *Cancer* 1993;71:3146–52.
25. Fedak MA, Pinshow B, Schmidt-Nielsen K. Energy cost of bipedal running. *Am J Physiol* 1974;277:1038–44.

26. Fox LE, King RR, Shi F, Kurzman ID, Macewen EG, Kubilis PS. Induction of serum tumor necrosis factor-alpha and interleukin-6 activity by liposome encapsulated muramyl tripeptide-phosphatidylethanolamine (L-MTP-PE). *Cancer Biother* 1994;9:329–40.

27. Elmslie RE, Ogilvie GK, Dow SW, Gasper P, Hoover EA, Cooper MF, et al. Evaluation of a biologic response modifier derived from Serratia marcescens: effects on feline macrophages and usefulness for the prevention and treatment of viremia in feline leukemia virus-infected cats. *Mol Biother* 1991;3:231–8.

28. Goitsuka R, Ohashi T, Ono K, Yasukawa K, Koishibara Y, Fukui H, et al. IL-6 activity in feline infectious peritonitis. *J Immunol* 1990;144:2599–603.

29. van Snick J, Cayphass S, Vink A, Uyttenhove C, Courie PG, Rubira MR, et al. Purification of NH2-terminal amino acid sequence of a T-cell derived cytokine with growth factor activity for B-cell hybridomas. *Proc Natl Acad Sci USA* 1986; 83:9679–83.

30. Hansen MB, Nielson SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989;119: 203–6.

31. Zicker SC, Rogers QR. Temporal changes in the concentrations of amino acids in plasma and whole blood of healthy neonatal foals from birth to two days of age. *Am J Vet Res* 1994;55:1020–9.

32. Zicker SC, Rogers QR. Use of plasma amino acid concentrations in the diagnosis of nutritional and metabolic diseases in veterinary medicine. In: Kaneko JJ, editor. Proceedings IVth Congress of the International Society for Animal Clinical Biochemistry University of California, Davis, CA, July 18–21, 1990.

33. IP C, Birt DF, Rogers AE, Mettlin C, editors. Dietary fat and cancer. *Prog Clin Biol Res* 1986;222:3-885.

34. Ohta A, Mayo MC, Kramer N, Lands WE. Rapid analysis of fatty acids in plasma lipids. *Lipids* 1990;25:742–7.

35. Littel RC, Milliken GA, Stroup, WW, Wolfinger RD. The SAS system for mixed models. Cary, NC: SAS Institute Inc., 1996: 87–134.

36. SAS. SAS/Stat users guide. Version 6.03. 4th ed. Cary, NC: SAS Institute Inc., 1989:1024–76.

37. Sokal RR, Rohlf FJ. Biometry. 3rd ed. New York: W.H. Freeman and Co., 1995:598–609.

38. Cox DR, Oates D. The analysis of survival data. New York: Chapman and Hall, 1984:1–23.

39. Begin ME, Ells G, Horrobin DF. Polyunsaturated fatty acids-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. *J Natl Cancer Inst* 1988;80:188–94.

40. Lefer AM. Significance of lipid mediators in shock states. *Circ Shock* 1989;27:3–12.

41. Abou-el-Ela SH, Prasse KW, Carroll R, Wade AE, Dhar-wad-kar S, Bunch OR. Eicosanoid synthesis in 7, 12-demthyl-benz(a)-anthracene-induced mammary carcinomas in Sprague-Dawley rats fed primrose oil, menhaden oil, or corn oil diets. *Lipids* 1988;23:948–54.

42. Noguchi M, Rose DP, Earashi M, Miyazaki I. The role of fatty acids and eicosanoid synthesis inhibitors in breast carcinoma. *Oncology* 1995;52:265–71.

43. Plumb JA, Luo W, Kerr DJ. Effect of polyunsaturated fatty acids on the drug sensitivity of human tumor cell lines resistant to either cisplatin or doxorubicin. *Br J Cancer* 1993; 67:728–33.

44. Holroyde CP, Skutches CL, Boden G, Reichard GA. Glucose metabolism in cachectic patients with colorectal cancer. *Cancer Res* 1984;44:5910–3.

45. Holroyde CP, Gabuzda TC, Putnum RC. Carbohydrate metabolism in cancer cachexia. *Cancer Treat Rep* 1975;35:3710–4.

46. Lawson DH, Richmond A, Nixon DW, Rudman D. Metabolic approaches to cancer cachexia. *Annu Rev Nutr* 1982;2:277–301.

47. Moley JF, Morrison SQ, Norton JA. Insulin reversal of cancer cachexia in rats. *Cancer Res* 1985;45:4925–31.

48. Schilling J, Vranjes N, Fierez W, Joller H, Gyurech D, Ludwig E, et al. Clinical outcome and immunology of postoperative arginine, omega-3 fatty acids, and nucleotide-enriched enteral feeding: a randomized prospective comparison with standard enteral and low calorie/low fat IV solutions. *Nutrition* 1996;12:423–9.

49. Bower RH, Cerra FB, Bershadsky B, Licari JJ, Hoyt DB, Jensen GL, et al. Early enteral administration of a formula (Impact) supplemented with arginine, nucleotides and fish oil in intensive care unit patients: results of a multicenter, prospective, randomized, clinical trial. *Crit Care Med* 1995; 23:436–49.

50. Daly JM, Lieberman MD, Goldfine J, Shou J, Weintraub F, Rosato EF, et al. Enteral nutrition with supplemental arginine, RNA, and omega-3 fatty acids in patients after operation: immunologic, metabolic, and clinical outcome. *Surgery* 1992;112:56–67.

51. Boudreau MD, Chanmugam PS, Hart SB, Lee SH, Hwang DH. Lack of dose response by dietary n-3 fatty acids at a constant ratio of n-3 to n-6 fatty acids in suppressing eicosanoid biosynthesis from arachidonic acid. *Am J Clin Nutr* 1991;54:111–7.

52. Rioux FM, Innis SM, Dyer R, MacKinnon M. Diet-induced changes in liver and bile but not brain fatty acids can be predicted from differences in plasma phospholipid fatty acids in formula- and milk-fed piglets. *Nutrition* 1997;127: 370–7.

53. Vaughn DM, Reinhart GA, Swaim SF, Lauten SD, Garner CA, Boudreaux MK, et al. Evaluation of effects of dietary n-6 to n-3 fatty acid ratios on leukotriene B synthesis in dog skin and neutrophils. *Vet Dermatol* 1994;5:163–73.