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DISSERTATION

THE ROLE OF SUPPLEMENTAL GLUTAMINE ON METABOLIC AND ENDOCRINE CHANGES IN CANINE PATIENTS WITH CRITICAL ILLNESS AND NEOPLASIA

Submitted by

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In partial fulfillment of the requirements
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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ELISA M. MAZZAFERRO ENTITLED "THE ROLE OF SUPPLEMENTAL GLUTAMINE ON METABOLIC AND ENDOCRINE CHANGES IN CANINE PATIENTS WITH CRITICAL ILLNESS AND NEOPLASIA" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE DOCTORATE OF PHILOSOPHY

Committee on Graduate Work

Adviser

Department Head

ABSTRACT OF DISSERTATION

THE ROLE OF SUPPLEMENTAL GLUTAMINE ON METABOLIC AND ENDOCRINE CHANGES IN CANINE PATIENTS WITH CRITICAL ILLNESS AND NEOPLASIA

Metabolic and endocrine changes have been documented in canine patients with critical illness and neoplasia. The study was divided into two parts. The first part of the study investigated endogenous protein synthesis (PSR), urinary loss of nitrogen, glucose flux, resting energy expenditure (REE), and respiratory quotient (RQ) in fifteen dogs with osteosarcoma (OSA) and 11 healthy female beagle dogs using stable isotope tracer (^{15}N-glycine, 6,6-deuterium-glucose) and indirect calorimetry techniques. Indirect calorimetry was performed before and after limb amputation surgery. Twenty-four hours after surgery, infusions of stable isotope tracers (4.5 mg/kg 15N-glycine intravenous bolus, and 6,6-deuterium-glucose 4.5 mg/kg IV bolus, followed by 1.5 mg/kg/hour as a constant rate infusion for 3 hours) was performed. Blood samples were obtained before the start of stable isotope infusion, and at 3 and 10 hours after infusion. Urine was collected for ten hours after infusion of tracers, and a pooled urine sample was used for sample analysis. Mass spectroscopy was utilized to measure the isotope or its metabolites in the serum and urine, then endogenous protein synthesis, urinary loss of nitrogen, and glucose flux were calculated using lean body mass values obtained from a dual energy x-ray absorptiometry examination performed on each patient while under general anesthesia. Dogs with OSA had statistically higher REE in the pre, but not post-operative time periods. There was no significant difference in

RQ from dogs with OSA before or after surgery, and the healthy beagle controls Post-operatively, dogs with OSA had significantly lower rates of endogenous protein synthesis, higher urinary loss of nitrogen, and greater glucose flux than the healthy beagle controls, indicating alterations in nitrogen balance and carbohydrate flux.

The second part of the study was performed on 36 dogs with a variety of critical illnesses and neoplasia (6 sepsis, 5 trauma, 12 surgery, 13 neoplasia) before and after 48 hours of supplemental enteral feeding with or without additional supplemental L-glutamine powder. The hypothalamic-pituitary-thyroid and -adrenal axes were examined in the pre- and post-feeding time periods by measuring endogenous thyroid stimulating hormone (eTSH), total thyroxine (T_4) and ACTH stimulation tests using 0.25 mg/patient IV Cosyntropin[1]. Basal energy requirements (BER) were calculated using the formula (30 x body weight in kg) + 70 = kcal/day BER. Stable isotope tracer analyses were performed in an identical manner as that performed on the dogs with OSA before and after 48 hours of supplemental enteral feeding. Patients were also randomly assigned to receive L-glutamine powder (0.24 g/kg/day) supplementation in excess of that found in the liquid enteral diet. A p-value of $p < 0.05$ was set as a level of significance. A Kolmogorov-Smirnov test was performed to assess normality of distribution of data. The data were normally distributed. Bartlett's test of homogeneity was performed to assess normality of variances of data. The variances were not homogeneous, and were log transformed for further statistical comparison. After log transformation of the data, an analysis of variance, with

Fisher's Least Significant Difference Test were performed for treatment effects and individual means comparisons. There was no significant difference in eTSH or T_4 values across disease categories, before or after supplemental feeding. Glutamine had no effect on thyroid axis function in any disease category. Euthyroid sick syndrome, characterized by low or normal eTSH and low T_4, was observed in 41.6% of patients before feeding, and 60% of patients after feeding. No significant difference in adrenocortical function was observed in any disease category. Glutamine supplementation had no effect on adrenocortical axis function. Endogenous protein synthesis was not statistically lower in any disease category before or after supplemental nutrition. Patients with sepsis had higher PSR after supplemental feeding. Overall urinary nitrogen loss statistically decreased in the post-feeding time period. Glutamine supplementation had no significant effect on PSR or urinary loss of nitrogen in any disease category. Glucose flux was not significantly lower in any disease category before or after supplemental feeding. Glutamine had no effect on glucose flux. The second part of the study demonstrated that euthyroid sick syndrome is a common occurrence in canine patients with critical illness and neoplasia, and that supplemental nutrition may prevent negative nitrogen balance during the course of illness and treatment. Key words: calorimetry, dual-energy x-ray absorptiometry, glutamine, critical illness, stable isotope tracer, metabolism

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PREFACE

This paper was written in standard thesis form using the conventions of the *American Journal of Veterinary Research*.

"In the middle of difficulty lies opportunity"..... Albert Einstein

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CHAPTER 1

INTRODUCTION

Metabolic derangements including changes in resting energy expenditure, impaired protein synthesis and carbohydrate utilization, and negative nitrogen balance have been documented in human and veterinary patients with neoplasia and critical illness. The metabolic changes observed may be associated with alterations in protein metabolism, suppression of thyroid axis activity and nutrient intake, release of glucocounterregulatory hormones during stress, and inadequate utilization of carbohydrates and protein sources for energy, including the futile cycling of energy substrates by the host. The use of stable isotope tracers has allowed researchers to investigate alterations in protein and carbohydrate metabolism that occur during states of stress and illness. Improvements in diagnostic endocrinology techniques have also led to a better understanding of hormonal axes within the body. The roles of the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary–adrenal (HPA) axes, in conjunction with alterations in protein and carbohydrate metabolism and utilization during critical illness and neoplasia are discussed.

The Hypothalamic-Pituitary-Thyroid Axis in Critical Illness

During states of health, the hypothalamic-pituitary-thyroid (HPT) axis plays a very important role in a variety of cellular functions, including stimulation of cellular oxygen consumption and basal metabolism, promotion of growth and development, regulation of cardiovascular indices, and regulation of carbohydrate and lipid metabolism.[1] The HPT axis

normally functions via negative feedback inhibition loops, insuring that hormones are released in sufficient quantities for normal body functioning.

The hypothalamus secretes thyrotropin releasing hormone (TRH) into the median eminence. Thyrotropin releasing hormone, in turn, acts on thyrotrophs, cells of the anterior pituitary gland, to stimulate the release of thyrotropin, or thyroid stimulating hormone (TSH). Thyrotropin (TSH) travels through the vascular system to the thyroid glands, stimulating the production and release of the thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). One hundred percent of T_4 is released directly from the thyroid glands in response to TSH. Approximately 10 - 20% of circulating T_3, the active form of thyroid hormone, is the result of direct release from the thyroid glands. Eighty to 90% of circulating T_3, however, is created via the action of a 5'-deiodinase enzyme in the peripheral tissues.[2] The deiodinase enzyme promotes monodeiodination of the outer ring of T_4 to form T_3. Both T_4 and T_3 are approximately 99% bound to prealbumin, albumin, and thyroid carrier proteins in the peripheral blood. Only a small quantity is "free" or unbound. Evidence supports that the free forms (i.e. FT_4 and FT_3) of circulating thyroid hormones have a negative feedback effect on the hypothalamus and pituitary gland to regulate the release of TRH and TSH, respectively.[1]

A variety of non-thyroidal systemic illnesses, including pneumonia, diabetes mellitus, hyperadrenocorticism, renal disease, liver disease, and neoplasia have been associated with euthyroid sick syndrome in dogs.[3-11] A combination of factors in euthyroid sick syndrome ultimately results in a decrease in circulating T_4 and T_3 with inappropriately low or normal TSH concentrations. Teleologically, down-regulation of thyroid hormone metabolism appears to reduce energy expenditure and protein loss during periods of stress and healing.[12] Euthyroid sick

syndrome in humans has been associated with impaired release of TRH from the hypothalamus, and decreased pituitary sensitivity to TRH in the median eminence, resulting in decreased TSH release.[13] Other mechanisms involved in the pathophysiology of euthyroid sick syndrome include decreased levels of thyroid binding proteins, decreased affinity of thyroid hormone for the carrier proteins, decreased conversion of T_4 to T_3 by impaired 5'-monodeiodinase function, and decreased affinity of T_3 for peripheral receptors. The combination of factors results in impaired thyroid hormone metabolism.

Glucocorticoids have been implicated as the primary mediators of euthyroid sick syndrome in both human and non-human animals. Administration of dexamethasone to normal euthyroid humans was found to decrease pituitary TSH release.[14,15] In dogs, administration of exogenous steroids has been demonstrated to decrease peripheral levels of thyroid hormone, as well as decrease thyroid response to exogenous TSH.[4,6,8] In addition, stress of concurrent non-thyroidal systemic illness is associated with euthyroid sick syndrome in acute[10] and chronic[3,5,9,11] illness in dogs. In one study, 67% of dogs admitted to a critical care unit demonstrated derangements of thyroid axis function, including decreased T_4 and decreased T_3 concentrations.[10] Low T_3 concentrations were associated with a significantly higher risk of mortality.[10] This is similar to findings of human studies that documented increased mortality in patients with severely decreased thyroid hormone concentration.[16,17] Improvement of clinical conditions was associated with similar improvements in thyroid axis function. In humans, failure to improve thyroid indices with improvement from non-thyroidal illness is correlated with an increased risk of mortality.[18] Baseline T_3 and T_4 levels have been demonstrated to be superior to APACHE II scores for positive predictors of mortality in humans admitted to critical care units.[19] Impaired

thyroid function is a common entity in both human and veterinary medicine. Given such results, investigation of thyroid indices in veterinary patients with critical illness is indicated. Recognition of severity of thyroid hormone derangements in veterinary patients may be useful in assessing severity of systemic illness, as well as predicting clinical outcome.

The Hypothalamic-Pituitary-Adrenal Axis in Stress and Critical Illness

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a normal physiologic response to stressors, including those associated with illness, injury, and infection. A variety of stressors, via neuroendocrine activation, ultimately causes the release of corticotropin releasing hormone (CRH) from the hypothalamus.[12] Corticotropin releasing hormone, in turn, acts on cells of the anterior pituitary gland to stimulate the release of adrenocorticotropic hormone (ACTH). Adrenocorticotropic hormone then travels through the vascular system to stimulate the adrenal cortex to release the mineralocorticoid and glucocorticoid hormones aldosterone and cortisol, respectively. Cortisol is a hormone vital to normal cellular functioning and maintenance of homeostasis. Functions of cortisol include regulation of total body water distribution, maintenance of vasomotor tone and vascular permeability, and potentiation of the vasoconstrictor effects of catecholamines.[20]

The HPA axis has been extensively studied, but a complete understanding of its function in critical illness remains poor.[21] Increases in basal cortisol levels in response to illness-induced stress have been well-documented in human patients with critical illnesses.[20] Characteristics associated with the HPA axis in human patients with a variety of critical illnesses include increases in cortisol levels with loss of normal diurnal variation, a biphasic corticotropin response with ACTH levels initially high then falling, decreased affinity of glucocorticoid

receptors, and decreased levels of cortisol binding proteins.[21] Severe increases in basal and ACTH-stimulated cortisol release have been associated with an increase in mortality. Elevations in basal cortisol levels upon admission into a critical care unit has been found to be a good discriminator of outcome prediction in humans, with non-survivors having significantly higher baseline cortisol concentrations than survivors.[19,20,22] The increase in cortisol following stress may reflect the severity of illness or injury.[23] However, very extreme elevations in cortisol levels have been associated with a preterminal state and increased mortality.[23]

In a small percentage of patients, however, adrenocortical burnout can occur following prolonged illness, resulting in severe hemodynamic compromise characterized by increased cardiac output, profound refractory hypotension, decreased systemic vascular resistance, and blunted response to ACTH administration. In one study, the overall incidence of adrenocortical burnout among 1054 patients with trauma, surgery, neoplasia, or gynecological or urological problems was just 0.66%.[24] Other studies have documented a severe increased risk of mortality with low baseline and ACTH-stimulated cortisol release in human patients with sepsis.[25] Overall mortality rate in affected patients ranges from 25 to 100% in various studies unless exogenous glucocorticoids are administered.[20,24]

Subsequent to the discovery that occult hypoadrenocorticism occurs in a small but noteworthy percentage of human patients with critical illness, researchers have begun to investigate the incidence of this syndrome in critically ill veterinary patients. Evaluation of the HPA axis in clinically stressed dogs revealed 27% of dogs with non-critical illnesses demonstrated an exaggerated cortisol response to ACTH stimulation, and failed to suppress with low-dose dexamethasone challenge.[26] A second study documented a 79% rate of false positives

when dogs with non-adrenal illness were screened for adrenocortical stimulation using a urinary cortisol:creatinine ratio.[27] A recent retrospective study investigated the hypothalamic-pituitary adrenal axis in 20 dogs admitted to a critical care unit. Approximately 37% of patients had elevated basal cortisol levels, and approximately 10% had high baseline ACTH-stimulated cortisol levels. There were no significant differences in basal cortisol or median ACTH-stimulated cortisol concentrations between survivors and non-survivors. Dogs with lower initial ACTH-stimulated cortisol levels had increased ACTH-stimulated cortisol responses over time. However, no dog developed occult hypoadrenocorticism over six consecutive days of hospitalization.[20] A second veterinary study investigated activation of the hypothalamic-pituitary-adrenal axis in dogs with less severe critical illnesses.[28] Approximately 34% of dogs demonstrated elevated basal cortisol concentration, and 14% had elevated ACTH-stimulated cortisol concentrations. There appeared to be no significant correlation between cortisol levels and endogenous ACTH in either study.[20,28] Therefore, there appears to be a poor correlation between endogenous ACTH levels and cortisol levels in dogs. This is possible due to the susceptibility of this hormone to degrade without proper handling. Alternatively, other substances, such as endothelin and interleukin-1, have also been implicated to contribute to cortisol release from the adrenal cortex independent of ACTH.[29,30] The results of two veterinary studies demonstrate that circulating cortisol concentration is not a predictor of mortality.[20] Since no dog demonstrated occult hypoadrenocorticism, the prevalence of this syndrome appears to be very low in this subpopulation of critically ill dogs.[20] The exact interactions between the degree of illness and interaction of the hypothalamic-pituitary-adrenal axis still remain unknown at this time.

Protein Metabolism, Nitrogen Balance, and Glucose Flux During the Stress of Critical Illness and Neoplasia

Energy metabolism in critically ill patients is not fully understood.[31] Stress caused by various types of injury and infection is associated with a catabolic state in which loss of structural and functional body proteins necessary for homeostasis can occur. [32] The release of inflammatory cytokine mediators, such as interleukin-1, interleukin-2, interleukin-6, and tumor necrosis factor, along with endogenous glucocounterregulatory hormones have all been implicated to play a role in the catabolic stress response.[32,33,34] Activation of the sympathetic nervous system during stress causes the release of catecholamines such as epinephrine and norepinephrine. Cortisol is also released from the adrenal cortex. Epinephrine indirectly stimulates hepatic gluconeogenesis and glycogenolysis. Muscle glycogen is also converted to lactate, which is then shuttled to the liver for gluconeogenesis via the energy consuming Cori cycle. Muscle alanine, too, is used for glucose synthesis via the hepatic glucose-alanine cycle.[35] Simultaneously, the actions of catecholamines inhibit insulin release, while glucagon release is promoted. The increase in glucagon relative to insulin favors glycogenolysis, gluconeogenesis, and ureagenesis, promoting a state of catabolism and net loss of endogenous protein, rather than anabolism.[35,36] Infusions of cortisol, epinephrine, and glucagon into healthy human volunteers has been shown to promote negative nitrogen balance by enhancing peripheral proteolysis and urinary nitrogen excretion. In stressed patients, accelerated protein catabolism in excess of five times greater than that observed with non-stressed starvation can occur.[37] Endogenous nitrogen loss in the urine of affected patients can become extreme, often losing 10 – 20 grams of

nitrogen/kg per day.[34] This contributes to the protein-calorie malnutrition often observed in critically ill patients.

During the stress response, nutrient utilization differs significantly than during periods of simple, non-stressed starvation. During non-stressed starvation, caloric requirements are obtained primarily from fat and carbohydrates, with minimal use of endogenous protein stores, to maintain blood glucose levels. States of prolonged uncomplicated ("non-stressed") starvation also is associated with an increase in rT3, to down-regulate metabolism in an attempt to conserve energy. This does not appear to hold true in all cases of complicated, or "stressed" starvation, during which nitrogen losses continue to occur. During states of critical illness, endogenous protein is not spared, with approximately 25 percent of calories obtained from utilization of endogenous proteins.[32] Muscle protein appears to be the primary source of amino acids used for fuel. Increased protein catabolism can deplete the body of functional proteins used for immunity, wound healing, and cardiorespiratory system function.[37] Additionally, preferential synthesis of acute phase proteins by the liver during stressed state can divert amino acid utilization towards acute phase protein synthesis and away from structural and functional protein synthesis. This can drain the body's store of amino acids from skeletal muscle and accelerate the loss of lean tissue[38,39,40]

Nitrogen balance is a function of nitrogen intake in the form of dietary protein and nitrogen loss.[38] Loss can occur in the urine, feces, and body cavity or wound exudates. Positive nitrogen balance occurs during periods of nitrogen retention, while negative nitrogen balance occurs when nitrogen loss exceeds intake.[33] Negative nitrogen balance ultimately reflects a state of protein catabolism, such as that observed in patients with critical illness. Often, nitrogen

balance is further worsened by inadequate consumption of nutrients, either due to a patient's anorexia or inability to eat, malabsorption of nutrients, or inadequate feeding orders by the primary clinician.[41] Researchers have demonstrated an increase in protein catabolism in human patients with advanced cancers, even when supplemental nutrition was administered.[42,43] Accelerated protein breakdown with impaired capacity for protein synthesis has also been documented in human patients with pancreatitis.[44] A number of other researchers have documented that even patients receiving total parenteral or enteral nutrition still remain in a state of negative nitrogen balance despite supplemental nutrition.[45,46] A study published by Michel et al[33] documented increased urinary nitrogen loss in the form of urinary urea nitrogen in a number of canine patients with a variety of critical illnesses, including trauma, surgery, infection, diabetes mellitus, and coagulopathies. Nitrogen loss in the canine patients corresponded to that observed in human patients with critical illness.[33]

Marked alterations in carbohydrate metabolism have also been documented in human and canine patients with various types of neoplasia, including carcinoma, sarcoma, lymphoma, and leukemia.[47,48,49,50] Abnormalities in hepatic glucose production, peripheral tissue glucose utilization, and whole body glucose oxidation and turnover occur. Inefficient utilization of carbohydrate as an energy substrate has been implicated to contribute to the state of cachexia observed in some cancer patients. Neoplastic cells preferentially consume carbohydrates as an energy source. Through anaerobic glycolysis, lactate is produced. Lactate, as stated previously, can then be shuttled back to glucose via the Cori cycle, an energy consuming process. The cycling of glucose through the Cori cycle, with a net loss of energy, is called a "futile cycle". Futile cycling of energy substrates through such pathways can increase the daily energy needs of

affected patients.[49] Ogilvie and others have documented hyperinsulinemia and glucose intolerance in patients with lymphoma, even after remission was achieved.[49] Other studies in human cancer patients have documented similar changes in serum insulin.[47] Additionally, serum lactate levels were found to be increased in canine patients with lymphoma[48] and non-hematopoeitic malignancies, even after removal of gross tumor burden.[50] Research conducted in human sarcoma patients has also demonstrated increased rates glucose turnover and recycling in addition to net protein catabolism[44,51]

Stress, such as that observed with critical illness, is associated with an increased flow of glucose from the liver to the periphery if the liver is functioning properly. With hypermetabolism, glucose is converted into three-carbon intermediates and shuttled back to the liver for synthesis of new glucose.[35] The glucocounterregulatory hormones epinephrine, cortisol, and glucagon released as part of the stress response increase the synthesis of pyruvate carboxylase and phophoenolpyruvate carboxylase, enzymes which favor the conversion of three-carbon intermediates to glucose.[35]. Entry of glucose into the tricarboxylic acid cycle is limited, favoring shuttling of glucose through other pathways, such as the Cori cycle or the glucose-alanine shuttle.[35]. Although glucose infusion has been shown to have a protein sparing effect during stressed conditions, further investigation is necessary to elucidate the types of energy substrates utilized during stress and critical illness.

