Dietary Carbohydrate Restriction Slows Prostate Tumor Growth

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Glucose metabolism remains an intensely explored topic of cancer biology since the initial discoveries of Otto Warburg nearly 80 years ago [1-2]. Many solid tumors metabolize glucose primarily to lactate despite the availability of oxygen, revealing a dependence on glycolysis that may serve as a basis for targeted therapy [3-5]. In particular, a diet devoid of carbohydrate may minimize the growth capabilities of glucose-dependent cancers. As our interests lie in prostate cancer, we examined whether a ketogenic diet devoid of carbohydrate (NCKD) would reduce the growth rate of tumors derived from human prostate cancer cell lines in a murine xenograft model.

Our initial experiments utilized the LAPC-4 cell line, a human androgen-sensitive prostate cancer cell line, in a SCID-mouse xenograft model to determine the effects of an NCKD on tumor growth and animal survival relative to two other diets: (1.) a Western-type diet (WD) reflecting consumption patterns of men diagnosed with prostate-cancer in the Western world and (2.) a low-fat diet (LFD) representing the present standard of care. Following this study, we conducted a second study utilizing a different human prostate cancer cell line (LNCaP) to assess whether our initial observations were robust across multiple prostate cancer tumor models and to also further explore the molecular underpinnings of our observations. Both studies revealed the NCKD leads to a reduction in tumor growth rate and greater overall mouse survival relative to the WD. In addition, the NCKD was equivalent in these parameters to the LFD. We also observed key

associations between survival and extent of urinary ketosis as well as favorable changes in insulin and insulin-like growth factor-1 (IGF-1) and gene expression that would be predictive of prolonged survival in mice consuming the NCKD.

We believe these data provide compelling evidence to consider a potential therapeutic role for dietary carbohydrate restriction in prostate cancer. We hope these results ultimately serve as a basis to conduct future clinical trials assessing whether dietary carbohydrate restriction, either alone or in combination with more conventional therapies, provides clinicians with an additional weapon against prostate cancer.

Acknowledgments

This work would not have been possible without the guidance and support of many people. First and foremost, I must sincerely thank Dr. Salvatore (Sal) Pizzo who showed immeasurable support and patience throughout my graduate school career. Sal allowed me to follow my research interests no matter where they led me, the ultimate testament to his faith in my abilities, curiosity, and resourcefulness which ultimately allowed me to create a network of collaborations that proved critical to the genesis of this thesis. Sal has taught me these and many other lessons about mentorship; suffice it to say here that I hope to be blessed someday to implement the same lessons when teaching the next generation of research scientists.

I must also sincerely thank Dr. Stephen Freeland for his willingness to welcome me as a major collaborator. Dr. Freedland's infectious passion for understanding the role of diet in prostate cancer provided the perfect environment for a motivated graduate student to get things done. He has taught me much about surviving as a young investigator in today's very competitive research environment, lessons I have no doubt will be useful when I start my own lab someday.

I also owe deep thanks to Dr. Eric Westman, who provided me with an invaluable opportunity to conduct clinical research that ultimately allowed me to bridge my basic and pre-clinical scientific experiences with the realm of clinical research. Through his words and deeds, Dr. Westman taught me to "follow the data, not the trend" despite

criticism and skepticism from colleagues stemming from bias and/or preconception. I was very fortunate to start my research collaborations with Dr. Westman near the start of his odyssey to demonstrate the benefits of low-carbohydrate nutrition and I hope to remain both a life-long collaborator and, more importantly, a close friend.

I must also thank Drs Dewhirst and Schroeder for their ongoing collaborations with me to further investigate the effects of diet on the tumor metabolic microenvironment It is our hope that such studies will serve to stimulate future investigations on the use of diet in cancer patients.

In addition, I thank the numerous researchers and friends who encouraged and helped me on a day-to day basis during my graduate experience by providing stimulating discussion on countless topics, including, on occasion, science. For this and much more I thank, in no particular order, Dr. Richard Feinman, Dr. Mary Vernon, Dr. Mario Gonzales-Gronow, Dr. George Cianciolo, Quintin Quinones, Timothy Robinson, Dr. Steven Kazowcka, Dr. Timothy Fields, Dr. Shahid Nimjee, Dr. Matthew McGirt, Dr. William Yancy, Dr. Matthew McGirt, Dr. Laura Young, Dr. Narayan Melgiri, and Dr. Sulene Chi. If I have forgotten to include someone in this list, please accept my sincerest apologies.

Lastly, I want to mention that my mother will be graduating from college shortly after my thesis defense. After more than three decades away from academia she returned to obtain a degree in Accounting. She has always served as a constant source of inspiration to me and I hope to honor her example in whatever the future holds for me.

Table Of Contents

List Of Tables

List Of Figures

List Of Abbreviations

1-LN,	murine inguinal lymph node-derived human prostate cancer cell line
2DG,	2-deoxyglucose
AcAc,	acetoacetate
ACTH,	adrenocorticotropic hormone
ATP,	adenosine triphosphate
β-OH,	beta hydroxybutyrate
c-Myc,	cellular homolog of the viral myelocytomatosis oncogene (transcription factor)
CoA,	co-enzyme A
DL-,	dextrorotatory and levorotatory mixture (racemic)
DU-145,	Duke-145 prostate cancer cell line
EDTA,	ethylene diamine tetra-acetic acid
ELISA,	enzyme-linked immunosorbent assay
g,	gram
G6P,	glucose-6-phosphate
GLUT-1,	glucose transporter (isoform 1)
GSEA,	gene set enrichment analysis
HDL,	high density lipoprotein
HIF-1α,	hypoxia inducible factor-1 subunit alpha (transcription factor)
IGF,	insulin-like growth factor

IGFBP,	insulin-like growth factor binding protein
IL-6,	interleukin-6
kcal,	kilocalorie
kg,	kilogram
KD,	ketogenic diet
KD-40,	ketogenic diet at 40% fewer calories than KD-AL
KD-AL,	ketogenic diet ad lib
KD-R,	ketogenic diet at 65-70% total calories of KD-AL
LAPC-4,	Los Angeles prostate cancer-4
LDL,	low density lipoprotein
LFD,	low fat diet
LFSD,	low fat standard diet
LFSD-40R,	low fat standard diet at 40% fewer calories than LFSD-AL
LFSD-AL,	low fat standard diet ad lib
LNCaP,	lymph node carcinoma of the prostate
MAC16,	murine adenocarcinoma 16
MAP-kinase,	mitogen activated protein kinase
MCT,	monocarboxylate transporter
µg,	microgram
mL,	milliliter
mM,	millimole

mm,	millimeter
mm^3,	cubic millimeter
NCKD,	no-carbohydrate ketogenic diet
NK-κB,	nuclear factor-κB (transcription factor)
p53,	protein 53 (transcription factor)
PAGE,	polyacrylamide gel electrophoresis
p-Akt,	phospho-Akt protein
PC-3,	prostate cancer-3 cell line
PET,	positron emission tomography
PhIP,	poly-aromatic hydrocarbon (2-Amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine)
PI3-kinase,	phosphatidylinositol 3-kinase
PTEN,	phosphatase and tensin homolog deleted on Chromosome 10
RANKL,	ligand of RANK (receptor activator of NF-κB)
RNA,	ribonucleic acid
RPMI,	Roswell Park Memorial Institute growth medium
SCID,	severe combined immunodeficient
SDS,	sodium dodecylsulfate
SEM,	standard error of the mean
t-Akt,	total Akt protein
TNF-α,	tumor necrosis factor alpha

VEGF, vascular endothelial growth factor

ω-3, omega-3 fatty acid

ω-6, omega-6 fatty acid

WD, western diet

1. Introduction

1.1 Glucose Metabolism In Cancer

The importance of glucose metabolism in the biology of cancer was first recognized by Otto Warburg nearly 80 years ago [1]. Warburg observed that cancer cells, unlike normal cells, metabolize glucose primarily to lactate in oxygenated environments. This landmark finding, eponymously referred to as the 'Warburg effect' (or 'aerobic glycolysis' or a 'glycolytic phenotype') was initially viewed as a hallmark of all cancers [2]. However, it was later discovered that the Warburg effect, while present in rapidly growing cancers [3, 4], did not occur in all cancers [5]. That regional differences in glucose distribution often exist within a single tumor [6] confirmed heterogeneity, even on a local scale, was the rule and not the exception in cancer metabolism. A key insight thought to explain these differences was provided by Judah Folkman, who emphasized the importance of angiogenesis to metabolite delivery in tumors [7]. These findings, combined with the subsequent discovery of a genetic basis for cancer, were ultimately viewed by some researchers as evidence that changes in metabolism were secondary phenomenona [8]. In this view, mutations in tumor suppressors and/or oncogenes promoted rapid cellular growth which outpaced the creation of new blood vessels needed to deliver adequate oxygen and metabolic fuels. Consequently, changes in metabolism were thought to result from nutrient shortages caused by earlier and, presumably, more fundamental mechanisms of cancer.

Recent discoveries, however, undermine this view by supporting a role for metabolism that is temporally on par with early mechanisms of cancer and not merely a response to them [9]. Aberrant proteins once thought to cause cancer by activating mitogenic signaling pathways are now also known to direct the intermediary metabolism of cancer cells. For example, constitutive activation of the Akt signaling protein, while increasing the rate of cellular proliferation, also promotes the Warburg effect [10] and increases the expression of phospho-fructokinase [11] and hexokinase [12], two critical rate-determining enzymes in glycolysis. Indeed, cells with activated Akt undergo rapid apoptosis in response to decreased glucose levels *in vitro* [10]. In addition, two frequently mutated genes in cancer, p53 [13] and c-*Myc* [14], have also been shown to induce aerobic glycolysis through effects on mitochondrial enzymes. Furthermore, the finding that *all* glycolytic enzymes as well as the GLUT-1 transporter are upregulated by HIF-1α [15], a key transcription factor in angiogenesis [16], underscores the importance of glucose metabolism to critical events in early stages of carcinogenesis. Together, these and other discoveries reflect a growing body of evidence supporting an integrated model of carcinogenesis where growth, angiogenesis, and metabolism are now viewed as coincident and interdependent events [17].