Nutritional support in critically ill patients is aimed at preventing the negative effects of starvation during the course of disease, and minimizing the effects of protein catabolism.[52] Preventing protein-calorie malnutrition has been suggested to help prevent the increase in metabolic rate observed in some traumatized animals.[53] A meta-analysis of human critical care

patients documented a decrease in septic morbidity and decreased catabolism in patients with trauma and pancreatitis when early interventional nutrition was used.[40] Additionally, animal studies documented a decrease in enteric bacterial translocation and improved nitrogen balance when early enteral nutrition was started.[40] An improved understanding of protein and carbohydrate metabolism in patients with critical illness is required for proper nutritional management of critically ill veterinary patients.

Stable Isotope Tracer Studies in Metabolism

A stable isotope is, by definition, a non-radioactive form of an element that has a different atomic mass than the form of the element most commonly found in the environment.[54] The stable isotope contains the same number of protons as the most common form of the element, but differs in the atomic mass due to the different number of neutrons present.[55] This difference allows researchers to measure the amount of stable isotope relative to its more common counterpart using mass spectometry analysis. Examples of stable isotopes include carbon (^{13}C), deuterium (^{2}H), nitrogen (^{15}N), and oxygen (^{18}O).

Substitution of the stable, non-radioactive isotope into a compound allows researchers to investigate metabolic processes involving carbohydrate, protein, and lipid metabolism.[54] In metabolic tracer studies, one or more atoms of the parent compound are replaced with a stable isotope. The parent compound is then administered either parentally or orally. Once the compound enters systemic circulation, the compound containing the stable isotope competes with endogenous substrate in the host via the same metabolic pathway. This competitive interaction allows researchers to measure and calculate how the host metabolizes the compound.

The information obtained can then be extrapolated to provide information how the host metabolizes endogenous proteins, carbohydrates, or lipids under various conditions.

Once a substance containing a stable isotope is absorbed, kinetic studies are utilized to measure either the substance itself with no metabolic alterations, or measure the appearance of one or more products of the substances metabolism. These processes are known as autogenic kinetics, and heterogenic kinetics, respectively.[54] In order for a substance containing a stable isotope tracer to provide information about a system under study, the substance containing the tracer must compete with endogenous substrate and be metabolized in a similar manner. Additionally, the known amount of tracer is very small, as to not interfere with the system being studied.

The stable isotope tracer enters the metabolic pool as either a bolus or as a constant rate infusion. Once the tracer mixes into the metabolic pool, eventually a steady state is reached upon which the rate of entry of the tracer and exit of the tracer or its metabolites is constant. In many cases, the rate of removal of the tracer or its metabolites from the metabolic pool follows first-order kinetics, in which the rate of removal is directly proportional to the concentration of that substance within the metabolic pool. When a bolus injection of tracer is followed by a constant rate infusion of a known amount of the substance, the rate of turnover or "flux" of the substance through the body can be determined.[56] The stable isotope deuterium (^{2}H) is lost at various stages of glycolysis and gluconeogenesis.[57] In this study, the compound 6,6-^{2}H-glucose was utilized to measure the degree of carbohydrate flux in patients with a variety of critical illnesses.

Problems in interpretation of isotope metabolism can occur when amount of tracer in the metabolic pool is perturbed and its specific activity is not constant, therefore, it does not reach steady-state kinetics. This can occur if the amount of tracer entry into the pool or rate of exit from the pool is changed. When the quantity of substrate entering the metabolic pool is known, administration of additional substrate (either labeled or unlabeled) will perturb the system and compete with the known amount of substrate previously administered. A second scenario in which the metabolic pool is changed is when quantitation of a product from a substrate is being measured. Should the substrate undergo alternate metabolism through a different metabolic pathway, the amount of product to be measured will thus decrease. A list of potential complications and interferences with stable isotope tracer analysis in our critically ill dogs is provided in Table 1.

In this study, ^{15}N-glycine was administered as a single bolus injection. Glycine is conjugated with benzoic acid in the liver to form hippuric acid, which is then excreted in the urine. Urine and serum is then analyzed for metabolites of the parent compound. When ^{15}N glycine is used as a tracer, whole body protein synthesis and breakdown rates can be calculated from urinary ammonia or the cumulative excretion of ^{15}N, primarily in urinary urea.[58] In this experiment, ammonia was isolated from urine in a series of reactions using potassium carbonate and sulfuric acid, with ammonium sulfate as the final end product. Nitrogen from ammonium sulfate was then measured using isotope ratio mass spectrometry. This provided a quantitative measure of urinary nitrogen loss. Further analysis of the urine was performed to extract and measure hippuric acid using a gas chromatograph-mass spectrometer. Measurement of the ^{15}N-glycine metabolite hippurate in the urine allows calculation of the fractional synthesis of protein.

A complete description of the methodology used in this experiment is provided in Appendix 1. This method estimates the precursor pool for liver-derived protein synthesis. This can further be extrapolated to represent total body protein synthesis; however, this accuracy of this technique has been questioned by some researchers.[59] The difference between methodologies of the urea method and the ammonia method in estimating whole body protein synthesis is that the urea method primarily reflects hepatic protein synthesis, whereas the ammonia method also reflects contribution of amino acids from muscle. The urea method thus is biased toward reflecting only hepatic protein metabolism, and the ammonia method towards muscle protein metabolism.[57] In combination, quantitation of whole body fractional protein synthesis and urinary nitrogen loss provides researchers with methods to assess whole body nitrogen balance.[60] . This diagnostic tool be then be utilized to not only to clarify the pathophysiology of catabolic states, but also allow clinicians to more accurately determine the nutritional needs of critically ill patients.[61] In order to accurately determine markers of protein and carbohydrate metabolism using stable isotope tracer analysis, the use of Dual-Energy X-ray Absorptiometry in used to determine exact lean body mass, the metabolic pool in which the tracers are metabolized.

DUAL ENERGY X-RAY ABSORPTIOMETRY

A number of methods have been developed to allow determination of total body composition in both humans and a variety of animal species. Such methods include measurement of total body water by isotopic dilution techniques, skin fold measurements, bioelectrical impedance, and hydrodensitometry (underwater weighing).[62,63] However, the usefulness of these techniques is often limited by being logistically difficult to perform, expense, risk to patient, and poor precision and accuracy.[2,63,64,65,66,67] Additionally, some methods involve

a risk of radiation exposure. In a clinical and research setting, DEXA was found to be superior to other noninvasive methods of determining body composition in humans.[65]

Dual-energy x-ray absorptiometry is a non-invasive, relatively simple technique which allows precise and accurate assessment of total body composition.[63]Grier et al, 1996). An additional advantage of DEXA scanning is that total body radiation dose to the subject is minimal, and is approximately equal to background radiation in the environment.[68] Absorptiometry techniques were first developed to measure radionucleotide sources absorbed by bone.[69] Further development resulted in the use of dual-energy x-ray absorptiometry to measure total body composition in a three compartment model of bone mineral content, lean body mass, and fat mass.[65] Dual energy x-ray absorptiometry uses x-rays of two energy sources (70 and 140 kvp) passing through the subject under study. The subject is placed (or lies) on a table which contains an x-ray source. The two energy levels of x-rays pass through the patient, and are detected by a detector located in an arm that passes over the study subject. Each patient must lie completely still during the scanning process, necessitating heavy sedation or general anesthesia in some non-human species. The energy sources passing through the study subject are attenuated differently by the different types of tissue within the body. The attenuation of the energy beams is roughly proportional to the amount of mass through which it passes.[68] The ratio of soft tissue attenuation of the high and low energy x-ray beams is measured as they pass through the body and the detector is able to calculate the type and quantity of tissue in each pixel scanned.[63] The pixels scanned from attenuation through the study subject are compared to a block of known densities, called a phantom. Computer software that simulates the attenuation

properties of fat and lean body mass then uses algorithms to calculate the amount of bone mineral content, lean body mass, and fat mass with both accuracy and precision.[65,70]

A number of studies have been conducted both in humans and non-human animals investigating the precision and accuracy of DEXA to assess total body composition.[62,64,65,66,67,70,71,72,73,74,75,76,77] Precision is determined by the repeatability of measurements. Accuracy is a measure of how each measurement deviated from the actual measured value. Unpublished data from the University of Illinois compared DEXA analysis with chemical composition analysis in piglets, and found a positive correlation of $r^2 > 0.9$ between the two techniques.[74] Additionally, other researchers have validated the accuracy of DEXA analysis in dogs, finding a coefficient of variation for total body composition of just 1.55%.[74] Validation of DEXA by comparison with chemical analysis of dogs and cats indicated that, on average, DEXA compared very well to chemical analysis of body composition, but that DEXA results can be skewed by total body water content.[75] However, precision studies of lean body mass were just 0.1% in another study[76] indicating that DEXA is an accurate research tool to measure lean body mass for research purposes.

Use of Indirect Calorimetry to Assess Energy Expenditure

Energy is obtained through oxidation of carbohydrate, fat and protein substrates that are either ingested in foodstuffs, or from breakdown of body stores. An organism's basal energy requirement is the number of calories (kcal) expended by an awake animal in a post-absorptive state while resting in a thermoneutral environment.[78] This calculation differs slightly from minimum energy requirements of the organism, as MER also accounts for caloric expenditure used during the prehension and utilization of food. Values for BER and MER have been

obtained largely from healthy animals or extrapolated from values obtained from human subjects.[79] Precise measurement of resting energy expenditure has become a fairly routine technique utilized in nutritional support in human medicine.[80]

It was previously through that all humans and animals with illness are in a hypermetabolic state, and therefore require caloric intake in excess of basal energy requirements for maintenance of energy balance during periods of stress or illness.[79,81] This presumption led to the routine use of multiplying the basal energy requirement (BER) by an arbitrary illness/infection/injury factor to account for the increase in calories required in stressed or injured animals. Recent work, however, documented no significant difference in the basal energy expenditures as measured by use of indirect calorimetry analysis performed in over 106 critically ill dogs compared with REE of healthy controls.[79] Others have found no significant increase in REE of canine patients with nonhematopoietic malignancies.[82] Surgery and anesthesia also did not increase REE in such patients compared with healthy controls.[83] Resting Energy Expenditure has been documented to change over the course of time with varying severity of disease.[81] For example, energy expenditure in human head trauma patient was found to be increased acutely following injury, but then decreased to nearly basal levels over the next 12 - 19 days.[81] Other researchers have documented an increase in energy expenditure in patients with sepsis[52] and various forms of neoplasia.[38,42,84,85,86,87] Depending on the time that REE is measured in a patient, if REE reflects a hypermetabolic state, nutritional support based on these guidelines may overestimate caloric requirements if a patient's energy requirements decrease during the course of an illness. Use of illness/injury/infection multiplication factor may actually overestimate a patients' actual caloric needs. Conversely, if REE is measured at a time point in

which the patient is hypometabolic, nutritional support may underestimate the patient's changing needs and contribute to malnutrition. Oversupplementation of carbohydrate sources in human critically ill patients has been found to cause respiratory acidosis, and increase patient morbidity and mortality.[88,89] Therefore, metabolic energy requirements should be assessed using a method that is readily available and reliable.[90] Indirect calorimetry is a non-invasive technique that has been utilized in both human and veterinary medicine to calculate REE and give insight about the type of substrates being used for energy, thus allowing the clinician to predict daily caloric needs of the patients.

Indirect calorimetry measures gas exchange at the level of the alveolus. Knowing that fuels oxidized for energy require a net consumption of oxygen and produce carbon dioxide as a by-product, the open-flow indirect calorimeter measures the amount of oxygen consumed and the amount of carbon dioxide produced over a given time period. The open-flow indirect calorimeter uses a face-mask placed over the patient's mouth and nose. Collection of all expired gases occurs. The face mask is connected to a closed collection system to which oxygen and carbon dioxide detectors are connected. The levels of oxygen consumed and carbon dioxide produced are measured, and can be used to calculate resting energy expenditure and respiratory quotient using the following formulas:

Modified Weir Formula[91]

REE (kcal/day) = (3.9{VO_2 ml/min} + 1.1 {VCO_2 ml/min})1.44. Respiratory Quotient = VCO_2/VO_2

True basal metabolic requirements are difficult to obtain, as a variety of confounding factors, including stress, noise, hormonal fluctuation, and diurnal variation can all influence

energy expenditure.[79] Ideally, indirect calorimetry should be performed in a non-stressed patient following an overnight fast, as post-prandial thermogenesis can alter the calculations significantly. The patient is placed in lateral or sternal recumbancy in a thermoneutral environment and then acclimated to the indirect calorimetry unit for a period of 15 - 20 minutes. The system then measures oxygen consumption and carbon dioxide production for a period of 15 minutes after the system has reached steady-state. This approach has been validated in several studies in normal and unhealthy dogs.[80,90]

Resting energy expenditure, as calculated by the modified Weir formula, can be expressed in terms of actual body weight (kcal/kg/day) or metabolic body size (kcal/$kg^{0.75}$/day). Respiratory quotient predicts that type of substrate primarily used for energy. Respiratory quotients of lipid, protein, and carbohydrate consumption are 0.7, 0.8, and 1.0, respectively. Stressed animal primarily utilize carbohydrates for energy, and subsequently, respiratory quotients are nearly equal to 1.0. However, in a study published in dogs with critical illness, no study group demonstrated respiratory quotients near 1.0, indicating that they were utilizing lipids and proteins as primary energy substrates.[79] The respiratory quotients of surgical[82] and cancer[83] patients were similar to those observed in the critical care dogs. This suggests that hospitalization and surgery, nor stress associated with the indirect calorimetry analysis does not cause undue stress and thus unduly influence substrate utilization for energy purposes. Appropriate use of calorimetry techniques, in conjunction with stable isotope tracer analysis, may allow a better understanding of the pathophysiology of metabolic stresses in canine patients with critical illness.

Table 1: Potential interferences with the metabolism of stable isotopes include sources of human and technical error, and potentially clinical condition of the patient.

Potential interferences with stable isotope tracer analyses

Clinical Condition
Lack of hepatic urea synthesis and metabolism in patients with hepatic disease (hepatoma, hepatic abscess, portosystemic shunts)

Collection
Inadequate urinary collection (cannot obtain all of nitrogen lost in urine)
Inadvertent loss/spillage of urine thus decreasing known volume and amount of hippurate and labeled ammonia

Competition of labeled and unlabeled substrate
Inadvertent feeding of patient during study by student, thus competition of labeled and unlabeled substrate
Need to administer glucose for hypoglycemic patients

Delivery
Inaccuracy of infusion pump delivery
Disconnection of lines, unknown volume of sample lost
Loss of catheter patency, thus excluded from study

Therapy
Change in volume of distribution due to fluid therapy

APPENDIX 1: Methodology for stable isotope tracer analysis ^{15}N-glycine, and 6,6-^{2}H-glucose

A jugular or a lateral saphenous central venous catheter placed for ease of sample collection in all subjects. A urinary catheter was placed and connected to a closed collection system for 10 hours post-infusion for ease of urine collection and quantification. Blood samples (3 mL each) were obtained at 0, 2, 2.5, 3, 6, and 10 hours post infusion. Samples were allowed to clot, and serum decanted following centrifugation at 3400 rpm. Serum samples were frozen at -70 degrees Celsius and stored until analysis. Total urine volume for 10 hours post-infusion was determined. A 3 mL aliquot of pooled urine was frozen at -70 degrees Celsius and stored until analysis was performed. All samples were shipped on dry ice to the University of Medicine and Dentistry of New Jersey and analyzed for stable isotope metabolites via mass spectrometry.

Blood urea nitrogen (BUN) was determined by the urease method. For determination of the isotopic enrichment of the BUN, 1 mL of water and urease solution (1.0 mL, 60 uM units urease/mL in 0.1 phosphate buffer, pH 6.5) was added to 2 mL plasma. After 30 minutes of incubation at 37 degrees Celsius, 2 mL K_2CO_3 and 8 drops 2-Octanol were added. The reaction results in the production of ammonia. Ammonia was then removed via aeration and collected in 0.1N H_2SO_4. Total urinary nitrogen was measured on 1 mL of urine via the Kjedahl method. ^{15}N enrichment of the BUN derived ammonia and Kjedahl digests were measured via isotope ratio mass spectroscopy using a VG SIRA II mass spectrometer.

The isotopic enrichment of plasma glucose was also determined. Plasma (0.2 mL) was deproteinized with barium hydroxide [$Ba(OH)_2$] (4.73%) and 1 mL zinc sulfate [$ZnSO_4$] (5.5%). The supernatant was lyophilized following centrifugation. The residue was then reconstituted

with 1 mL deionized water and run through a column of Dowex 50 and Dowex 1 ion exchange resin (0.7 g each). The glucose was then eluted with 2 mL water. The sample was then frozen, lyophilized and converted to the penta-acetate derivative by reaction with excess acetic anhydride:pyridine (2:1, 0.2 mL) at 60 degrees Celsius for 10 minutes. The mixture was evaporated almost to dryness under a stream of nitrogen gas and reconstituted with dichloromethane (0.2 mL). The isotopic enrichment of the glucose was determined by a Hewlett Packard 5970 gas chromatograph-mass spectrometer in the selective ion monitoring mode. The ions at 200/202 were monitored.

Whole body protein synthesis rate (PSR) was calculated from the total amount of ^{15}N excreted. The amount of the administered dose excreted (*e) was calculated by measuring the amount of ^{15}N excreted in the 10 hour period and the ^{15}N remaining in the body urea pool at 9 hours. The latter was calculated from the blood taken at the end of the sampling period (10 hour sample). The body urea pool size was estimated by assuming total body water = body weight*0.65. The amount of isotope remaining in the BUN at ten hours was less than 5%. The following calculations were then used:

15N in urea pool = UDS(in liters)*BUN(g/L)*BUN15N (APE*0.01)

PSR = ET(*d/*e-1)

Where *d = ^{15}N given in g N 10 hr

*e = ^{15}N excreted (urine + BUN) in g N 10/hr

ET = N excretion in g N 10/hr

PSR = rate of protein synthesis in g N 10/hr.

The rate of glucose production was also calculated and used to determine glucose flux. For 6,6-(deuterium)-glucose, the rate of appearance (Ra) in the plasma was calculated using the formula:

$$Ra = F^* (APEinfusate/APRplasma - 1)$$

Where F = isotope administration rate, APE infusate and APE plasma are the isotopic enrichments in the infusate and plasma, respectively.

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CHAPTER 2[1]

METABOLIC ALTERATIONS IN DOGS WITH OSTEOSARCOMA

Objective: To evaluate changes in resting energy expenditure (REE) as well as protein and carbohydrate metabolism in dogs with osteosarcoma (OSA).

Animals: Fifteen weight-stable OSA dogs that did not have other concurrent metabolic or endocrine illness and twelve 1-year-old sexually intact female Beagles (control dogs).

Procedures: Indirect calorimetry was performed on all subjects to determine resting energy expenditure (REE) and respiratory quotient (RQ). Stable isotope tracers (^{15}N-glycine, 4.5 mg/kg IV and 6,6-deuterium-glucose 4.5 mg/kg IV followed by a continuous rate infusion 1.5 mg/kg hour) were used to determine rate of protein synthesis (PSR) and glucose flux in all dogs. Dual energy X-ray absorptiometry (DEXA) scans were performed to determine total body composition.

Results: Accounting for metabolic body size, REE in OSA patients, was significantly higher before and after surgery, compared with REE of healthy control dogs. The RQ values did not differ significantly between groups. Dogs with OSA also had decreased rates of protein synthesis, increased urinary nitrogen loss, and increased glucose flux during the post-operative period.

Conclusions and Clinical Relevance: Alterations in energy expenditure, protein synthesis, urinary nitrogen loss, and carbohydrate flux were evident dogs with OSA, similar to results

documented in humans with neoplasia. Changes were documented in REE as well as protein and carbohydrate metabolism in dogs with OSA. These changes were evident even in the absence of clinical cachexia.

Key Words: metabolism, carbohydrate, protein, neoplasia, osteosarcoma, energy expenditure

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[1] Mazzaferro EM, Hackett TB, Stein TP, Ogilvie GK, Wingfield WE, Walton J, Turner AS, Fettman MJ. Metabolic alterations in dogs with osteosarcoma. *Am J Vet Res* 2001; 62(8):1234-1239.

Introduction

Human and other domestic animals affected by cancer have alterations in protein and carbohydrate metabolism, including impaired carbohydrate utilization, altered lipid patterns, catabolism of endogenous proteins and changes in resting energy expenditure (REE).[1-11] Indirect calorimetry has become standard for use in human and veterinary research to determine REE and metabolic energy requirements (MER).[12-16] Metabolic energy requirements for healthy dogs have been known for many years;[17-19] However, MER have only recently been determined directly from animals with neoplasia.[12-14]

Assessment of total body composition is important for determining MER. Techniques to assess MER include evaluation of markers of muscle metabolism, measurement of total body potassium, densitometry, isotope dilution procedures, and anthropometry. These techniques can be time-consuming, invasive, and subjective in nature.[20] The newer technique of dual energy x-ray absorptiometry (DEXA) analysis is considered the standard for determining total body composition in human medicine.[21] Dual energy x-ray absorptiometry scans are gaining popularity among researchers involved in small animal research because of the ease of rapid, non-invasive, precise and repeatable measurements of total body composition.[22] Precise measurement of body fat, muscle and bone content can be used to determine lean body mass. These measurements can then be used to calculate more accurate estimates of REE and MER.

Reports of increased energy requirements with critical illness, neoplasia or trauma have led to the use of arbitrary multiplication factors to adjust REE to MER and to account for a presumed hypermetabolic state. However, conflicting reports exist in both human and veterinary literature regarding increases in REE attributable to disease. Several studies in human[23-25] and

veterinary[12-14,26] patients have found little or no difference in REE of patients with critical illness or neoplasia. For example, Ogilvie et al[12] observed a decrease in REE and oxygen consumption in dogs with lymphoma. Further, Walton et al[26] had equivocal results when determining REE in over 100 dogs that required critical care. Differences in REE were not observed in dogs that had surgical trauma, acquired trauma, or neoplasia, compared with 20 healthy control dogs. However, dogs with documented sepsis were found to have elevated REE compared with controls.

Increases in REE in tumor-bearing humans have been documented. Fredrix et al[27] found significantly higher REE in 60% of people with newly diagnosed non-small cell lung cancer. The same study documented hypermetabolism in only 14 of 104 (13%) of patients with gastric or colorectal cancer. Therefore, 87% of patients with colorectal or gastric cancer, and 40% of patients with non-small cell lung cancer were either eumetabolic or hypometabolic. Dempsey et al[28-29] also documented increases in REE in human patients with gastrointestinal or colorectal neoplasms. Peacock et al[30] documented similar results in human patients with sarcoma. Therefore, not all people with neoplasia are hypermetabolic, as previously believed. In fact, metabolic changes may represent a continuum of normometabolic, hypometabolic to hypermetabolic states, depending on the type and degree of illness at the time of diagnosis.

Analysis of results of studies[12,26] reveals that critically ill dogs or dogs with neoplasms may not differ substantially from those of clinically normal dogs, suggesting that previously utilized methods for predicting energy requirements may overestimate the actual needs of these dogs. An adequate understanding of the nutrient and energy requirements of cancer patients is necessary when considering nutritional therapy.[31] Alterations in metabolic pathways exist even

before the onset of clinical cachexia.[1,3,6-8,10,31-33] Use of stable isotope tracers has allowed researchers to obtain valuable information about metabolic derangements in patients with neoplasia.[1,34-36] The purpose of our study was to document changes in REE or respiratory quotient (RQ) in dogs with osteosarcoma (OSA) using indirect calorimetry techniques, and to determine whether the same dogs had measurable alterations in protein or and carbohydrate metabolism prior to the onset of clinical signs of anorexia or cachexia.

Materials and Methods

Patient Selection

Dogs with OSA: Fifteen client-owned dogs with OSA were included in the study. The OSA involved a single limb of each dog. Each owner consented to inclusion of their dog in the study. The study protocol was previously approved by a university animal care and use committee. A CBC, serum biochemistry analyses, and urinalysis was performed on each dog to rule out other underlying illness. Dogs were excluded from the study when they had underlying hepatic, renal or endocrine disease, had received steroid therapy or undergone anesthesia within 30 days prior to referral to our facility, or had historical or physical evidence of anorexia, weight loss or cachexia at the time of referral. During the study, general anesthesia was induced in each dog, and the affected limb was amputated, as described elsewhere.[37] Dogs had *ad libitum* access to water and were fed a commercial prescription diet[a] twice daily.

Healthy control dogs: Eleven healthy 1-year-old sexually intact female Beagle were selected for use as control dogs for comparison purposes. Each control dog was housed in a 2.4 m x 2.4m pens with 2 other beagles. Dogs had *ad libitum* access to water and were fed a commercial prescription diet (Hill's C/D, W/D or K/D)[a] twice daily.

Stable Isotope Tracers: Stable isotope tracers are widely used in human medicine to evaluate a variety of metabolic processes.[38-39] Analysis of stable isotope tracers was used to observe the characteristics of substrate flow in various metabolic pathways to determine carbohydrate and protein metabolism. Each dog was administered ^{15}N-glycine (4.5 mg/kg of body weight, IV)[b] and 6,6,-(deuterium)-glucose (4.5 mg/kg IV as a bolus, followed by constant rate infusion at 1.5 mg/kg/hour for 3 hours)[c].

Collection and analysis of blood and urine samples- a catheter[d] was placed in a jugular or a lateral saphenous of each dog for collection of blood samples. A urinary catheter[e,f] was inserted and connected to a closed collection system for 10 hours after infusion of stable isotopes to enable collection and quantification of urine. Blood samples (3 mL) were obtained at time 0 (just prior to start of isotope infusion), and 2, 2.5, 3, 6, and 10 hours after the start of isotope infusion. Samples were allowed to clot, and were centrifuged (3,000 x g), and serum was decanted. Serum samples were frozen at -70 C[g] and stored until analysis. Urine volume for the 10 hour period after infusion was determined. A 3 mL aliquot of pooled urine was frozen at -70 C and stored until analysis was performed. All samples were shipped on dry ice to a laboratory at the University of Medicine and Dentistry of New Jersey and analyzed for stable isotope metabolites, using mass spectrometry[h].

The BUN concentration was determined by use of the urease method [i]. For determination of the isotopic enrichment of the BUN, 1 mL of water and urease solution (1.0 mL, 60 μM units urease/mL in 0.1 phosphate buffer, pH 6.5) was added to 2 mL of serum. After incubation at 37 C for 30 minutes, 2 mL of K_2CO_3 and 0.4 ml of 2-Octanol were added. The reaction results in the production of ammonia, which is removed via aeration and collected in 0.1N H_2SO_4. Total

urinary nitrogen was measured on 1 mL of urine, using the Kjedahl method. ^{15}N enrichment of the BUN derived ammonia and Kjedahl digests were measured via isotope ratio mass spectroscopy using a mass spectrometer[40].

Isotopic enrichment of plasma glucose was determined as described elsewhere[41]. Serum (0.2 mL) was deproteinized with barium hydroxide [$Ba(OH)_2$] (4.73%) and 1 mL of zinc sulfate (5.5%). After centrifugation, the supernatant was lyophilized. The residue then was reconstituted with 1 mL of deionized water and separated through a column of Dowex 50 and Dowex 1 ion exchange resin[j] (0.7 g each). Glucose was eluted with 2 mL of water. The sample was frozen, lyophilized and converted to the penta-acetate derivative by reaction with excess acetic anhydride:pyridine (2:1, final volume of 0.2 mL) at 60 C for 10 minutes. The mixture was evaporated almost to dryness under a stream of nitrogen gas and reconstituted with 0.2 mL dichloromethane. Isotopic enrichment of the glucose was determined by use of a gas chromatograph-mass spectrometer[g] in the selective ion monitoring mode. The ions at 200/202 were monitored.[41]

Whole body protein synthesis rate was calculated from the total amount of ^{15}N excreted.[42-43] Amount of the administered dose excreted (*e) was calculated by measuring the amount of ^{15}N excreted in the 10 hour period and the amount of ^{15}N remaining in the body's urea pool at 9 hours. The latter was calculated from the blood taken at the end of the sampling period (sample obtained at 10 hours). Size of the body's urea pool was estimated by assuming total body water was equivalent to body weight x 0.65. The amount of isotope remaining in the BUN at ten hours was < 5%. Amount of ^{15}N in the urea pool was calculated, using the following equation:

^{15}N in urea pool = UDS (in liters)*BUN(g/L)*BUN ^{15}N (APE*0.01), where UDS is the urea distribution space, and APE is the percent excess.

Protein synthesis rate was calculated as follows:

PSR = ET(*d/*e-1)

Where *d = ^{15}N given in g N 10 hr

*e = ^{15}N excreted (urine + BUN) in g N 10/hr

ET = N excretion in g N 10/hr

PSR = rate of protein synthesis in g N 10/hr.

The rate of glucose production also was calculated and used to determine glucose flux. For 6,6-(deuterium)-glucose, rate of appearance (Ra) in serum was calculated using the following formula:

$$Ra = F^* (APE_{infusion}/APR_{plasma} - 1)$$

Where Ra is the rate of appearance in the serum, F* is the isotope administration rate, $APE_{infusion}$ and APE_{plasma} are the amount of isotopic enrichment in the infusate and serum, respectively.

Calorimetry

Indirect calorimetry was performed to determine REE and RQ for healthy Beagles and for dogs with OSA before and after limb amputation. Indirect calorimetry was performed in dogs with OSA before and 24 hours after anesthesia and amputation. Each control dog was acclimated to the indirect calorimetry unit[k] by use of once weekly sessions for 8 consecutive weeks. Food was then withheld overnight, and serial samples were obtained during a period of 10 – 15 minutes. The acclimation period and overnight withholding of food were used to

decrease the potential of stress-induced or postprandial increases in REE. Values for REE were estimated, using an open-flow indirect calorimetry system.[12-15,27,44-45] Rate of carbon dioxide production (VCO_2 ml/min/kg) and oxygen consumption (VO_2 ml/min/kg) were determined, and REE was then calculated by use of the following abbreviated Weir formula.[46] REE (kcal/day) =(3.9{VO_2 ml/min} + 1.1 {VCO_2 ml/min})1.44. This formula does not account for incomplete oxidation of protein; however, < 2.5% error is observed by use of this simplification.[44,46] The RQ is the ratio of VCO_2 to VO_2 and is used to estimate the type of substrates being used for energy. Values of RQ approaching 1.0 indicate glucose is the principal substrate for energy metabolism, whereas RQ values of 0.7 and 0.8 indicate that fat and protein, respectively, are the principal energy substrates.[16,44]

All calorimetry studies were conducted by the same two investigators (TBH, JW). Each dog was placed in lateral recumbancy in a thermoneutral (20 to 22 C) and relatively quiet environment to minimize stress. This method is similar to that described for humans and dogs in other studies.[12-15,27,29] For all evaluation periods, each dog was allowed to acclimate to the environment, handling and the collection mask for 5 to 15 minutes prior to sample collection.

To ensure that all expired gases were collected, the total flow of room air was 10 times the estimated basal VO_2. The percentage of O_2 in a dried aliquot (100 mL) of the effluent gas was measured continuously by an O_2 sensor[l] and O_2 analyzer[m] using electrochemical cell techniques. Percentage of CO_2 in a similar aliquot was also measured by a CO_2 sensor[n] and CO_2 analyzer[o], using infrared absorption. Medical grade 100% N_2 and 10% CO_2 were used to calibrate the unit immediately following each measurement. Before calibration, gas content of each tank was verified using mass spectrometry. A Brook's volumeter was used to calibrate flow

meters each month. VO_2 was calibrated using Fedak's nitrogen dilution technique, [15] whereas VCO_2 was calibrated by infusing a known flow of CO_2 into the mask. A minimum of 10 minutes was allowed for equilibration; once the system reached a steady state after that time, data were collected for an additional 15 minutes in order to calculate mean VO_2 and mean VCO_2 for the time period.

Dual Energy X-ray Absorptiometry

A DEXA scan[p] was performed on each dog with OSA while it was anesthetized for surgery to determine total body composition. Description and validation of DEXA analyses have been reported elsewhere .[22] Each control dog was anesthetized by administration of Propofol® (4 - 7 mg/kg IV as a bolus, followed by constant rate infusion to reach the desired effect)[q]. For dogs that weighed < 10 kg, infant whole-body software was used, whereas for larger dogs, software for adult human patients was used.

Statistical Analyses: Data were determined to be normally distributed by use of the Kolmogorov-Smirnov test, and differences between dogs with OSA and healthy control dogs were analyzed to detect statistical differences using an unpaired t-test[r]. Values for REE in dogs with OSA and healthy control dogs in our study were compared with values obtained for control dogs conducted by Walton et al.[26] Those differences were evaluated by ANOVA, followed by a Fischer's least significant difference test for comparison of means[r]. A value of $p < 0.05$ was used to define significant differences. All results are presented as mean ± standard deviation.

Results:

Age and sex of dogs with OSA: The 15 dogs represented 8 breeds (3 Rottweilers, 3 Golden Retrievers, 3 Labrador Retrievers, 2 Doberman Pinchers, 1 Irish Setter, 1 Bernese Mountain dog,

1 Australian Shepherd, and 1 Greyhound). Age at the time of diagnosis ranged from 4 to 11 years (mean ± SD, 9.07 ± 1.87 years). Eight dogs were spayed female, 1 was a sexually intact female, and 6 were neutered males. Breed distribution of these dogs was representative of the population of dogs with OSA typically seen at Colorado State University Veterinary Teaching Hospital during one year.

Body weight and composition: Dogs with OSA weighed 35.10 ± 9.10 kg, and had a significantly ($p < 0.001$) higher body fat percentage than control dogs. Body fat of dogs with OSA ranged from 10 to 40% (24.00 ± 8.62%), whereas for control dogs it ranged from and 6.40 to 17.20% (10.20 ± 3.25%).

Results of calorimetry - The REE of dogs with OSA was significantly higher before (120%; $p = 0.01$), and after (112%, $p = 0.04$) surgery, compared with values for control dogs (Table 1). Also, REE of dogs with OSA reported here was significantly ($p = 0.006$) higher than that of control dogs published elsewhere (75.80 ± 18.30).[26] The REE for our control dogs did not differ significantly ($p = 0.90$) from that of control dogs published elsewhere.[26] When corrected for lean body mass, REE obtained before surgery in dogs with OSA was still significantly ($p = 0.02$) higher 115% than that of control dogs. However, REE obtained after surgery for dogs with OSA did not differ significantly ($p = 0.35$), compared with values for control dogs. The RQ values from OSA dogs following withholding food before ($p = 0.26$) or after ($p = 0.50$) surgery were not significantly different from that of healthy control dogs.

Protein synthesis rate- Protein synthesis rate of dogs with OSA during the post-operative period was 59.70% of the rate of healthy control dogs. Mean value for dogs with OSA after surgery (7.97 ± 1.99 g/kg/day) was significantly ($p < 0.001$) less than for control dogs (13.40 ± 3.33

g/kg/day). Correcting for metabolic body size, protein synthesis rate of dogs with OSA was 72.70% of that observed for control dogs.

Mean value for dogs with OSA after surgery was 16.30 ± 7.99 g/kg$^{0.75}$/day, which was significantly (p = 0.048) less than the value for control dogs (22.50 ± 6.03 g/kg$^{0.75}$/day). Urinary nitrogen excretion was 165% significantly (p = 0.003) higher in dogs with OSA after surgery (0.76 ± 0.15 g/kg$^{0.75}$/day), compared with the value for control dogs (0.46 ± 0.26 g of protein/kg$^{0.75}$/day.

Glucose flux- Glucose flux of dogs with OSA was 178% that of healthy control dogs. Mean glucose flux in dogs with OSA after surgery (328.80 ± 124.30 mg/kg/hour) was significantly (p = 0.008) higher, compared with the mean value for control dogs (185.00 ± 94.90 mg/kg/hour).

Discussion

Analysis of the results of the study reported here revealed that changes in protein and carbohydrate metabolism, indicated by a decreased rate of protein synthesis, increased rate of urinary nitrogen loss, and increased glucose turnover were evident in dogs with OSA even though they did not have clinical signs of cachexia. In contrast to other studies conducted by our laboratory group on dogs with lymphoma or dogs requiring critical care in which the animals were hypometabolic and normometabolic,[12,14,26] dogs with OSA in this study were hypermetabolic prior to and following surgical removal of the tumor and affected limb, as determined on the basis of metabolic body size. When corrected for lean body mass, REE obtained after surgery for dogs with OSA was not significantly different from the value obtained before surgery or from the value for control dogs. Other researchers have described continuation of a hypermetabolic state even after surgical removal of tumor mass.[12,14,45,47] Removal of gross

tumor burden potentially may have cancelled out changes in REE after surgery that were attributable to the stress of anesthesia and surgery. However, other studies conducted by our laboratory group revealed that REE did not change following removal of gross tumor burden, including OSA,[14] or following elective surgeries.[13] Furthermore, although gross tumor burden was eliminated with amputation, microscopic tumor burden is likely to persist in dogs with OSA because of the propensity of this tumor type to metastasize.

Factors other than tumor burden alone are responsible for changes in REE and have been implicated in contributing to cancer cachexia, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-gamma), interleukin-1-beta (IL-1β), and interleukin-6 (IL-6).[36,48-50] Because we did not detect a significant difference in REE before and after surgery, it appears that REE did not increase as a result of the stress of anesthesia and surgery. This is similar to other findings by our laboratory group in which we documented that REE did not change in healthy dogs and dogs with malignancies before and after invasive surgery.[14]

One consideration is that dogs with OSA were not compared with age-matched controls. The healthy control dogs were younger in age and also had substantially lower percentage of body fat than dogs with OSA. However, it would be expected that younger, leaner dogs would have higher REE, compared with REE of clinically affected dogs. It is possible that control dogs had lower REE because of previous acclimation to the calorimetry unit and, therefore, were less stressed than clinical patients naïve to the apparatus. However, comparison of our control population did not differ significantly published control dogs.[26] Furthermore, variables that were dependent on body size were also analyzed relative to metabolic body size, using indexing on the basis of $kg^{0.75}$, and lean body mass. These are accepted methods for minimizing effects

of differences in mass and body composition that may be breed or age-dependent effects. The control population used in another study[26] was slightly older large-breed dogs. When corrected for lean body mass, REE in the study reported here was higher in dogs with OSA during the pre-operative but not post-operative period. Therefore, the discrepancy in REE of the dogs with OSA before surgery cannot be explained by age and body composition alone. Analgesics were required after surgery, and may have contributed to the decrease in REE observed in dogs with OSA. Alternatively, REE (when corrected for lean body mass) may have been significantly higher in before surgery in dogs with OSA as a result of some degree of discomfort or pain and initial stress of hospitalization. Because REE increases with stress, the decrease in REE in the post-operative period, compared with REE in the pre-operative period, could be associated with previous acclimation to the calorimetry unit. However, other research conducted by our laboratory group has not detected significant differences in REE or RQ in dogs with nonhematopoeitic malignancies before and after tumor removal.[13] Because a difference was not detected in that group of dogs patients following repeated calorimetry over time, it seems unlikely that acclimation to the calorimetry unit and decreased stress caused the decrease in REE observed in our dogs with OSA in the study reported here.

Furthermore, we did not detect significant differences in RQ before and after surgery in dogs with OSA. If excitement or stress had artificially increased energy expenditure before surgery, it would be expected to shift to greater carbohydrate oxidation, and a higher RQ than values obtained after surgery. After surgery, patients are often mildly overhydrated due to high crystalloid fluid rates during anesthesia. This transient increase in total body water could artifactually increase lean body mass, and contribute to the lack of significant differences in

REE between dogs with OSA and controls dogs. Finally, the decrease in REE during the post-operative period may have been associated with the body's normal adaptive mechanisms of the thyroid gland hormone axis to down-regulate metabolism and concentrate on healing. Many patients with critical illness or neoplasia have documented euthyroid sick syndrome. Clearly, investigation of thyroid axis activity will be required to determine whether changes can be temporally related to changes in REE.

An increase in REE can develop as a result of futile cycling of substrates for energy.[3,32-33,51] Tumors preferentially use carbohydrates for energy, converting glucose to lactate in the process.[3,32-33,51] In turn, lactate must be converted back to glucose by the liver via the Cori cycle. Conversion via the Cori cycle is an example of futile cycling by which the host converts one substance to another for energy using an energy-consuming pathway. The process is detrimental in that there is net energy loss by the host. Daily energy requirements may increase by as much as 20% as a result of futile cycling of glucose in the Cori cycle.[32-33,51] Increases in glucose flux have also been documented in humans with other types of neoplasia.[1,52-53] The increase in glucose flux in our dogs with OSA after surgery suggests increased cycling of glucose, possibly within the Cori cycle or due to increased hepatic gluconeogenesis and the production of three-carbon intermediates.[48,54] Increased rates of glucose recycling and hepatic gluconeogenesis can lead to depletion of certain amino acids, such as glutamine and alanine. An increase in glucose flux was detected after surgery. This change was even observed in dogs that lacked an increase in REE.

Values for RQ are used traditionally to document the primary substrate used for energy. The RQ values did not differ significantly between OSA and control dogs, suggesting that dogs

with OSA have normal relative rates of utilization of endogenous protein, carbohydrate and fat substrates for energy. The RQ values of approximately 0.75 suggest that lipid oxidation was the primary energy source, which is expected for animals following withholding of food overnight.

Even in dogs that lacked clinical signs of cachexia or weight loss, dogs with OSA were apparently in a state of less positive nitrogen balance. The typical adaptive response to reduced protein intake or starvation is a reduction in protein synthesis and a reduction of nitrogen loss. In cancer patients, however, this normal adaptive mechanism is lost, and protein synthesis declines with concomitant increases in nitrogen loss, resulting in a negative nitrogen balance.[31,55] As tumor synthesis of proteins increases, competition with the protein synthesis of the host may develop, resulting in decreased muscle protein synthesis by the cancer-bearing host.[56] The protein synthesis rate of dogs with OSA were significantly lower than that of control dogs. This may have been attributable to catabolism of amino acids for oxidative purposes, and may reflect a loss of muscle protein. Because anorexia was not documented in any of the dogs with OSA, the decrease in the rate of protein synthesis cannot be explained by a lack of protein intake alone. Additionally, documentation of increased urinary excretion of nitrogen in dogs with OSA reflects increased protein turnover and greater protein catabolism in those dogs, compared with control dogs. Our results are similar to those of other who document increased protein catabolism in humans with neoplasia.[1,9,31,55] The urinary N excretion of the dogs with OSA was quantitatively similar to that reported for other dogs requiring intensive care.[57] To our knowledge, this if the first study to document changes in REE and metabolic pathways in dogs with OSA. Additional research must be conducted to evaluate whether the changes observed progress with time, regress with analgesic agents after surgery, or regress with chemotherapy.

Because microscopic tumor burden is still present even after removal of tumor mass, it is reasonable to assume that metabolic changes may continue to progress over time, and therefore contribute to cachexia. Many factors are thought to contribute to metabolic changes, including cytokines (such as TNF-α, IFN-gamma, IL-1β), shifts in the thyroid axis, and the production of acute phase proteins. Clearly, studies must be conducted to determine the influence of these factors on metabolic changes seen in animals with neoplasia. A better understanding of the dynamic changes in metabolism in animals with neoplasia is essential for proper nutritional intervention.

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Footnotes

a. Hill's Pet Food, Topeka, KS, USA

b. 15N-glycine, Cambridge Isotope Laboratories, Inc., Andover, MA

c. 6,6-deuterium-glucose, Cambridge Isotope Laboratories, Inc., Andover, MA

d. Venocath®, 16 gauge, Abbott Laboratories, North Chicago, IL 60064

e. Foley catheter, Medline Industries Inc., Mundelein, IL 60060

f. Argyle Feeding Tube, Sherwood Medical St. Louis, MO 83103

g. Revco Scientific Incorporated, Asheville NC

h. VG-Micromass, Cheshire, England

i. Sigma Diagnostic Kit #640; Sigma Chemical Company, St. Louis, MO

j. VG Instruments, Cheshire, England

k. Oxymax Software 4.1, Columbus Instruments International Corp., 950 N Hague Ave. Columbus, OH 43204, USA

l. O_2 sensor N-22, Ametek, Philadelphia, PA

m. O_2 analyzer S-3A/I, Ametek, Philadelphia, PA

n. CO_2 sensor, P-61B, Ametek, Philadelphia, PA

o. CO_2 analyzer, CD-3A, Ametek, Philadelphia, PA

p. Hologic QDR-1000™/W whole body x-ray bone densitometer, Hologic, Inc. 35 Crosby Drive, Bedford, MA 02154 USA)

q. Propofol®, Abbott Laboratories, North Chicago, IL, 60064

r. Statview 5.0, SAS Institute, Inc, SAS Campus Drive, Cary, NC 27513

Table 1: Results (mean ± SD) of indirect calorimetry to determine resting energy expenditure (REE) and respiratory quotient (RQ) for 15 dogs with osteosarcoma (OSA) before and after surgery to amputate the affected limb, 12 healthy control Beagles, and control dogs reported in another study[26]

	REE		
Group	**kcal/kg$^{0.75}$**	**kcal/kg LBM**	**RQ**
OSA pre-op	92.60±16.20[a]	53.20±4.30[a]	0 .74±0.04
OSA post-op	86.10±10.0[a]	48.60±7.90[a,b]	0.75±0.07
Beagle	76.60±8.30[b]	45.80±4.30[b]	0.77±0.06
Other control dogs	75.80±18.20[b]	41.30 ± 9.40[b]	0.84±0.06

[a,b]Within each column, values with different superscript letters differ significantly ($P < 0.05$).

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CHAPTER 3[2]

ROLE OF GLUTAMINE IN HEALTH AND DISEASE

Introduction

Glutamine, the most abundant amino acid in plasma and the extracellular fluid compartment[1], constitutes the largest labile source of nitrogen in the body[2]. Traditionally classified as a non-essential amino acid, glutamine serves a wide variety of functions in healthy individuals, including transporting nitrogen and carbon between tissue[2-4], regulating protein synthesis[5-6], generating substrates for renal ammoniagenesis[7], synthesizing nucleic acid, and providing fuel for gastrointestinal[8], renal tubular[9], immune[10], and vascular endothelial cells[11]. Glutamine also plays a central role in carbohydrate metabolism as a gluconeogenic precursor.[5] Because of its involvement in a variety of metabolic events, glutamine is essential for optimal cell growth and function.

The classification of glutamine as a non-essential amino acid is misleading because numerous studies have demonstrated that it is indispensable during critical illness. In disease states, glutamine becomes a conditionally essential amino acid.[12] In human medicine, there is an intense interest in glutamine metabolism. This paper describes glutamine synthesis and degradation, glutamine flux between tissue, consequences of glutamine depletion during critical illness, and potential benefits of glutamine therapy in critically ill animals.

Glutamine Synthesis and Degradation

In animals, glutamine is readily synthesized from glutamic acid and ammonia in an ATP-dependent reaction catalyzed by glutamine synthetase, an enzyme found in most tissue (e.g., muscle, liver, lung, brain, adipocytes, lymphocytes, heart, and small intestine).[13] In humans, skeletal muscle is the main site of glutamine synthesis and storage in the post-absorptive state.[2] Under normal conditions, intramuscular glutamine synthesis and proteolysis balance the release of glutamine into circulation, where it is transported for use by other tissue.[13]

Glutamine is degraded by the enzyme glutaminase. Most organs have glutamine synthetase and glutaminase activity and are, therefore, capable of synthesis and degradation.[2] In most cases, the activity of one of the enzymes predominates, thus making the organ a net producer or net consumer of glutamine. In healthy humans, intracellular glutamine synthesis exceeds glutamine use during states of health, resulting in a net production of glutamine.[13] Organs that consume glutamine include the GI tract, pancreas, kidney, and immune cells.[2] Depending on metabolic conditions, the liver can be a net producer or net consumer of glutamine. Under normal physiologic conditions during states of health, the balance of glutamine synthesis and breakdown by the liver is almost equal.[2]

Glutamine Flux

Circulating glutamine concentration is dependent on relative rates of glutamine uptake, synthesis and release.[3] During states of health, the plasma glutamine pool is maintained at a fairly constant level. In mammals, the plasma glutamine concentration normally ranges between 0.6 - 0.9 mmol/liter.[1] Intracellular glutamine concentration (20 mmol/liter) in humans is approximately 30 times its serum concentration.[14] Catabolic states (e.g., metabolic acidosis,

sepsis, starvation) elicit significant changes in interorgan glutamine flow and can cause the redistribution of glutamine between tissue.[15]

Normal Glutamine Functions

Nitrogen transport

Glutamine contains two amine groups that allow the transportation of carbon and nitrogen through the body. Glutamine reactions serve to scavenge and transport ammonia in a non-toxic form from peripheral tissue to the liver and kidneys, where gluconeogenesis and ureagenesis occur, respectively.[2-3,16-17] Glutamine also plays a role in renal acid-base balance by transporting nitrogen and acting as a buffer, thereby facilitating excretion of acid equivalents (e.g., ammonium) in the urine.[17]

Gastrointestinal Function

The importance of glutamine as a "competence factor" for enterocytes is unequivocal.[18] Glutamine is the main metabolic substrate that exerts trophic effects on enterocytes, thereby supporting their normal function (Figure 2). Enterocytes can extract as much as 25% of glutamine from circulation, or can obtain it via luminal absorption.[19] A small amount of glutamine synthesis can also occur within enterocytes. The overall synthetic capacity, however, is small, and often inadequate to meet the metabolic needs of the enterocyte, particularly during states of illness or stress. The maintenance of intestinal mucosal integrity, therefore, is dependent primarily on an adequate supply of glutamine from other sources.[20] Glutamine nitrogen is used for hexosamine synthesis, which serves as a precursor for carbohydrate molecules used to form intracellular tight junctions needed for mucosal barrier function.[21]

Gastrointestinal glutamine is also used for synthesis of a protective mucus gel, which provides the first line of defense against luminal pathogens.[22]

Immune Function

Glutamine is an essential nutrient for proper function of immune cells such as macrophages, lymphocytes, and neutrophils.[23] It provides precursors for purine and pyrimidine synthesis during phagocytic cell activation, antigen-presenting cell stimulation and differentiation, lymphocyte blastogenesis, expression of cell-surface markers, and antibody production.[23] Glutamine also upregulates activation of cytotoxic T-cells, which play a central role in defense against bacterial infection.[24-25] Furthermore, glutamine is required for synthesis of the inflammatory cytokines interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interferon-γ (IFN-γ), and tumor necrosis factor- alpha (TNF-α).[14,26]

Alterations in Glutamine Metabolism

Critical Illness

In critical illness, glutamine metabolism is altered in tissue. Profound changes in amino acid distribution occur as plasma and intracellular glutamine concentrations fall.[2] The release of glucocorticoids and inflammatory cytokines (e.g., IL-1β and TNF-α) results in a unidirectional flux of glutamine from muscle and lung in excess of glutamine production.[27-28] The release of glucocounterregulatory hormones (e.g., epinephrine, glucagon) during stress and disease stimulate glutamine uptake and use by the GI mucosa.[29] The accelerated export of glutamine in excess of its synthesis depletes muscle glutamine concentrations by 30% or more, causing protein catabolism and muscle wasting. Ultimately, body glutamine stores can become depleted.[14,30] This occurrence has been documented in humans with trauma, sepsis and

necrotizing pancreatitis.[31] When glutamine synthesis does not meet disease requirements, it becomes a conditionally essential amino acid which must be supplemented.[14] (Figure 3).

Sepsis

Gut-specific nutrients (e.g., glutamine) are important for normal GI homeostasis and immune function.[32] Glutamine depletion, therefore, can lead to dysfunction. Healthy dogs given parenteral glutaminase to deplete circulating glutamine develop emesis, diarrhea, intestinal villous atrophy, mucosal ulceration, and necrosis.[33] In vitro, glutamine-starved intestinal cells upregulate protein synthesis, inducing apoptosis, or programmed cell death.[34]

Deterioration of the gut mucosal barrier and increased intestinal permeability have been reported in various critically ill humans with endotoxemia, multiple trauma and major burns.[31] In states of health, the intestinal epithelium normally restricts the passage of bacteria and toxic macromolecules.[35] Glutamine depletion can result in increased intestinal mucosal permeability, allowing migration of intestinal bacteria into the bloodstream. The circulating bacteria can then stimulate mesenteric mononuclear cell activation. Known as the "second hit" theory, this event may play a role in the development of multiple organ dysfunction syndrome (MODS) and systemic inflammatory response syndrome (SIRS) in response to sepsis.[24,36] (Figure 4)

Cancer

In cases of neoplasia, the cause of glutamine depletion is multifactorial (e.g., increased utilization of glutamine, abnormal glutamine metabolism).[1] Although host glutamine depletion is normally a characteristic of advanced malignancy, depletion occurs early in the course of disease while the patient still appears healthy and still has a good appetite.[1] Fibrosarcoma, mammary carcinoma, and other tumors can consume glutamine as their principal amino acid source, thus

acting as "glutamine traps". Changes in interorgan glutamine metabolism occur because malignant cells import glutamine faster than do non-malignant cells.[3] In an adaptive response to increased glutamine uptake and degradation by neoplastic cells, muscle glutamine synthetase activity increases to maintain adequate circulating stores. Early in neoplasia, TNF-α stimulates enhanced glutamine release from hepatocytes, causing the liver to switch from an organ of net glutamine extraction to one of net synthesis and release.

Over time, tumors become the major tissue for glutamine uptake, extracting as much as 50% of glutamine from the circulating pool.[1] Tumor growth is positively correlated with increased glutaminase activity.[37-40] With progressive tumor growth and advanced malignancy, muscle glutamine synthetic capacity and hepatic glutamine stores become exhausted.[1,41] In human cancer patients, glutamine transport activity into the tumor is maintained even at the expense of the host when cachexia is present. Tumor glutaminase activity increases even when intestinal glutamine extraction decreases, depleting the supply of glutamine needed for normal enterocyte function.[42,43] The resulting defective gastrointestinal mucosal integrity can lead to increased bacterial translocation.

Potential Benefits of Glutamine Supplementation

Cancer

Feeding glutamine-enriched diets to human cancer patients and some animal models has been shown to have significant positive effects, including repleting host glutamine stores, increasing glutamine synthetase activity, normalizing host catabolic changes, and improving clinical outcome.[43-44]

Glutamine is required for the synthesis of glutathione, which in turn is needed for IL-2 activation of cytotoxic T-cells and natural killer cell activity.[25] Oral glutamine supplementation during exposure to radiation or chemotherapy increases glutathione levels in the gut, liver, heart, kidney, and muscle.[45] In rat fibrosarcoma cells, glutamine supplementation is associated with increased tumor cell glutathione levels, resulting in increased susceptibility to chemotherapy and decreased tumor expansion.[46] Oral glutamine supplementation to tumor-bearing rats up-regulated host glutathione synthesis and natural killer cell activity in a dose-dependent manner. This activity may improve host defense against blood-borne metastasis[25] and decrease tumor growth.[47]

Glutamine supplementation in cancer patients may enhance tumoricidal effectiveness of antitumor drugs and improve the patients' tolerance to toxic effects of chemotherapy and radiation therapy.[48] Numerous studies in humans undergoing chemotherapy have demonstrated a significantly decreased incidence of mucositis and stomatitis with glutamine supplementation.[44,49] Other studies have failed to produce similar results.[50-52] Supplemental glutamine increases tumor glutamine concentrations and appears to decrease the efflux of methotrexate from tumor cells.[43] This may help prevent the development of drug resistance. Clinical studies[53,54] investigating supplemental glutamine in animals with cancer are few in number and have demonstrated equivocal results. Marks and colleagues[53] found that glutamine supplementation proved no benefit in cats with methotrexate-induced enterocolitis. Other studies[54] have demonstrated that glutamine supplements given to dogs undergoing radiation therapy showed positive effects in reducing mucositis[a].

Critical Illness

In critically ill human and animals, decreased food intake is deleterious to proper GI function and integrity. A growing trend has developed in human medicine toward the use of supplemental nutrients that can become selectively depleted during catabolic states.[55] These supplements can improve clinical outcome in critical illness. The dose of supplemental glutamine varies widely. In human enteral and parenteral formulas, glutamine supplementation (dose 0.285 – 0.36 g/kg/day) has been shown to increase peripheral leukocyte numbers, increase fractional protein synthesis by the liver, restore muscle glutamine levels, and improve overall nitrogen balance.[55-57] These supplements have also been shown to reduce the incidence of infection, improve recovery from illness, decrease the length of hospital stay, and increase the 6-month survival in critically ill humans after MODS.[57] (See Potential Benefits of Glutamine Supplementation)

In experimental models associated with bacterial translocation and sepsis, glutamine supplementation improved intestinal barrier function by decreasing intestinal villous atrophy and increasing intestinal IgA levels.[12,45,48] Although numerous experimental models have demonstrated that glutamine supplementation may be beneficial, other studies have found little benefit.[59] Beneficial results have also been demonstrated when glutamine has been added to total parenteral nutrition (TPN) formulations of humans with multiple trauma, surgical trauma, neoplasia, and inflammatory bowel disease. The use of TPN in patients with normally functioning gastrointestinal tracts is controversial because TPN may not provide enough trophic stimuli to prevent enterocyte atrophy, even with glutamine supplementation.[60] In patients with normally functioning GI tracts, enteral nutrition is preferred.

Oral glutamine exerts trophic effects on the GI tract by increasing DNA content and mucosal protein synthesis, both of which may serve to improve growth and repair of small bowels and reduce the incidence of bacterial translocation.[43,45,61] The incidence of bacterial pneumonia, bacteremia and sepsis are subsequently decreased.[62] Oral glutamine supplementation in human colorectal surgery patients has been shown to prevent the mononuclear cell activation, which contributes to excessive production of inflammatory cytokines andSIRS.[63]

Recommendations for Glutamine Therapy

Previously, glutamine was not routinely included in most parenteral and enteral formulations due its instability during storage.[20] However, the advent of heat-stable glutamine dipeptides (e.g., L-alanine-L-glutamine and glycyl-L-glutamine) which are stable in solution and are readily hydrolyzed following infusion, has made it possible for glutamine to be added to human enteral or parenteral formulas, provided that sterile technique is used during preparation of TPN solution.[64] Furthermore, recent evidence has demonstrated that L-glutamine is stable in TPN solution for at least 22 days at room temperature.[65]

Glutamine is an essential nutrient during periods of stress and critical illness. Studies have validated its use as a nutriceutical in human critical care and cancer patients as well as animal models of critical illness (e.g., sepsis). Thus the concept that glutamine may be beneficial in animals is not without reason. Its use in veterinary medicine has not yet been emphasized.

Recommendations for glutamine therapy in veterinary medicine are speculative, because only a limited number of studies have investigated the use of glutamine supplementation in

animals with equivocal results.[53,54] Dosages of glutamine used in animals have largely been extrapolated from those recommended in human literature. Cats may require larger doses of glutamine or may be resistant to the potential benefits of its supplementation at a dose of 1.08 g/kg/day. One study[54] has shown that L-glutamine (4 g/m^2/day) in suspension was beneficial to dogs undergoing radiation therapy, suggesting that L-glutamine was adequately absorbed in the GI tract. Further, glutamine infusion in anesthetized dogs failed to produce any detrimental effects, particularly to the liver or kidneys[66], indicating its safety as a nutriceutical in dogs.

Additional clinical research must be conducted to validate its use in critically ill animals. Potential benefits are promising and merit further investigation. The doses we have used have been extrapolated from those recommended for humans; therefore, further study is needed to determine the efficacy in small animals. The addition of glutamine to enteral or parenteral formulations at a dose of 0.24 – 0.32 g/kg/day may potentially have a positive effect by improving nitrogen balance and immune function, and decreasing morbidity and mortality in critically ill animals. Its use, therefore, may be beneficial in a variety of illnesses, including acquired or surgical trauma, inflammatory conditions (e.g., sepsis, pancreatitis, SIRS), disease states that promote ileus and subsequent bacterial translocation, and conditions associated with negative nitrogen balance (e.g., cancer).

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Footnotes

a. Khanna C, Klausner JS, Walter P, et al: A randomized clinical trial of glutamine versus placebo in the prevention of radiation-induced mucositis in dogs. In: Proceedings of the Veterinary Cancer Society, 15th Annual Conference (abstract):46 – 47, 1995.

b. Clinicare®, Abbott Laboratories, North Chicago, IL, 60064, USA. Clinicare® contains 1300 mg glutamine per 237 ml of Clinicare® (550 mg per 100 kcal as fed)

c. L-glutamine, Professional Compounding Centers of America, Inc. 9901 South Wilcrest Houston, TX 77099, 1-800-331-2498, and L-glutamine PhysioLogics 12755 Claude Ct. Thornton CO 80241.

d. Reprinted with permission. Souba, W. In: Glutamine: Physiology, Biochemistry, and Nutrition in Critical Illness, RG Landers Company.

Table 1: Potential Benefits of Glutamine Supplementation

Glutamine supplementation in enteral and parenteral formulations may improve function of the immune system and gastrointestinal tract, improve nitrogen balance, and improve survival.

Potential Benefits of Glutamine Supplementation

Immune System

- Stimulates macrophage and lymphocyte function
- Improve natural killer (NK) cell activity

Nitrogen Balance

- Increase muscle and liver protein synthesis

Gastrointestinal Tract

- Improve intestinal barrier function
  - Increase mucosal IgA levels
  - Increase mucosal DNA synthesis
  - Mucin production
  - Decrease bacterial translocation
    - Decrease incidence of bacteremia/sepsis

Human Cancer Patients

- Increase host glutathione production
  - Enhance free radical scavenging
  - Decrease chemotherapy-induced cardiotoxicity
- Decrease stomatitis and mucositis
- Enhance tumoricidal effects of chemotherapeutic agents

Critical Care Human Patients

Decrease infections and MODS

Decrease morbidity and mortality

Decrease length of hospital stay

Improve 6 month survival

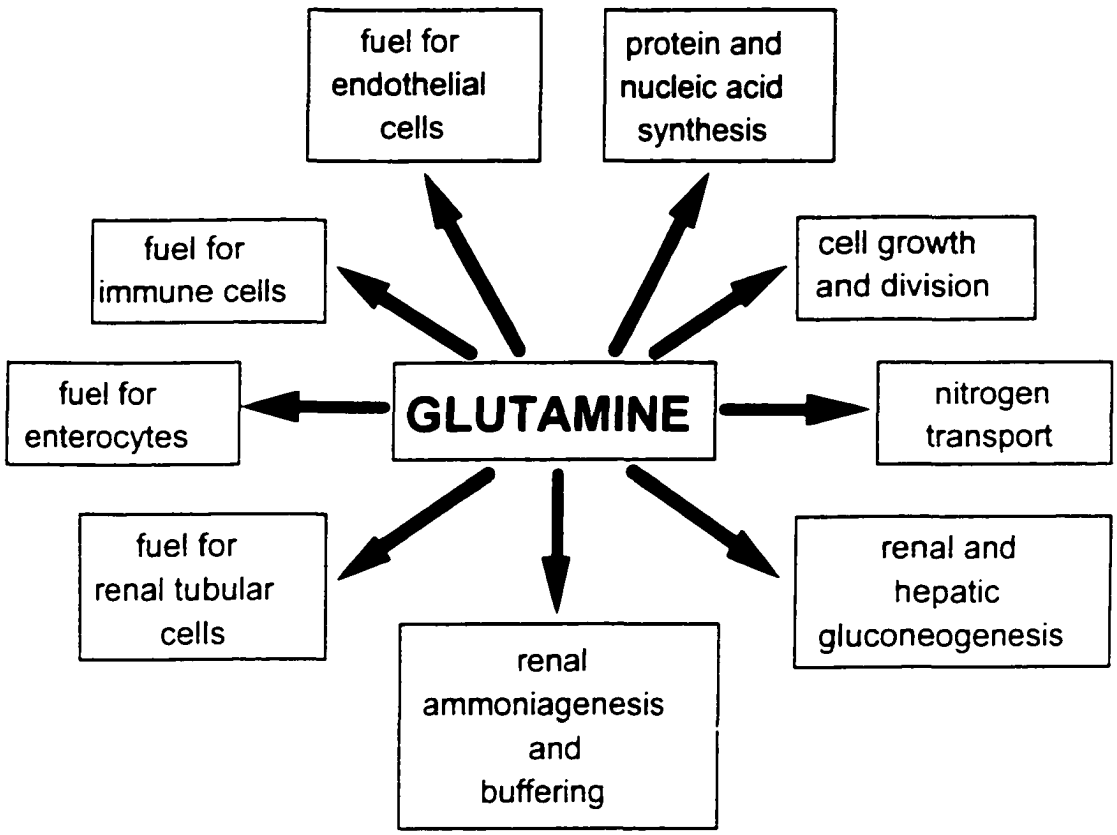

Figure 1: Functions of Glutamine in States of Health

Glutamine serves a variety of functions during states of health, including protein and nucleic acid synthesis, cell growth and division, renal and hepatic gluconeogenesis, nitrogen and carbon transport, as well as providing fuel for immune cells, endothelial cells, enterocytes, and renal tubular cells.

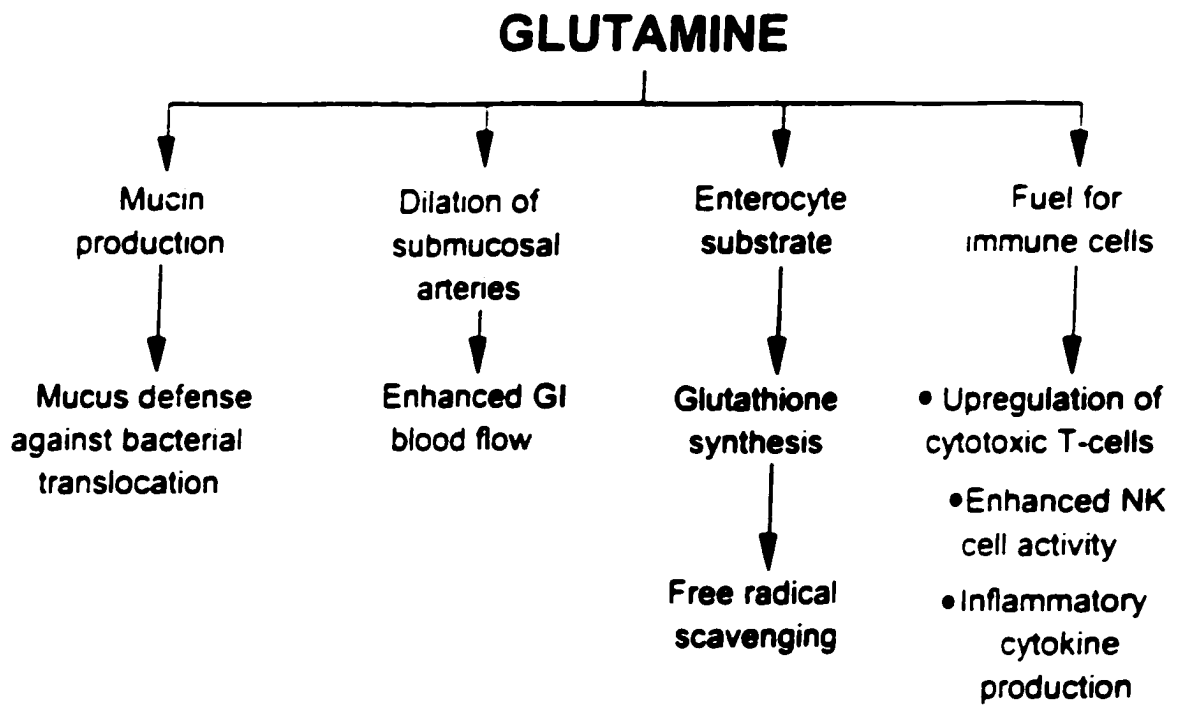

Figure 2

Functions of Glutamine in the Intestinal Tract

Glutamine, which is required for normal enterocyte health and function, is used as a primary fuel for enterocyte and immune cells and plays a role in glutathione and mucin production.

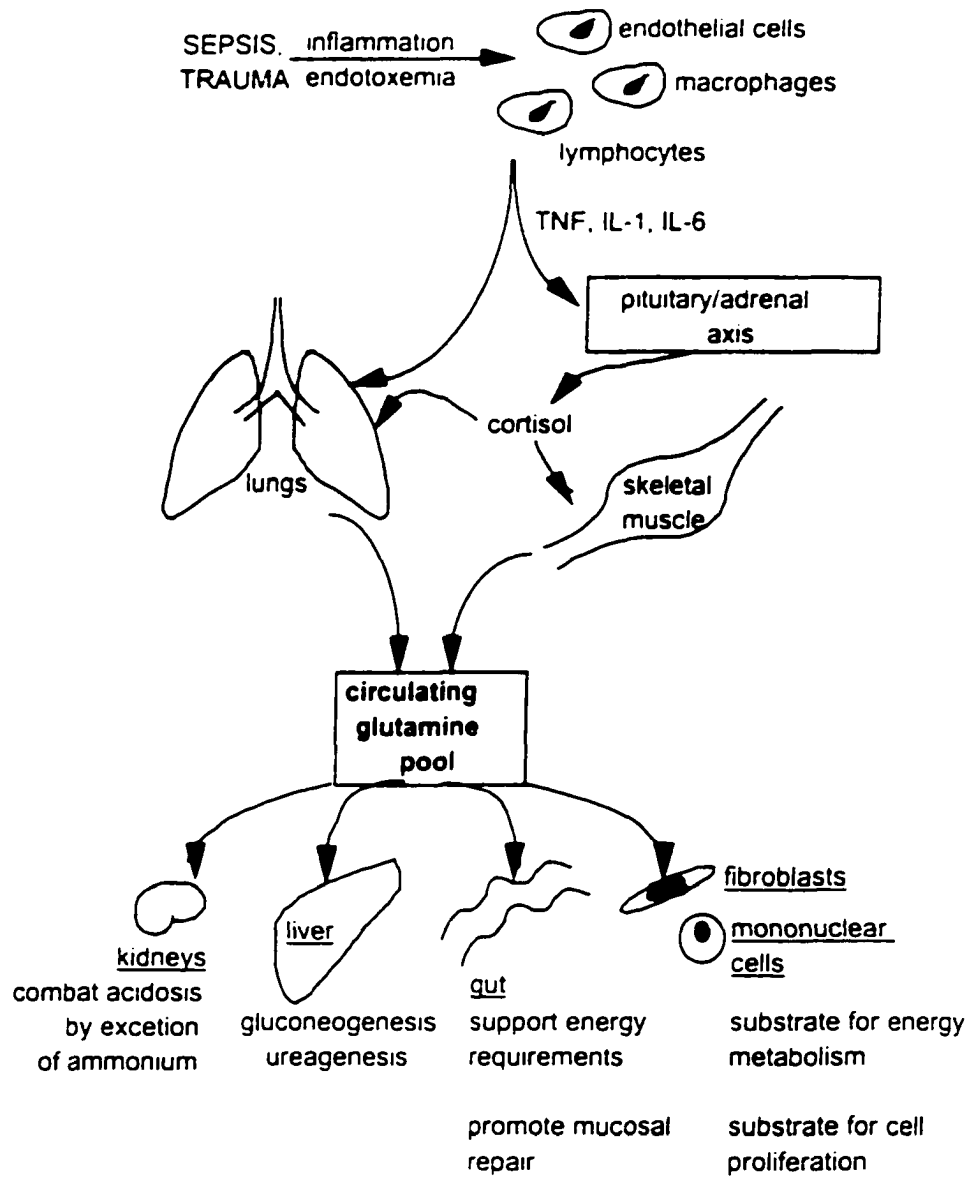

Figure 3: Role of Glutamine in Critical Illness and Neoplasia

During critical illness and neoplasia, glutamine requirements often exceed synthesis; glutamine thus becomes a conditionally essential amino acid. Maintenance of glutamine stores supports normal intestinal and immune function, renal ammoniagenesis and buffer mechanisms, and whole body protein synthesis. (Modified from Souba WW: *Glutamine: Physiology, Biochemistry and Nutrition in Critical Illness*. Georgetown, TX, RG Landes, 1992, p 84; with permission.)

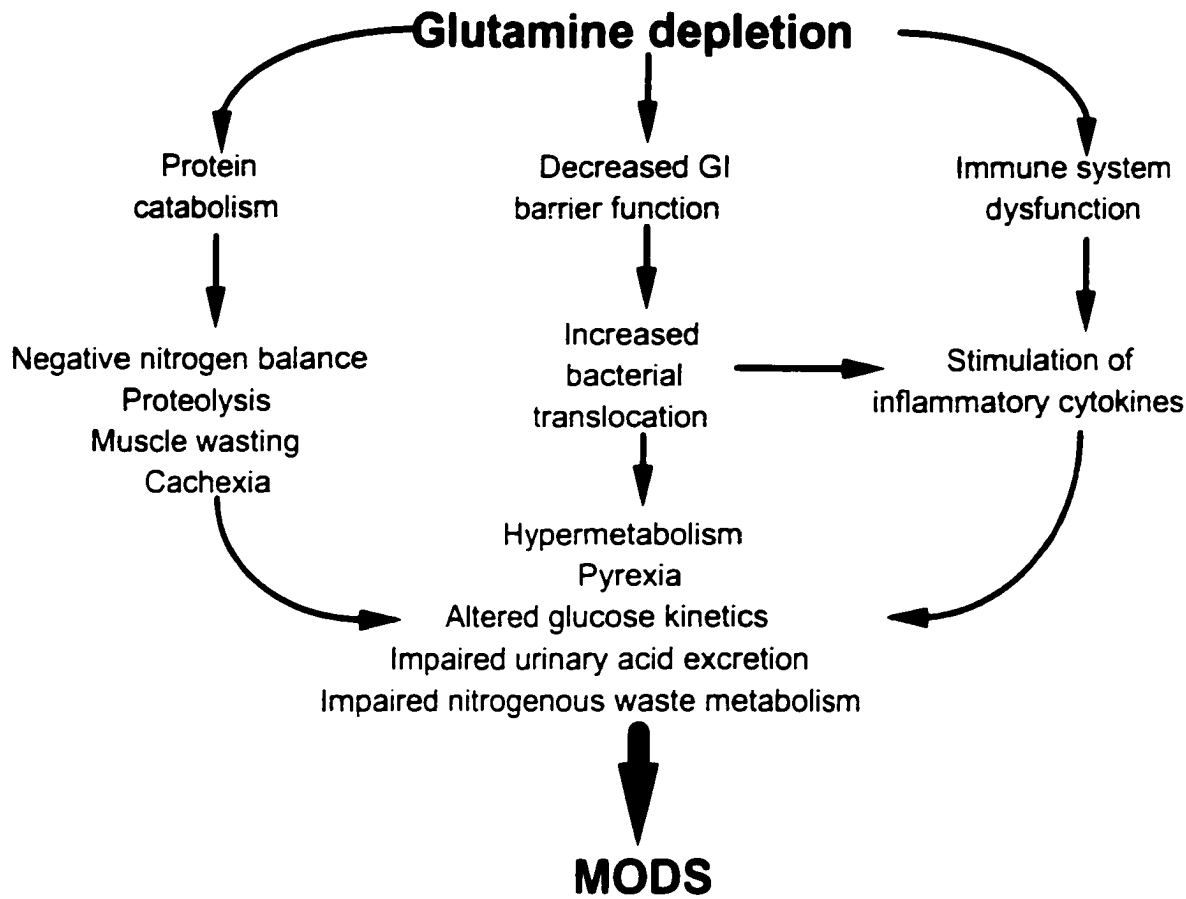

Figure 4: Consequences of Glutamine Depletion

Glutamine plays a critical role in a variety of metabolic pathways throughout the body.

Glutamine depletion can lead to many negative consequences, including multiorgan dysfunction.

CHAPTER 4

THE ROLE OF GLUTAMINE ON METABOLIC AND ENDOCRINE CHANGES IN CANINE PATIENTS WITH CRITICAL ILLNESS AND NEOPLASIA

ABSTRACT

Objective To evaluate the effect of L-glutamine supplementation in excess of that found in the diet on carbohydrate and protein metabolism, thyroid axis, and adrenal axis in canine patients with critical illness and neoplasia.

Animals Thirty-six dogs with a variety of critical illnesses (6 sepsis, 5 trauma, 12 surgery, 13 neoplasia) and eleven 1-year-old sexually intact female Beagles (control dogs).

Procedures Stable isotope tracers (^{15}N-glycine, 4.5 mg/kg of body weight, IV; 6,6-deuterium-glucose, 4.5 mg kg of body weight IV, followed by a constant rate infusion, 1.5 mg kg/hour for three hours) were used to determine rates of protein synthesis and glucose flux in all dogs. Dual energy x-ray absorptiometry (DEXA) scans were performed on all dogs to determine total body composition. Serum samples were analyzed for total thyroxine (TT_4), endogenous thyroid stimulating hormone (eTSH) and cortisol before and after adrenocorticotrophic hormone (ACTH) stimulation, to evaluate the thyroid and adrenal axes.

Results Across disease categories, serum thyroxine levels decreased during hospitalization and therapy. The incidence of euthyroid sick syndrome was 42% of clinical cases before feeding, and 60% after feeding. No significant change in adrenal axis function was apparent before or after feeding in any disease category. Analysis of nitrogen balance revealed endogenous protein

synthesis to be elevated in surgery patients before and after feeding, although this result may be artifactual. Urinary nitrogen excretion significantly decreased across all disease categories after feeding. There was no significant increase in urinary nitrogen excretion in any disease category, suggesting the nitrogen balance was normal in all patients. There was a trend for glucose flux to decrease in all disease categories after feeding. Glutamine supplementation had no significant effect on changes in thyroid axis, adrenal axis, nitrogen balance (protein synthesis or urinary nitrogen loss) or glucose flux in any disease category.

Conclusions and Clinical Relevance This study documents that euthyroid sick syndrome is a common entity in dogs with critical illness and neoplasia. Recovery from primary disease may not be immediately reflected by changes in thyroid axis function. Adrenocortical burnout does not appear to occur within the first four days of critical illness in dogs. Normal rates of endogenous protein synthesis rates and urinary loss of nitrogen suggest that negative nitrogen balance may not occur in all dogs with critical illness and neoplasia. Feeding appears to decrease the amount of urinary nitrogen loss and cycling of substrates through energy-consuming processes, as evidenced by a substantial decrease in glucose flux post-feeding. At the dose and route selected for this study, supplemental glutamine in excess of that found in the diet appeared to have no effect on hormone axes, nitrogen balance, or glucose flux in dogs with critical illness and neoplasia, and is not advocated at this time.

INTRODUCTION

The metabolic response to many illnesses consists of a complex cascade of neurohumoral mechanisms that help the host adapt to stress and hopefully improve survival. In the early stages of non-stressed starvation, adaptations occur which favor utilization of fat for energy, sparing

carbohydrate for glucose-dependent tissues and diverting host amino acids and protein to healing processes.[1] However, with the stress of trauma or illness, normal adaptive mechanisms to spare protein are ineffective, resulting in utilization of host protein stores for energy via gluconeogenic pathways. Amino acids are no longer available for *de novo* protein synthesis, and may become depleted. Numerous studies have demonstrated that amino acids, including glutamine, can become rapidly depleted during stressed conditions, leading to a variety of secondary illnesses, including impaired immune and gastrointestinal function, delayed wound healing, and increased risk for sepsis.[2,3,4,5,6,7]

Alterations in host substrate metabolism and endocrine pathways have been documented in both human and veterinary patients with critical illness and neoplasia.[5,8,9,10,11,12] Previous work conducted by this laboratory in canine patients with osteosarcoma has documented changes in protein and carbohydrate metabolism, including decreased rates of *de novo* protein synthesis, increased urinary nitrogen loss, and increased rates of glucose flux.[12] Similar changes in host protein metabolism have been documented in human patients with pancreatitis[13] and cancer.[14] Early interventional nutrition with selected nutrients, including glutamine, has been found to improve nitrogen balance, decrease the incidence of infection, and decrease patient morbidity in select studies.[15,16,17,18,19]

The changes observed in metabolic pathways are intimately related to changes in the hormonal axes, including suppression of thyroid hormone activity and host release of glucocounterregulatory hormones, including cortisol. The euthyroid sick syndrome, characterized by low serum thyroxine (T_4), low triiodothyronine (T_3), and normal to low endogenous thyroid stimulating hormone (eTSH), has been described in a variety of non-

thyroidal illnesses.[8,9,10,11,20,21,22,23] including canine patients with critical illness.[11,21,22,23] The exact mechanisms resulting in impaired thyroid hormone function and utilization remain unclear, but may be associated with impaired peripheral conversion of T_4 to T_3, or impaired binding of thyroid hormone to carrier proteins. Recent evidence suggests that early in critical illness, hypothalamic release of thyrotropin releasing hormone (TRH) may be impaired, resulting in decreased TSH secretion, and an inability to stimulate T_4 release by the thyroid gland.[24] The decrease in thyroid hormone activity has been found to correlate with increased patient morbidity and mortality.[11,25]

Other researchers have documented changes in the hypothalamic-pituitary-adrenal axis in response to the stress of critical illness. Early increases in serum cortisol concentrations have been used as a positive predictor of morbidity and mortality in human patients with critical illness.[25] Activation of the hypothalamic-pituitary-adrenal axis is necessary as part of the "fight-or-flight" response to stress. The release of glucocorticoids from the adrenal glands in response to stress appears to be intimately involved in the changes observed in thyroid hormone activity during the stress of non-thyroidal illness. Endogenous and exogenous glucocorticoid adminstration has been shown to decrease 5'-monodeiodinase enzyme activity, thus impairing peripheral conversion of T_4 to T_3.[26,27,28] The adaptive mechanism of glucocounterregulatory hormone release during stress can thus affect metabolic function by suppressing thyroid hormone activity. Some researchers consider this to be an adaptive response by which metabolism is down-regulated in an attempt to conserve the body's resources for tissue repair. Research conducted by this laboratory has similarly demonstrated that energy expenditure in dogs with

critical illness does not increase, but rather, remains normal to decreased,[29] supporting a role for down-regulation of metabolism with critical illness.

The use of stable isotope tracers has allowed researchers to investigate the changes in metabolic pathways that occur in a variety of clinical conditions in both humans and animals.[12,30,31,32,33] The purpose of this investigation was to use stable isotope tracers to investigate metabolic changes in canine patients with critical illness, to document changes in thyroid and adrenal hormone axes in these patients, and to determine whether early interventional nutrition with supplemental L-glutamine powder has any effect on patient metabolism or hormone axes.

MATERIALS AND METHODS

Patient selection

Dogs with critical illness Thirty-six client-owned dogs admitted to the critical care unit for a variety of illnesses were included in the study. Each owner consented to inclusion of their dog in the study. The study protocol was approved by a university animal care and use committee.

A complete blood count (CBC) and serum biochemistry profile were performed on each dog. Dogs were excluded from entering the study if they had preexisting known endocrine illness such as hyperadrenocorticism, hypo- or hyperthyroidism, or had been treated with glucocorticoids or anticonvulsant therapy, or had received general anesthesia within 30 days of referral to our facility. During the study, dogs were held without food for a period of twelve hours prior to infusion of stable isotope tracers and subsequent blood sampling. Following the

first sampling period, dogs were fed the commercially available liquid diet Clinicare® for a period of 48 hours.

Healthy control dogs- Eleven healthy 1-year-old sexually intact female Beagles were selected for use as control dogs for comparison purposes. Each control dog was housed in a 2.4 x 2.4-m pen with two other beagles. Dogs had *ad libitum* access to water and were fed a commercial prescription diet[a] twice daily.

Stable isotope tracers- Stable isotope tracers are widely used in human medicine to evaluate various metabolic processes.[33,34] Analysis of stable isotope tracers was used to observe characteristics of substrate flow in various metabolic pathways to determine protein and carbohydrate metabolism. Each dog was administered ^{15}N-glycine[b] (4.5 mg/kg of body weight, IV) and 6,6-deuterium-glucose[c] (4.5 mg/kg of body weight, IV as a bolus followed by constant rate infusion at 1.5 mg/kg/hour for 3 hours).

Collection and analysis of blood and urine samples- A catheter[d] was placed in a jugular or lateral saphenous vein of each dog for collection of blood samples. A urinary catheter[e] was inserted and connected to a closed collection system for 10 hours following infusion of infusion of stable isotopes to enable collection and quantification of urine. Blood samples (9 ml) were obtained at time 0 (just prior to start of isotope infusion), and at 1, 2, 2.5, 3, 6, and 10 hours after the start of isotope infusion. Following collection of the time 0 blood sample, a synthetic ACTH analogue (Cosyntropin[f], 0.25 mg, IV) was administered for ACTH stimulation test of the hypothalamic-pituitary adrenal axis. Blood samples were allowed to clot and were centrifuged (3,000 x g), and serum was decanted. Serum samples were frozen and were analyzed within 72 hours of collection for cortisol, endogenous thyrotropin-releasing hormone (eTSH), and

thyroxine (T_4) concentrations. Cortisol, eTSH, and T_4 were measured using commercially available test kits.[g,h]

Serum hormone analysis- For serum T_4, endogenous TSH, and cortisol, all hormones were analyzed using a commercially available solid-phase chemiluminescent competitive radioimmunoassay. In theory, an unknown amount of hormone in patient serum is added to a sample cup that contains antibodies directed toward the hormone under study, known as the analyte. The unknown concentration of hormone is allowed to incubate with the known amount of antibody and a known concentration of labeled-analyte. The labeled analyte competes with the unlabelled analyte in patient serum for antibody sites. Following incubation, addition of alkaline phosphatase reagent causes an enzymatic degradation of the chemiluminescent labeled analyte, causing a photon of light to be emitted. A photomultiplier tube in the Luminometer detects the light in counts per minute (cpm). The amount of light emitted is directly proportional to the amount of labeled substrate bound to the solid-phase antibody in the sample well. Less light will be emitted with higher concentrations of hormone in the patient's serum, while more light will be emitted with lower concentrations of hormone. Thus the amount of bound labeled hormone complex, and the amount of photon output is inversely proportional to the concentration of hormone in the patient sample. The light in cpm are then compared with a standard curve, and a computer software package calculates the concentration of hormone in the patient's serum.

Canine Total Thyroxine (T_4) - Serum T_4 was measured using a commercially available solid-phase chemiluminescent competitive radioimmunoassay. Samples were allowed to thaw to room

temperature, and mixed by gently inversion. Thirty (30) uL of serum was added to the sample well and incubated with chemiluminescent substrate for 30 minutes.

The sample cup was then washed with probe wash and then alkaline phosphatase enzyme added. Following an additional wash cycle, the amount of photon emission from the sample cup was analyzed by the photomultiplier tube. The amount of TT_4 in patient serum was then calculated by computer software package comparing cpm of light emitted to a standard curve. Known standards are also used for control purposes for each run. Samples were analyzed for precision and accuracy. A total of 40 runs with 80 replicates yielded coefficients of variation (CV) of 3.9% - 10.8% for within-run, and CV 5.2-13.8% CV overall.

Endogenous TSH (eTSH) – Serum endogenous thyroid stimulating hormone (eTSH) was measured using a commercially available solid-phase chemiluminescent assay. Twenty-five (25) uL of patient serum thawed to room temperature was added to the sample well and incubated with chemiluminescent substrate for 60 minutes. The sample cup was then washed with probe wash and then alkaline phosphatase enzyme added. Following an additional wash cycle, the amount of photon emission from the sample cup was analyzed by the photomultiplier tube. The amount of eTSH in patient serum was then calculated by computer software package comparing cpm of light emitted to a standard curve. Known standards are also used for control purposes for each run. Intraassay precision was calculated for samples from the results of 20 replicates in a single run, and were found to have CV ranging from 3.8 – 5.0%. Interassay precision was also analyzed for assays from 10 different runs. Coefficients of variation ranged from 6.3- 8.2%.

Cortisol - Serum cortisol was measured using a commercially available competitive immunoassay as previously described. Ten (10) µL of patient serum thawed to room

temperature was added to the sample well and incubated with chemiluminescent substrate for 30 minutes. The sample cup was then washed with probe wash and then alkaline phosphatase enzyme added. Following an additional wash cycle, the amount of photon emission from the sample cup was analyzed by the photomultiplier tube. The amount of cortisol in patient serum was then calculated by computer software package comparing cpm of light emitted to a standard curve. Known standards are also used for control purposes for each run. Intraassay CV ranged from 7.3-10.0%.

PSR and glucose flux- Aliquots of each blood sample were also frozen at -70 C and stored until analysis. Urine volume for the 10-hour period after infusion was determined. A 3 ml aliquot of pooled urine was frozen at -70 C and stored until analysis was performed. All samples were shipped on dry ice to a laboratory at the University of Medicine and Dentistry of New Jersey and analyzed for stable isotope metabolites, using mass spectrometry.[k]

The BUN concentration was determined by use of the urease method.[l] For isotopic enrichment of BUN, 1 ml of water and urease solution (1.0 mL, 60 μM units of urease/mL in 0.1 mL of phosphate buffer, pH 6.5) was added to 2 mL of serum. After incubation at 37 C for 30 minutes, 2 mL of K_2CO_3 and 0.4 mL of 2-octanol were added. The reaction results in the production of ammonia, which is removed via aeration and collected in 0.1N H_2SO_4. Total urinary nitrogen content was measured on 1 mL of urine, using the Kjedahl method. Nitrogen enrichment of the BUN-derived ammonia and Kjedahl digests was measured by isotope ratio mass spectroscopy, using a mass spectrometer[m]. The ammonia method reflects primarily muscle protein synthesis, whereas the total nitrogen out, or urea method, more accurately reflects hepatic protein synthesis.

Isotopic enrichment of serum glucose was determined as described elsewhere.[55] Serum (0.2 mL) was deproteinized with barium hydroxide (4.73%) and 1 mL of zinc sulfate (5.5%). After centrifugation, the supernatant was lyophilized. The residue was then reconstituted with 1 mL of deionized water and separated through a column of Dowex 50 and Dowex 1 ion exchange resin[n] (0.7 g of each). Glucose was eluted with 2 mL of water. The sample was frozen, lyophilized, and concerted to the penta-acetate derivative by reaction with excess acetic anhydride:pyridine (2:1; final volume of 0.2 mL) at 60 degrees for 10 minutes. The mixture was evaporated almost to dryness under a stream of nitrogen gas and reconstituted with 0.2 mL of dichloromethane. Isotopic enrichment of glucose was determined by use of a gas chromatograph-mass spectrometer in the selective-ion monitoring mode. Ions at 200/202 were monitored.

Whole body protein synthesis rate was calculated from the total amount of ^{15}N excreted. Amount of the administered dose excreted during the 10 hour collection period and the amount of ^{15}N remaining in the body's urea pool was calculated from the blood sample collected at the end of the blood sampling period (sample obtained at 10 hours). Size of the body's urea pool was estimated by assuming total body water was equivalent to body weight x 0.65. The amount of isotope remaining in the BUN at 10 hours was < 5%. Amount of ^{15}N in the urea pool was calculated, using the following equation:

^{15}N in urea pool =UDS X BUN X ^{15}N in BUN X (APE X0.01)

where UDS is the urea distribution space, and APE is the atom percent excess.

Protein synthesis rate was calculated as follows:

Protein synthesis rate = ET(*d {e-1})

Where ET is the grams of nitrogen excreted during the 10 hour period. *d is the amount of ^{15}N administered, and *e is the amount of ^{15}N excreted in the urine and BUN during the 10-hour period.

Rate of glucose production was calculated and used to determine glucose flux. For 6,6-(deuterium)-glucose, rate of appearance in serum was calculated, using the following formula:

$$R_a = F^*(APE_{infusion} / \{APE_{serum} - 1\})$$

Where R_a is the rate of appearance in serum, F* is the isotope administration rate, and $APE_{infusion}$ and APE_{serum} are the amount of isotopic enrichment in the infusion and serum, respectively.

Dual Energy X-ray Absorptiometry- A DEXA scan[o] was performed on each dog under general anesthesia to determine total body composition. Description and validation of DEXA analyses have been reported elsewhere.[30] Each dog was anesthetized by administration of Propofol[p] (4 - 7 mg/kg, IV as a bolus, followed by constant-rate infusion to achieve the desired effect). For dogs that weighed < 10 kg, infant whole-body software was used, whereas for larger dogs, software for adult humans was used.

Feeding- Following the 10 hour blood and urine sample collection period, each dog with critical illness was fed its basal energy requirements ($30 \times BW_{kg}$) + 70 = kcal day for a period of 48 hours. Each patient was randomly assigned to receive Clinicare[r,s] (Table 1) with or without supplemental L-glutamine powder[t]. A known volume of Clinicare[r] corresponding to each patient's energy requirements was administered daily. L-glutamine powder (0.24 g kg/day) was mixed with the Clinicare[r] by swirling and gentle inversion until the powder dissolved. Clinicare[r] (1 kcal/mL) was chosen because it could be fed orally or through naso-or jejunostomy tubes in patients that were inappetant. Calculated values of glutamine contained in Clinicare[r]

are 1300 mg of glutamine per 8 fl oz, which translates to 550 mg of glutamine per 100 kcal on an as fed basis.[5] Following 48 hours of feeding, the blood and urine sampling, along with stable isotope tracer infusion were repeated.

Statistical analyses- A modified Kolmogorov-Smirnov test[1] was used to assess normality of distribution of data, and a Bartlett's test of homogeneity to assess equality of variances among groups to determine distribution characteristics and whether actual data could be used for parametric analysis. All continuous parameters were compared between groups by an analysis of variance (ANOVA) for treatment effects, with repeated measures for time effects. Fisher's least significant difference test was used to identify individual group/time differences. Associations between continuous variables were assessed by standard linear regression analysis. Values of $p < 0.05$ are considered significant.

Following ANOVA, sepsis, surgery, cancer, and trauma patients were compared with control dogs published in a previous study.[12] Due to dissimilarities in variances between disease categories and control dogs, as determined by Bartlett's test for homogeneity of variances, log transformation of the data was performed prior to ANOVA comparison of protein synthetic rates and urinary nitrogen excretion rates among disease categories and controls.

RESULTS

Animals Thirty-six dogs were admitted into the study. Thirteen dogs had neoplasia, 6 had sepsis, 5 had trauma, and 12 had abdominal surgery not involving neoplasia. A list of patient signalment and individual diseases is listed in Table 2. A total of 16 breeds were represented. Twenty dogs were male (14 neutered, 6 intact) and 15 dogs were female (15 spayed, 0 intact). One patient was represented twice over a period of two years, once for peritonitis, and once for

abdominal exploratory surgery for a intestinal foreign body. The average age of patients was 5.4 +/- 3.0 years (range 1 - 12 years). A significant age difference was observed between disease categories, with cancer patients being significantly older than surgery patients ($p = 0.01$). The ages of sepsis and trauma patients were statistically similar to both cancer patients and surgery patients ($p > 0.05$). Body composition analysis was also performed (Table 3). Sepsis and trauma patients were statistically similar ($p > 0.05$) with 27.4% (± 13.6) and 27.7% (± 6.8) body fat, respectively. Cancer patients had body fat percentage (22.2 ± 6.9) statistically similar ($p > 0.05$) and intermediate to sepsis, surgery, and trauma patients. Surgery patients had significantly lower percent body fat (17.7 ± 7.7%) than sepsis or trauma patients, which may have been due to the younger age of most surgery patients.

Hypothalamic-pituitary-thyroid axis Serum total thyroxine (TT_4) and endogenous thyroid stimulating hormone (eTSH) were analyzed at the onset of the study and after 48 hours of supplemental feeding. A simple regression analysis was performed to investigate any effect of age on TT_4 levels. No significant interaction between age and TT_4 was observed. Mean TT_4 and eTSH levels, before and after feeding, are provided in Table 4. Across disease categories, serum TT_4 concentrations were significantly lower ($p = 0.0363$) after supplemental feeding than before feeding. Patients with sepsis and trauma had increased serum TT_4 values in the post-feeding time period, while post-operative surgery and neoplasia patients had absolute decreases in serum TT_4 post-feeding. Endogenous TSH levels were similar in the pre- and post-feeding time periods in all disease categories.

The incidence of euthyroid sick syndrome, defined as low TT_4 with normal to low eTSH was determined. No dog with neoplasia demonstrated euthyroid sick syndrome at the onset of

the study. However, 46% of dogs with neoplasia demonstrated euthyroid sick syndrome after 48 hours of feeding. Fifty per cent of surgery patients demonstrated euthyroid sick syndrome before surgery. Following surgery, the incidence was slightly higher, at 54%. Eighty per cent of trauma patients demonstrated euthyroid sick syndrome before feeding. The incidence increased to 100% after feeding in this disease category. Alternatively, 100% of septic patients had euthyroid sick syndrome before feeding. The prevalence dropped to 60% after feeding in this disease category. Overall prevalence of euthyroid sick syndrome was 41.6% before feeding, and 60% after 48 hours of supplemental nutrition. Supplemental glutamine had no significant effect on thyroid hormone levels or the occurrence of euthyroid sick syndrome in any disease category.

Hypothalamic-pituitary-adrenal axis ACTH stimulation tests were performed in all patients before and after 48 hours of supplemental feeding. As expected, all patients except one patient with sepsis secondary to hypoadrenocorticism demonstrated a significant ($p < 0.05$) increase in post-ACTH stimulation cortisol release. Mean baseline and post-ACTH stimulation serum cortisol concentrations before and after supplemental feeding are shown in Table 5. There were no significant differences in baseline or post-ACTH stimulation cortisol levels in any disease category before or after supplemental feeding ($p > 0.05$). The change in serum cortisol levels from baseline following ACTH stimulation (Δ cortisol) was also investigated, and no significant difference in Δ cortisol was observed in any disease category before or after supplemental feeding. No dog demonstrated adrenocortical burnout, or inadequate ACTH stimulated cortisol release at any time during the study. A simple regression analysis was performed investigating any possible interaction between baseline cortisol levels and TT_4. No correlation existed, indicating that there was no significant interaction between TT_4 and serum cortisol levels, and

that factors other than cortisol levels alone caused the increased incidence of euthyroid sick syndrome observed in these patients.

PSR and nitrogen loss Protein synthetic rate (PSR) before and after supplemental feeding was measured (Table 6). Overall, there was no significant difference in PSR pre- versus post-feeding across all treatment groups ($p = 0.321$). Before feeding, the PSR (g/kg/day) of surgery patients was significantly higher than the control beagles ($p = 0.025$), sepsis ($p = 0.012$), cancer ($p = 0.010$), and trauma ($p = 0.002$) patients both in the pre- and post-feeding time periods. Before feeding, the PSR (g/kg/day) of the control beagles, cancer, sepsis, and trauma patients were statistically similar ($p > 0.05$). After feeding, PSR (g/kg/day) of surgery patients was still significantly higher than that of cancer ($p = 0.470$) and trauma ($p = 0.027$) patients. The PSR of septic patients increased slightly, and was not significantly different than any other disease category or control dogs. When correcting for metabolic body size (i.e. PSR in $g/kg^{0.75}$ day), before feeding, surgery patients still had significantly ($p > 0.05$) higher PSR than control dogs and all other disease categories. Statistically, overall, for all treatment groups, glutamine appeared to significantly increase PSR ($p = 0.009$), however this effect may be artifactual.

Nitrogen loss was also measured, and was analyzed as a function of g/kg day urinary nitrogen loss (Table 7). There was no significant difference urinary nitrogen loss when expressed in g/kg day. Overall, when correcting for metabolic body size (nitrogen loss in $g/kg^{0.75}$ day), nitrogen loss for all disease categories was significantly lower ($p = 0.039$) post-feeding than pre-feeding. However, glutamine supplementation in excess of that found in Clinicare® had no significant effect on urinary nitrogen loss ($p > 0.05$).

Carbohydrate Metabolism Glucose flux was measured in mg/kg/hour for all dogs (Table 8). Overall, there was no significant difference in glucose flux before or after feeding between disease categories ($p = 0.090$). However, there was an overall trend for a decrease in glucose flux after feeding compared with glucose flux before feeding. Glutamine supplementation in excess of that found in Clinicare® appeared to have no significant effect on glucose flux in any disease category.

DISCUSSION AND CONCLUSIONS

The results of this study are similar to the findings of Elliot et al[11] and Scott-Moncrieff et al,[23] who documented a high incidence of euthyroid sick syndrome in dogs with both critical and chronic non-critical illnesses. Vail and others[37] also documented a significant increase in the incidence of low T_4, T_3 and FT_3 in canine patients with cancer cachexia, and cachexia associated with a variety of diseases other than cancer. A noteworthy observation is that no dog with neoplasia, a chronic illness, demonstrated euthyroid sick syndrome at the onset of the study, yet 60% of dogs with neoplasia developed euthyroid sick syndrome while in the hospital. In this study, 42% of dogs with acute critical illnesses (surgery, trauma, and sepsis) demonstrated euthyroid sick syndrome upon entry into the study. The syndrome continued to be present in the majority of those cases, except for two dogs with sepsis, whose serum TT_4 values normalized with advancing time. This prevalence is slightly lower than that reported by Elliot et al.[11] Numerous diseases were represented in the study by Elliot et al,[11] including pneumonia, megaesophagus, immune-mediated hemolytic anemia, and laryngeal paralysis. Our study differs from that of Elliot et al,[11] in that a large percentage of our cases were surgical in nature, with approximately half of surgical cases also involving some neoplastic process.

Vail et al[37] demonstrated a higher incidence of low T4 in canine patients with cancer cachexia, but not in patients that were weight-stable at the time of entry into the study. Specifically, the incidence of euthyroid sick syndrome was not investigated, as endogenous TSH levels were not measured. In that study, dogs demonstrating cancer cachexia with concomitant low T4 syndrome had neoplasia other than appendicular OSA. The majority of cases demonstrating low T4 had some form of carcinoma. The demographics of these patients differs greatly from the majority of patients in our study, who were weight stable dogs with OSA at the time of entry into the study. In Vail's study, OSA and sarcoma patients were listed in the non-cachectic group, who had normal T4 levels. Lymphoma patients were equally represented in both cachexia and non-cachexia groups.

In this study, the presence of normal serum TT_4 concentrations in dogs with neoplasia may represent a continuum of the disease process, in which the body is no longer stressed and therefore no longer has to down-regulate metabolic processes. The majority of patients with neoplasia were admitted into the critical care unit pre-operatively for protein and carbohydrate tracer analyses. The majority of patients in this disease category were stable at the time of entry. Following surgery, the increased incidence of euthyroid sick syndrome following 48 hours of hospitalization and feeding is important, as treatment for the primary neoplasia (i.e. surgical tumor removal) did not appear to prevent changes in thyroid hormone activity from developing. Many of the patients with advanced neoplasia underwent surgical treatment for their primary disease in between the first sampling period and the second sampling period, 48 hours following supplemental feeding. An increase in the occurrence of euthyroid sick syndrome was also observed in patients operated for non-neoplastic diseases. This suggests that the stress of

anesthesia and surgery is a confounding factor that can cause down-regulation of the thyroid axis, with the subsequent development of euthyroid sick syndrome. These findings are similar to that of Wheeler et al (unpublished data), who found the thyroid axis to become depressed in canine patients following elective surgery.[38] No significant interaction was observed between baseline serum cortisol and TT_4 levels before or after feeding, suggesting that factors other than elevated cortisol alone were responsible for dysregulation of the thyroid axis. Other factors thought to be involved in the development of euthryoid sick syndrome include dysregulation of thyroid hormone binding proteins, increased production of inactive T_3 (rT_3), and peripheral receptor unresponsiveness to thyroid hormone. A limitation of this study is that rT_3 and T_3 levels were not examined. However, TT_4 and eTSH are highly stable and used routinely for diagnostic purposes. Therefore, the diagnosis of euthyroid sick syndrome in affected animals appears to be appropriate. Without subsequent hormonal analyses, however, the exact mechanism by which euthyroid sick syndrome occurred remains unknown at this time.

No significant abnormalities were observed in the hypothalamic-pituitary-adrenal axis of all clinical cases except for one case of documented hypoadrenocorticism associated with sepsis. The results of our study were similar to the findings of Prittie et al.[39] who documented no significant decrease in adrenocortical responsiveness to ACTH stimulation over the course of critical illness in dogs. In human studies, adrenocortical burnout did not occur before day 6 of hospitalization. A confounding factor in this study, as well as that of Prittie et al.[39] is that ACTH stimulation testing was performed in all cases before day 6 of hospitalization. In this study, diagnostic testing was usually performed within 24 hours of hospitalization, and again 48 - 72 hours after the first series of blood tests. Unlike the human patients with critical illness, canine

patients in this study and that of Prittie et al[39] were not hospitalized in the critical care unit for longer than 7 days. In the latter study, the average length of stay in the critical care unit was just 2.0 days. Prittie et al[39] also documented a 35% mortality rate in the canine patients. In that study, there was no significant difference in basal cortisol levels in survivors versus non-survivors. In our study, discharge rate from the critical care unit was 100% (thus, mortality rate was 0%). Therefore, unlike human patients with critical illness, activation of the hypothalamic-pituitary adrenocortical axis does not appear to be a sensitive prognostic indicator of outcome in critically ill dogs. The observation that the degree of adrenocortical sensitivity to ACTH stimulation appears to change with time in human patients warrants further investigation of the hypothalamic-pituitary adrenal axis in veterinary patients with long-term critical illnesses, after day 6 of hospitalization.

The results of this study suggest that endogenous protein synthesis is not significantly different in trauma, septic, or cancer patients compared with healthy dogs. Surgery patients had significantly higher PSR in both the pre- and post-feeding time periods, before and after correcting for metabolic body size. The PSR of septic patients increased following treatment and feeding, and was intermediate to trauma and cancer patients, and surgery patients and control dogs. No disease category demonstrated lower PSR than that of control dogs.

Urinary nitrogen loss was not increased in any disease category. After feeding, urinary nitrogen loss significantly decreased when corrected for metabolic body size. The combined effect of PSR and a decrease in urinary nitrogen loss data suggest that nitrogen balance was normal in this subset of critically ill dogs, both before and after feeding, and that recovery from illness and feeding may decrease urinary nitrogen loss, thus improving nitrogen balance.

Endogenous protein synthesis appeared to increase slightly in trauma, sepsis, and cancer patients after feeding, but this effect was not statistically significant. Endogenous protein synthesis was slightly lower in the surgery patients after the 48 hour feeding period, but PSR was still significantly higher than other disease categories. The dramatic increased rate of endogenous protein synthesis in the surgery group both before and after surgery and feeding, is likely not actually significant of improved protein synthesis, but rather, is due to tremendous variability of data. Although the data were normally distributed, there was an extremely large standard deviation of PSR in this disease category. Additionally, at the time of entry into the study, most surgery patients were active and mobile, thus making it difficult to maintain urinary catheterization. Incomplete collection of urine throughout the sample collection period can artifactually increase the PSR by inadequate collection of the tracer isotope. Alternatively, examining each individual patient's data, the patients with the largest PSR values were young, outwardly healthy animals both before and after surgery. The urease method using ^{15}N-glycine measures hepatic protein synthesis. A number of the patients with markedly elevated PSR rates were entered into the study for portosystemic shunts. Therefore, it would be expected that hepatic synthesis of urea would be low patients with portosystemic shunting. However, analysis of results indicate that the dogs with portosystemic shunting had the highest PSR. This is likely artifactual, as a result of incomplete collection of urine despite urinary catheterization at the end of the collection period. The younger, healthier patients also had relatively lower per cent body fat and relatively larger percent lean body mass. This may have artifactually elevated the PSR calculations relative to the older patients that were less lean and skewed the results for the dogs

undergoing surgery. The most likely cause of increased PSR in this group likely was due, however, to incomplete collection of urine.

Previous research conducted by our laboratory group[12] revealed an increased rate of glucose flux in dogs with osteosarcoma. The cancer patients, as well as the other patients requiring critical care, in this study demonstrated no increase in glucose flux before or after feeding compared with healthy control dogs. This suggests that futile cycling of substrates was not occurring in this subset of critically ill patients with sepsis, trauma, surgery, and various forms of cancer. The relative change in glucose flux following feeding suggests that feeding channels substrates through energy-yielding, not energy-consuming pathways, despite the lack of statistical significance. With low number of clinical cases, it is possible that statistical significance would be reached by inclusion of additional patients into this study. Interventional nutritional support during times of metabolic stress may provide energy substrates that can be utilized for repair processes. A limitation of this study is that no patient was withheld from food. Therefore, it remains unknown whether withholding of food in these patients would affected glucose flux differently than in the patients that had been fed for 48 hours. Additionally, clinical improvement from the primary presenting illness may also have resulted in a decrease in glucose flux in these patients as a result of a decrease on metabolic stress. It would be expected that an increase in metabolic stress over the time of supplemental feeding would have been reflected in an elevated delta cortisol after 48 hours of feeding, which was not the case in this study. Additionally, direct comparison of the patients in our study with the first study[12] cannot be performed, as the timeline of blood sampling post-operatively differs enormously. The glucose flux and protein synthesis of dogs with OSA in the first study was performed 24 hours post-

operatively, whereas the stable isotope tracer analyses were performed on the third post-operative day in this study in dogs having surgery for cancer or other non-neoplastic processes. It is possible that evaluation of the dogs with OSA on the third post-operative day would have differed from that obtained in the immediate post-operative time period.

Glutamine supplementation in excess of that provided in Clinicare® had no effect on decreasing glucose flux in any disease category. Although the additional glutamine supplementation appeared to have a statistically significant effect on PSR after feeding, this effect is likely erroneous, as PSR in individual treatment groups showed a mild increase post-feeding, but were statistically similar both before and after feeding ($p = 0.330$). Additionally, the slight increase in PSR was also observed in groups that did not receive glutamine. The appearance of a positive glutamine effect was largely due to the surgery patients having elevated PSR before and after feeding in the group that received supplemental glutamine. The lack of glutamine effect on other disease categories suggests that glutamine supplementation in excess of that found in Clinicare® had no effect on nitrogen balance in these subsets of critically ill dogs. These findings are similar to the findings in cats with methotrexate-induced enterocolitis.[40] One limitation of this study is that no dog received an enteral diet without any glutamine, as Clinicare® contains approximately 550 mg 100 kcal as fed. Another limitation of this study was that serum glutamine levels after feeding were not measured. Possible causes for the apparent lack of effect of additional glutamine supplementation include inappropriate dosage, incomplete absorption of the L-glutamine powder, and incomplete utilization of the "conditionally essential" amino acid. The dose of glutamine used in this study was extrapolated from that published in human literature. It is therefore possible that the glutamine dose was too low to be effective. The

dose of glutamine used in one study that showed a statistically significant decrease in radiation-induced mucositis in dogs undergoing radiation therapy for nasal tumors.[41] the dogs of glutamine was 4 g/m^2/day, a slightly lower dose than that used in this study. Given that Khanna's group demonstrated a positive effect of glutamine, it seems plausible that the dose used in our study would be more than adequate (i.e. 3 times that used by Khanna's groups), if systemic absorption occurred. A second theory is that the positive glutamine effect in Khanna's study was not due to systemic absorption at all, but rather, was due to a local effect of glutamine on decreasing mucositis by providing a glutathione precursor to decrease oxidative damage induced by radiation therapy.

An alternate theory is that the glutamine powder supplemented in the diet of the patients was not completely absorbed. Although L-glutamine has been shown to be stable in solution, breakdown in the gastrointestinal tract may occur, making absorption incomplete. Alternatively, the intraluminal glutamine may be used exclusively by the enterocytes, and thus not absorbed in sufficient amounts to be utilized for other purposes, including endogenous protein synthesis. Other forms of glutamine supplementation, including via total or partial parenteral nutrition solutions, may be a better method of supplementing glutamine, as this modality bypasses utilization of intraluminal glutamine completely by the enterocytes.

Ileus is often a secondary complication observed in many veterinary patients with critical illness. Most patients, particularly in the post-operative period, are on opioid drugs which may impair gastrointestinal motility and thus predispose to ileus and incomplete nutrient absorption. Thus, glutamine absorption may be impaired. Finally, glutamine depletion may not be present in canine patients with critical illness and neoplasia. Although whole-body glutamine depletion has

been documented in experimental animals and humans with a variety of illnesses, little is known about the role of glutamine in the canine species. Alternatively, if whole-body glutamine depletion is present in critically ill dogs, the glutamine dose selected may be inadequate to replenish body glutamine stores in amounts necessary to improve endogenous protein synthesis. Utilization of glutamine in canine patients with illness remains unknown at this time. Another limitation of this study is that the role of supplemental glutamine on endogenous protein synthesis and nitrogen loss was not examined in healthy patients. This may lend insight into whether supplemental glutamine is an effective means of supplementing glutamine in the dog. Finally, it is possible that glutamine is not a preferred fuel for canine enterocytes. Beaulieu et al[42] recently demonstrated that canine enterocytes may prefer other energy substrates, including glucose, and the short chain fatty acid butyrate, rather than glutamine. Measurement of serum glutamine concentration following glutamine administration is indicated in future studies. Glutamine supplementation appeared to have no negative effect, even in patients with compromised hepatic function secondary to hepatic surgery or portosystemic shunts. Until further research is performed investigating the absorption and utilization of glutamine in healthy dogs, glutamine supplementation in canine patients with critical illness may not be beneficial in improving nitrogen balance and hormonal changes.

FOOTNOTES

a. C/D, W/D or K/D, Hill's Pet Food, Topeka, KS

b. ^{15}N-Glycine, Cambridge Isotope Laboratories, Andover, MA

c. 6,6-(deuterium)-glucose, Cambridge isotope Laboratories, Andover, MA

d. Venocath, 16 gauge, Abbott Laboratories, North Chicago, IL

e. Foley catheter, Medline Industries, Inc., Mundelein, IL

f. Cortrosyn, Organon Inc., West Orange, NJ

g. Immulite Cortisol test, Diagnostic Products Corporation, Los Angeles, CA

h. Immulite Canine THS, Diagnostic Products Corporation, Los Angeles, CA

i. Immulite Canine Total T_4, Diagnostic Products Corporation, Los Angeles, CA

j. Immulite Chemiluminescent Immunoassay System, Diagnostic Products Corporation, Los Angeles, CA

k. Hewlett Packard Quadrupole, H-P Inc, Palo Alto, CA

l. Diagnostic kit no. 640, Sigma Chemical Co, St. Louis, MO

m. Sira-II isotope ratio mass spectrometer, VG Instruments, Cheshire, England

n. Dowex 50 and Dowex 1 ion exchange resins, Applied Membrane Inc, Vista, CA

o. Hologic QDR-1000 W whole-body x-ray bone densitometer, Hologic Inc, Bedford, MA

p. Propofol, Abbott Laboratories, North Chicago, IL

q. Clinicare®, Abbott Laboratories-Animal Health, North Chicago, IL

r. L-glutamine powder PCCA, Houston, TX

s. Personal communication, Abbott Laboratories technical support services, North Chicago, IL

t. Statview 5.0, SAS Institute, Inc., Cary, NC

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TABLE 1: Diet composition of Canine Clinicare®, Abbott Laboratories, North Chicago, IL. Analysis of Canine Clinicare®, including crude protein, fiber, and fat content, caloric balance, elemental and molecular formulations, and amino acid composition. Although not separately listed, Canine Clinicare® contains glutamine, primarily within casein.

Guaranteed analysis	
Crude protein min	5.40%
Crude fat min	6.20%
Crude fiber max	0.05%
Moisture, max	82.00%
Ash, max	1.20%
Calcium, min	0.17%
Phosphorus, min	0.13%
Potassium, min	0.15%
Sodium, min	0.05%
Chloride, min	0.09%
Magnesium, min	0.01%

Calorie Distribution	
Protein	20%
Fat	55%
Carbohydrate	25%

Ingredients

Water	Potassium citrate	DL-methionine
Maltodextrin (corn)	Soy lecithin	Potassium phosphate dibasic
Sodium caseinate	Calcium carbonate	Carrageenan
Soy oil	Potassium chloride	Ferrous sulfate
Butter	Potassium phosphate monobasic	Taurine
Dried whey protein	Choline chloride	Zinc sulfate
Egg yolks	Magnesium chloride	Calcium phosphate dibasic
Calcium phosphate tribasic	L-arginine	Ascorbic acid
Riboflavin	Pyridoxine hydrochloride	Folic acid
Vitamin D_3	Potassium iodide	Sodium molybdate
Vitamin B_{12}	Mn-S04	Cu SO4
A-tocopherol	Biotin	Niacinamide
D-calcium pantothenate	Sodium selenite	Thiamine hydrochloride
Vitamin A palmitate		

TABLE 2: Patient information, including signalment (age, gender, and breed) and specific disease information. A total of 16 breeds of dog were represented. Thirteen dogs had neoplasia, 6 had sepsis, 5 had non-surgical trauma, and 12 had abdominal surgery not involving neoplasia. Twenty dogs were male (14 neutered, 6 intact). And 15 dogs were female (all spayed). Average patient age was 5.40 +/- 3.0 years. Cancer patients were significantly older than surgery patients ($p = 0.01$).

Patient ID	Disease Category	Disease	Age (yrs)	Gender	Breed
1	**Neoplasia**	OSA	8	fs	greyhound
2		OSA	7	mn	mix
3		OSA	9	fs	Mix
4		OSA	9	fs	Labrador Retriever
5		OSA	8	fs	Mix
6		OSA	8	mn	Mix
7		OSA	7	fs	Rottweiler
8		OSA	5	fs	Rottweiler
9		OSA	6	mn	Mix
10		OSA	4	mn	Rottweiler
11		soft tissue sarcoma	10	fs	Golden Retreiver
12		soft tissue sarcoma	9	m	Brittany Spaniel
13		leiomyosarcoma	7	m	Siberian Husky
14	**Sepsis**	pancreatitis/pneumonia	4	mn	Sheltie
15		addisonian crisis	2	fs	Great Pyrenees
16		peritonitis	3	m	Skye Terrier
17		lymphoma/post-chemo	2	fs	Beagle
18		peritonitis	4	mn	Labrador Retriever
19		peritonitis	2	fs	Golden Retriever
20	**Trauma**	hit by car/multiple fractures	5	mn	Bichon Frise
21		hit by car/pneumothorax	6	mn	mix
22		hit by train/degloving	2	fs	Alaskan Malamute
23		hit by car/pelvic fractures	11	fs	mix
24		hit by car/multiple fractures	9	mn	Spitz

TABLE 2 continued

25	**Surgery**	pancreatitis/exploratory	2	mn	Akita
26		renal abscess/exploratory	1	fs	German Shepard
27		laryngeal paralysis/tie-back	8	fs	Great Pyrenees
28		portosystemic shunt/exploratory	1	m	Bernese Mt. Dog
29		hiatal hernia repair	6	mn	Cocker Spaniel
30		portosystemic shunt/cystotomy	1	mn	Border Collie
31		thoracotomy/lobectomy	2	mn	Golden Retriever
32		pelvic fracture repair	9	fs	Corgi
33		pelvic fracture repair	2	m	Labrador Retriever
34		intestinal foreign body removal	3	fs	Golden Retriever
35		visceral epilepsy/exploratory	6	mn	Mix
36		hepatoma removal	8	mn	Chessie

OSA = osteosarcoma, PSS= portosystemic shunt; Chessie = Chesapeake Bay Retriever, M= male (intact), MN= neutered male, FS = spayed female.

TABLE 3: Body Composition, with percent body fat shown as mean ± standard deviation for each disease category. Patients with trauma and sepsis had significantly higher percent body fat than surgery patients . Cancer patients had percent body fat intermediate to the other disease categories, and were statistically similar.

Group	Percent body fat	
	Mean	Std dev
Cancer	22.2[ab]	6.8
Sepsis	27.4[a]	13.6
Surgery	17.6[b]	7.7
Trauma	27.7[a]	6.8

[a,b,ab]Within each column, values with different superscript letters differ significantly ($P < 0.05$).

TABLE 4: Mean ± SD for serum thyroxine (TT_4), endogenous TSH (eTSH) for each disease category, before and after 48 hours of supplemental feeding. No significant ($P > 0.05$) difference was observed in TT_4 or eTSH between disease categories, or before and after supplemental feeding. Euthyroid sick syndrome, defined as a low TT_4 with normal to low eTSH, was present in all disease categories except animals with cancer before feeding. After feeding, the prevalence of euthyroid sick syndrome in trauma patients decreased to 0. The prevalence of this syndrome in cancer patients increased to 50% after feeding. Glutamine had no effect on TT_4, eTSH, or the prevalence of euthyroid sick syndrome.

	TT4			
	Before feeding		After Feeding	
Disease Category	mean	std dev	mean	std dev
Cancer	1.82	0.51	1.20	0.15
Sepsis	0.32	0.20	0.64	0.49
Trauma	0.57	0.44	0.67	0.25
Surgery	1.13	0.51	1.02	0.51
	eTSH			
	Before Feeding		After Feeding	
	mean	std dev	mean	std dev
Cancer	0.16	0.14	0.15	0.08
Sepsis	0.24	0.28	0.52	0.72
Trauma	0.12	0.10	0.18	0.07
Surgery	0.11	0.05	0.15	0.10

TABLE 5: Hypothalamic-pituitary-adrenal axis. Mean ± SD baseline and post-ACTH stimulation cortisol levels are given, before and after supplemental feeding for all disease categories. There was no significant difference in the magnitude of the change in cortisol (delta cortisol) levels after ACTH stimulation (delta cortisol) in any disease category, before or after supplemental feeding. Supplemental glutamine in excess of that found in Clinicare® had no effect on the hypothalamic-pituitary-adrenal axis responsiveness to ACTH stimulation.

	Before Feeding			
	Pre-ACTH Cortisol		Post-ACTH Cortisol	
Disease Category	mean	std dev	mean	std dev
Cancer	5.24	2.34	15.38	4.75
Sepsis	4.40	3.24	16.98	10.34
Surgery	4.52	3.67	9.96	3.40
Trauma	4 43	4.02	16.86	10.51

	After Feeding			
	Pre-ACTH Cortisol		Post-ACTH Cortisol	
	mean	std dev	mean	std dev
Cancer	4.82	2.38	16.75	7 11
Sepsis	11.97	10.97	23.12	13.06
Surgery	3.30	0.67	12.68	4.40
Trauma	5.9	7.28	15.03	7.96

TABLE 6: Mean = SD for Protein Synthetic Rate (PSR), expressed in g/kg/day and in reference to metabolic body size, as $g/kg^{0.75}$/day. Endogenous protein synthesis after feeding increased in septic and cancer patients when corrected for metabolic body size . Supplemental glutamine in excess of that found in Clinicare® had no significant effect on PSR in any disease category.

	Pre-feeding			
Disease	**PSR g/kg/day**		**PSR $g/kg^{0.75}$/day**	
	mean	**std dev**	**mean**	**std dev**
Beagle controls	4.62	2.50	7.99	4.23
Cancer	11.55	5.82	28.78	16.08
Sepsis	9.67	4.67	20.6	10.12
Surgery	23.22	21.27	47.84	41.10
Trauma	5.42	3.79	11.67	9.57
	Post-Feeding			
	PSR g/kg/day		**PSR $g/kg^{0.75}$/day**	
	mean	**std dev**	**mean**	**std dev**
Beagle controls	4.62	2.50	7.99	4.23
Cancer	10.68	5.65	23.68	14.11
Sepsis	11.60	11.00	23.94	22.04
Surgery	16.50	9.44	35.43	21.88
Trauma	4.89	3.07	17.30	8 48

TABLE 7: Mean ± SD for urinary nitrogen loss for all disease categories. Across disease categories, nitrogen loss decreased significantly ($p = 0.039$) in the post-feeding time period. However, there was no significant decrease in urinary nitrogen loss after feeding within disease groups. Glutamine supplementation in excess of that found in Clinicare® had no significant effect on urinary nitrogen loss.

	Total Urinary Nitrogen Loss Pre-feeding			
	g/kg/day		$g/kg^{0.75}$/day	
Disease category	mean	std dev	mean	std dev
Beagle controls	0.27	0.09	0.46	0.15
Cancer	0.26	0.19	0.63	0.46
Sepsis	0.36	0.36	0.71	0.81
Surgery	0.37	0.2	0.77	0.41
Trauma	0.46	0.59	0.84	0.94

	Post-feeding			
	g/kg/day		$g/kg^{0.75}$/day	
	mean	std dev	mean	std dev
Beagle controls	0.27	0.09	0.46	0.15
Cancer	0.18	0.13	0.45	0.32
Sepsis	0.26	0.26	0.5	0.4
Surgery	0.26	0.13	0.52	0.31
Trauma	0.41	0.52	0.76	0.83

TABLE 8: Mean ± SD for glucose flux in mg/kg/hour. There was a non-significant trend toward a decrease in glucose flux across disease categories after feeding ($p = 0.09$). Glutamine supplementation in excess of that found in Clinicare® had no significant effect on glucose flux in any disease category.

	Before feeding mg/kg/hour		After feeding mg/kg/hour	
Disease category	mean	std dev	mean	std dev
Beagle controls	184.81	101.44	171.84	101.44
Cancer	171.23	57.38	147.77	137.17
Surgery	146.26	156.01	93.61	48.34
Sepsis	238.56	158.05	107.27	114.2
Trauma	201.82	63.25	141.74	82.15

CHAPTER 5

DISCUSSION AND CONCLUSIONS

Analyses of the results of this study suggest that metabolic and endocrine alterations occur in subsets of dogs with critical illness and neoplasia. In the first subset of dogs with osteosarcoma (OSA), changes in resting energy expenditure (REE), glucose flux, endogenous protein synthesis and urinary loss of nitrogen occurred in the absence of clinical signs of cachexia and after removal of gross tumor burden, compared with healthy control dogs. Dogs with OSA had lower rates of endogenous protein synthesis, higher urinary nitrogen loss, and higher glucose flux.

Before surgery, when tumor burden was greatest, resting energy expenditure was higher in dogs with OSA compared with healthy control dogs and the same dogs with OSA after removal of gross tumor burden. The decrease in REE after surgery appeared to not be associated with analgesics used or previous acclimatization to the calorimetry apparatus, since a different study demonstrated that canine patients undergoing elective neutering procedures had no significant increase in REE immediately after and 7 days after surgery.[2] Other factors associated with a decrease in metabolism, such as down-regulation of the hypothalamic-pituitary-adrenal axis, were not measured in this group of dogs with OSA. Extrapolating from the information obtained in the second part of the study, the groups of dogs with illness requiring critical care, we observed a marked increase in the incidence of euthyroid sick syndrome in patients with cancer in the post-feeding time period, after surgery was performed to remove gross tumor burden. It is possible that euthyroid sick syndrome may have been partially responsible for the apparent

decrease in REE observed in this group of dogs with OSA. Therefore, a limitation of this study is that evaluation of the thyroid hormone axis activity was not performed.

After surgery, although REE was not significantly elevated, glucose flux was higher than that of healthy animals. Glucose flux, or cycling of substrates through energy consuming pathways, can result in an increase in REE. The anesthetic protocol of infusing a large volume of intravenous fluids may have artifactually increased apparent lean body mass in the dogs with OSA during the peri- and intraoperative period, causing an artifactual decrease in REE observed. Other research conducted by Ogilvie's laboratory group[2] has demonstrated REE to not be significantly increased or decreased in other dogs after general anesthesia and elective neutering procedures. Additionally, REE was also measured in the subset of patients having elective neutering procedures at the time of suture removal, and was not significantly different from REE obtained in the immediate post-operative time period. However, the changes in REE were observed after correcting for lean body mass, therefore a relative increase in lean body mass due to infusion of high rates of crystalloid fluids during surgery cannot fully explain the relative decrease in REE observed in our dogs with OSA after surgery and removal of gross tumor burden.

Decreased rates of endogenous protein synthesis and increased urinary nitrogen loss were observed in dogs with OSA even after removal of gross tumor burden. A limitation of this study was that dogs with OSA did not have stable isotope tracer analysis performed prior to surgical removal of the limbs affected with OSA; therefore, PSR, urinary nitrogen loss, and glucose flux analyses before and after removal of gross tumor burden were not obtained. Analysis of results of stable isotope tracer studies suggest that this subset of dogs with OSA were in a state of less

positive nitrogen balance during the immediate post-operative period than the healthy control dogs, even when the legs affected with tumor had been removed. Additionally, dogs with OSA had higher rates of glucose flux in the evaluation period after surgery. Glucose flux reflects the cycling of metabolic substrates through energy consuming pathways, and may increase apparent REE in some patients. The changes in PSR, urinary nitrogen loss, and glucose flux were concomitant with an apparent decrease in REE, which may suggest a down-regulation of whole-body metabolism during the healing process. Despite a decrease in REE, changes likely to be maladaptive to the host were occurring.

A limitation of this study was that thyroid hormone parameters were not analyzed before and after surgery. The euthyroid sick syndrome is a common entity in human and veterinary patients with cancer[3] and other forms of illness requiring critical care.[4] Down-regulation of the thyroid axis and euthyroid sick syndrome may be a possible explanation of a decrease in protein metabolism after surgery. Another limitation of this study is that inflammatory cytokine levels such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), inteurleukin-6 (IL-6), and others were not evaluated. Further, changes in the adrenocortical axis in response to stress were not evaluated. The dogs with OSA were presumably not stressed, as evidenced by a lack of significant difference in their respiratory quotients (RQ) from that of healthy control dogs. A significant stress response would presumably increase the utilization of carbohydrates for energy, yielding RQ values approaching 1.0. Because the RQ values of dogs with OSA were not significantly different from those of control dogs before or after surgery, stress and of the responses to glucocounterregulatory hormones such as epinephrine and cortisol were likely not excessive in this subset of dogs, however, their specific hormone levels were not measured.

The results of the first study were exciting, and prompted further investigation of protein and carbohydrate metabolism in relation to possible adaptations in the thyroid and adrenocortical axes in subsets of dogs with neoplasia and other diseases requiring critical care. Analysis of the second study indicates that euthyroid sick syndrome appears to be a ubiquitous phenomenon in canine patients with neoplasia and illnesses requiring critical care. This is similar to the findings of Elliot and others[4] who demonstrated a high incidence of euthyroid sick syndrome in dogs admitted to critical care units. Thyroid hormone manifestations consistent with euthyroid sick syndrome were apparent in all subsets of dogs with cancer, sepsis, trauma, and surgery. The thyroid axis down-regulated even during the healing process, and the incidence of euthyroid sick syndrome increased over time of hospitalization in most subsets of critically ill dogs, including those with cancer. Therefore, our earlier conclusion that the apparent decrease in REE after surgery in our first subset of dogs with OSA may have been associated with a decrease in thyroid hormone axis activity secondary to euthyroid sick syndrome may have been appropriate. It is also possible that the incidence of euthyroid sick syndrome would decrease later in the post-operative period. Specifically comparing trauma versus surgery patients (including surgery for neoplastic and non-neoplastic causes), the second evaluation of the thyroid axis was performed on the fourth day post-trauma in the trauma group, but was performed on the third day after surgery in the surgery patients. It is possible that the incidence of euthyroid sick syndrome would further decrease when evaluated later in the healing process.

The apparent decrease in thyroid hormone activity was not associated with significant changes in the hypothalamic-pituitary adrenal axis. Although we observed a trend towards a mildly increased response to exogenous ACTH in the post-feeding time period, the change in

post-ACTH cortisol was not significantly different from the change before feeding. This suggests that increased circulating glucocorticoids were not altogether responsible for the change in thyroid axis activity observed in the subsets of dogs with cancer and illness requiring critical care. No dog received exogenous glucocorticoid except one dogs with hypoadrenocorticism and one dog treated with Prednisone for lymphangectasia at the time of entry into the study. Therefore, exogenous administration of glucocorticoids was not responsible for the change in thyroid hormone activity observed. Additionally, no dog exhibited apparent burnout of the adrenal cortex, or lack of response to exogenous ACTH administration, during the course of the study, except one dog with true hypoadrenocorticism. This is similar to the recent findings of Prittie et al[5] who also found no decrease in adrenocortical activity in over 20 dogs hospitalized for illnesses requiring critical care. This suggests that the syndrome of adrenocortical insufficiency rarely observed in human patients with critical illness does not apparently occur in dogs. However, this interpretation may be overly simplistic, as none of the dogs in our study or Prittie's study were analyzed past four days of hospitalization. In one study conducted in human critically ill patients, the first incidence of adrenocortical insufficiency did not manifest itself until the 6th day of hospitalization.[6,7] Therefore, it is possible that ACTH stimulation testing performed later during the course of hospitalization would have demonstrated apparent adrenocortical insufficiency necessitating exogenous glucocorticoid administration in some patients.

Analysis of results of ^{15}N-glycine tracer studies of endogenous protein synthesis and urinary nitrogen balance demonstrated no statistical difference between patients requiring critical care and healthy control dogs. Endogenous protein synthesis was numerically higher in the dogs

with cancer from the second study than the dogs with OSA in the first study. Careful analysis of these dogs suggested that there should not have been any difference, as the patients were very similar clinically. One explanation is that a major difference between subsets of patients with cancer in study one and study two is that in the first study, PSR was evaluated 24 hours after surgery. However, in the second study, PSR was performed on the third post-operative day, after the 48 hours of supplemental feeding. It is possible that the PSR observed in the immediate post-operative period (e.g. 24 hours after surgery) was different that observed on the third post-operative day. In our second study, no patient was anorexic at the time of entry into the study, and none exhibited signs of weight loss or cachexia. This was further supported by the absence of differences in per cent body fat between dogs with cancer and other disease categories, including sepsis and trauma - diseases that were more acute in nature than cancer. There is no obvious explanation for the difference in protein synthesis among these subsets of dogs with cancer. A second explanation is that there is a wide variability across a disease category, and it may be difficult to predict based on clinical signs alone which patients are in negative nitrogen balance. No cancer patient in the second study was cachexic or anorectic. During stressed starvation, body protein is not spared despite body's compensatory mechanisms, such as alterations in the thyroid hormonal axis.[1] Infusion of cortisol into healthy human volunteers has been shown to promote negative nitrogen balance by enhancing proteolysis and urinary nitrogen excretion. Activation of the hypothalamic-pituitary-adrenal axes was not excessive in any subset of dogs with critical illness and neoplasia. Although stress associated with hospitalization, unfamiliar environment, and therapy may have occurred in our dogs with critical illness, care was taken to adequately manage discomfort and pain. Analgesics are routinely used after

surgery, thus negating any pain-induced cortisol release. In some cases, anxiolytic drugs such as acepromazine were used in combination with analgesics, to promote restful activity and to decrease anxiety that may have been detrimental to the dog's healing and overall well-being. Anxiolytic agents, too, can suppress release of catecholamines and cortisol by decreasing stress. If analgesics and anxiolytics were not used in our patients, the stress response likely would have been greater and may have produced negative nitrogen balance. However, this approach to therapy is unconventional and cruel, and would be purely academic. The use of analgesic and anxiolytic agents in the animals in this study was appropriate and justified, as our goal of investigation was to determine whether alterations in protein and carbohydrate metabolism and hormonal axes occur in patients using appropriate standard-of-care therapy. Standard-of-care therapy was also changed according to each patient's needs and individual clinician preferences, and therefore included other confounding variables that may have altered results. The criteria for exclusion from the study were diagnosis of underlying metabolic or endocrine abnormalities such as true hypothyroidism requiring thyroxine supplementation, seizure history necessitating antiseizure medication that can potentially interfere with thyroid hormone metabolism and hepatic protein synthesis, and glucocorticoid administration for any purpose other than hypoadrenocorticism. Although one patient received prednisone for lymphangectasia, the steroid dose was very low, and only approached physiologic steroid levels. Therefore, this patient remained in the study. Further studies in inappetant, stressed and cachectic patients are warranted to investigate endogenous protein metabolism in other subsets of more critically ill patients and those with end-stage neoplasia.

Feeding, with or without glutamine supplementation in excess of that found in Clinicare®, had no substantial effect on nitrogen balance in this subset of dogs with critical illness and neoplasia, despite an apparent significant increase in PSR in patients that received the amino acid supplement. The increased PSR in patients that received glutamine supplementation in excess of that found in Clinicare® was likely artifactual, and was caused by unusually high rates of protein synthesis in the subset of patients requiring surgery, as no effect was observed in any other disease category.

The higher rate of endogenous protein synthesis in the subset of patients requiring surgery was surprising. The urea method for estimation of PSR most closely reflects hepatic protein synthesis. In our hands, the patients with the highest rates of hepatic protein synthesis were dogs with portosystemic shunts. Although each patient had an abdominal exploratory laparotomy to repair the shunt, current methods for shunt ligation involve slow closure of the anomalous vessel over four to six weeks time. Therefore, shunting around the liver still occurs in the immediate post-operative period. It would be expected that hepatic protein synthesis in these patients would be low, not high. One of the markers for impaired hepatic protein synthesis is decreased serum albumin concentration. None of the dogs with portosystemic shunts had low serum albumin concentration, and none were severely ill at the time of surgery. This created a confounding variable in the study, because it was difficult to maintain urinary catheterization in these apparently healthy, very active young dogs for complete collection of urine samples for the 10 hours after infusion of stable isotope. Incomplete collection of urine can artifactually increase the apparent PSR. Dogs that pulled their urinary catheters then urinated in the cage had incomplete collection of all the infused isotope. Additionally, human error caused by inadvertent

spillage of urine during free catch collection of the sample sometimes occurred. The exact volume of urine spilled was difficult to measure, and had to be estimated.

No dog in this subset of critically ill patients had loss of excessive amounts of nitrogen in their urine, before or after feeding. Urinary nitrogen loss significantly decreased in the post-feeding time period across disease categories. Although negative nitrogen balance is commonly reported in human patients with critical illness and neoplasia, and experimental animals, less positive nitrogen balance was not apparent in this study, except in the first group of dogs with OSA. Again, despite the apparent decrease in REE in the dogs with OSA after surgery, it may be difficult to predict nitrogen balance on clinical signs alone.

No dogs in the second study had increased rates of glucose flux when compared with healthy control dogs. This suggests that not all patients with critical illness have futile cycling of substrates, or shuttling of substrates through energy consuming processes. Dogs with OSA demonstrated increased glucose flux compared with the healthy controls. An exciting observation is the apparent decrease in glucose flux after feeding, despite the lack of statistical ($p = 0.09$) significance at the time of this writing.

Feeding a balanced diet early in the course of hospitalization may have spared protein catabolism and favored maintenance of normal rates of protein synthesis and decreased rates of urinary nitrogen loss in these patients. Secondly, a lack of increase in glucose flux suggests that feeding may favor appropriate utilization of substrates for energy, and prevent utilization of the body's proteins for hepatic gluconeogenesis through energy-consuming processes such as the Cori cycle. The absence of an unfed control group is a technical limitation of this study. Therefore, it remains unknown whether the decreased rate of glucose flux observed after feeding would have

been eliminated in patients that had received no supplemental nutrition. It would have been inappropriate to withhold nutritional support, because inadequate nutrient stimulation of the enterocytes in the lumen of the gastrointestinal tract is associated with their atrophy and may increase the incidence of bacterial translocation. Appropriate interventional nutrition is necessary in treating dogs with critical illness.

A very large limitation of our study was that no patient with critical illness severe enough to make the patient a high anesthetic risk was entered into the study. General anesthesia was required in most cases for DEXA scans to be performed. Clients were often apprehensive about general anesthesia in their dog, frequently declining entry into the study for this reason alone. Another limitation of the study was that Survival Prediction Index (SPI).[8,9,10] was not performed in any patient, allowing categorization of the apparent severity of disease at the time of entry into the study. If a patient was not likely to survive the four days of hospitalization of the study, they were not included. This decision was made based on the apparent increased risk of anesthesia required for DEXA scan, as well as an apparent increase in risk associated with loss of blood volume and need for repeated blood sampling. The need for frequent blood sampling, although not likely to cause problems in patients with normal red blood cell counts, can decrease oxygen delivery in patients with severe anemia. Therefore, severely anemic patients were not included in the study due to the increased risk to their health that would be incurred by our need for frequent blood samples. Although our subsets of patients had illness requiring some form of critical care, the most severely ill patients, including those with the poorest prognoses, were specifically not included in this study.

Use of clinical patients in research involves many confounding variables which make observations difficult and may alter results. Other limitations of this study include not measuring inflammatory cytokine levels before and after feeding. Cytokine analysis is important in obtaining a more complete understanding of each patient's clinical condition, and possible correlations with alterations in metabolism. Another severe limitation of this study is the lack of measurement of glutamine in patient serum after supplementation. The lack of an apparent glutamine effect of protein metabolism may have been due to inadequate dose used, inadequate nutrient absorption from the gastrointestinal tract, or instability of glutamine powder in solution thus causing its breakdown before it could be absorbed effectively. It is also possible that utilization of glutamine by enterocytes limited its absorption into the systemic circulation for extra-intestinal effects. It is also possible that glutamine is not a preferred substrate for canine enterocytes. A recent study[11] documented preferential utilization of butyrate and glucose over glutamine by canine enterocytes. Measurement of serum glutamine concentration may have provided insight as to the apparent absence of a glutamine effect in this subset of critically ill dogs.

Future directions for this area of research include using alternate forms of glutamine, such as alanyl-glutamine, in enteral and total parenteral nutrition solutions. Measurement of serum glutamine concentration following its supplementation is also necessary, to determine whether enteral glutamine is absorbed from the gastrointestinal tracts of dogs. Additionally, the doses used in this study were extrapolated from those provided in human literature. The dose used by Khanna's group[12] was approximately 3 times lower than that used in our study, yet they found a subjectively lower incidence of radiation-induced mucositis in the dogs that received

glutamine. The apparent glutamine effect in that study[12] may have been due to a local effect, possibly as a glutathione precursor thus decreasing oxidative damage caused by radiation therapy, and not due to systemic absorption of glutamine at all. Other amino acids, such as arginine, are also currently being examined as supplemental nutrients in veterinary patients.

Survival Prediction Indexes (SPI) also is necessary to categorize the degree of illness in various subsets of dogs with illnesses requiring critical care. Stable isotope tracer analyses can be performed with minimal blood sampling, even in the most critically ill dogs. Use of a newer DEXA scanner can allow DEXA scans to be performed in just 2.5 minutes, rather than the 10 - 15 minutes necessary for the Hologic scanner used in this study. This may be a faster, safer alternative to obtaining body composition measurements in the most critically ill patients, and likely will only require sedation, not general anesthesia. Additionally, we have not observed a significant difference in endogenous protein synthesis when calculated based on metabolic body size versus lean body mass. Therefore, it seems unnecessary to perform DEXA analyses at all to calculate stable isotope tracer data, when metabolic body size calculations appear to be adequate.

Measurement of inflammatory cytokines using ELISA or competitive PCR can allow both quantitative and qualitative measurements of inflammatory mediators in dogs with critical illness. Other hormones, such as insulin, can also be measured and related to substrate utilization, as stress can induce a state of insulin resistance in some patients. Finally, use of stable isotope tracer analyses in a larger group of normal healthy dogs is warranted, for comparison purposes across different age and body composition categories.

TABLE 1: The limitations and sources of potential error of this study are listed below, and include human error, sources of technical difficulty, effects of treatment on selected variables. The need and potential for further research is also listed.

Human Error	
	Spillage of urine during free catch sampling
	Failure to collect all of isotope infused
	Failure to collect sample at scheduled time period
Technical error	
	Lack of patient cooperation, inability to maintain urinary catheter collection
	Disconnection of intravenous line during infusion of isotope
	Catheter thrombosis
Treatment Effects	
	High rates of IV administration causing an apparent increase in lean body mass
	Intravenous fluids diluting volume of distribution of stable isotope
Future Research	
	Collection of thyroid hormone samples from dogs with OSA
	Qualitative and quantitative measurement of inflammatory cytokines
	Measurement of serum glutamine concentration
	Stable isotope tracer analyses in dogs with OSA before surgery
	Large sample size
	Shorter DEXA scan times therefore not requiring general anesthesia
	Stable isotope tracer analyses in inappetant patients without supplemental nutrition
	Survival Prediction Index in all patients
	Analysis of stress hormones, T_4, eTSH, and isotope tracer studies in more critically ill patients
	Measurement of serum insulin concentrations
	Stable isotope tracer analysis in a normal dogs more similar to dogs with critical illness

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