1.2 Metabolite Distribution In Solid Tumors

The creation of targeted therapies against the glycolytic phenotype remains a goal of scientific research. A key to successfully identifying such therapies relies on exploiting differences in metabolite distribution in solid tumors.

Solid tumors are characterized by extensive spatial heterogeneity with regard to glucose and oxygen distribution [6]. Typically, highly perfused tumor regions contain high levels of both oxygen and glucose as these regions receive a steady influx of nutrients from surrounding blood vessels. Such regions exhibit the "Pasteur Effect," switching from glycolysis to oxidative phosphorylation as a primary mode of ATP production in response to adequate oxygen tensions [6]. In contrast, tumor regions distant from growing blood vessels receive oxygen and glucose by diffusion and therefore exhibit a marked deficiency in levels of both substrates. A major consequence of chronic oxygen deficiency is the establishment of hypoxia [18], a hallmark of solid tumors associated with a reliance on glycolysis for energy production. In some hypoxic regions, glucose can be depleted to undetectable levels [6] rendering these regions particularly sensitive to perturbations in glucose availability. As hypoxia increases rates of mutation, angiogenesis, local recurrence, and metastasis [19], there is clearly a need to devise therapies that target such regions.

Restriction of dietary carbohydrate may prove therapeutic in this regard as hypoxic tumor regions would be dependent primarily on endogenous glucose production

in this setting. Whether such tumor regions would ultimately remain viable during dietary carbohydrate deprivation remains unknown.

1.3 The Eclectic Palate Of Cancer: A Barrier To Diet Therapy

While glucose is likely the most important, and certainly the most studied, fuel with regard to cancer metabolism, other substrates may be also utilized by cancer cells to generate ATP. Indeed, metabolic adaptation is a hallmark of cancer cells that confers a survival advantage in a dynamic physiologic environment characterized by sporadic fluctuations in oxygen and glucose availability. In light of these facts, two issues must be discussed with regard to utilizing dietary carbohydrate restriction as cancer therapy: (1.) normal alterations in whole-body metabolism resulting from dietary carbohydrate deprivation (2.) non-glycolytic mechanisms of ATP synthesis as adaptive responses by cancers to changes in whole-body metabolism stimulated by dietary carbohydrate restriction.

1.3.1 Ketogenic Diet: Metabolism & Physiology

Any diet containing little to no carbohydrate will induce the biosynthesis of ketone bodies and can therefore be classified as a "ketogenic diet." A typical macronutrient ratio for a ketogenic diet is 6 to 8 grams of fat for every 1 gram of protein and 1 gram of carbohydrate, although greater allowances for carbohydrate intake are

possible. Humans consuming such diets must synthesize glucose endogenously to sustain tissues dependent, either primarily or absolutely, on glycolysis such as cornea, lens, renal medulla, erythrocytes, and the central nervous system. While the breakdown of glycogen from liver, muscle, and brain [20] serves to maintain serum glucose levels shortly after ingestion of a ketogenic diet, the eventual depletion of available glycogen, typically within 12 hours [21], induces the synthesis of glucose via gluconeogenesis. Provided the protein content of a ketogenic diet leads to an intake greater than 1.5 g protein per kg body weight per day [22], the human body can sustain nitrogen balance to preserve and/or grow muscle mass while using excess amino acids as substrate for gluconeogenesis [23].

Concurrent hydrolysis of endogenous and ingested triacylglycerols activates the synthesis of ketone bodies by liver [24], colon [25], and brain [26,27] to further stabilize blood glucose levels during low or no carbohydrate intake. Two ketone bodies, acetoacetate and β-hydroxybutyrate, are derivatives of dimerized acetyl-coenzyme-A and serve as circulating energy-yielding metabolites for use by any cell with functional mitochondria. Within such a cell, acetoacetate and β-hydroxybutyrate are enzymatically reconverted to acetyl-coenzyme-A for entry either into the citric acid cycle [28] or, less commonly, pathways for membrane lipid synthesis [29]. While useful for a variety of tissues and cell-types [28], ketone bodies are particularly important for the central nervous system [30,31], which typically accounts for 20% to 50% of resting metabolic energy expenditure [30]. By inducing the synthesis of ketone bodies as a supplementary

fuel to glucose in conditions of low carbohydrate intake, a ketogenic diet sustains the central nervous system by reducing its dependence on gluconeogenesis [31,32]. In this way, catabolism of amino acids is minimized, ultimately preserving the normal structure and function of biological tissues [33].

In summary, a ketogenic diet stimulates three compensatory metabolic pathways: glycogenolysis, gluconeogenesis, and ketogenesis. All three pathways ultimately serve to normalize circulating glucose levels, although the specific mechanism by which this is achieved differs for each pathway. In particular, hepatic glycogenolysis releases endogenous stores of glucose into the circulation while ketogenesis and gluconeogenesis utilize fats and amino acids, respectively, to maintain blood glucose levels.

1.3.2 Potential Metabolic Adaptations Of Cancer To Carbohydrate Restriction

While glucose availability remains a critical aspect of viability and growth for hypoxic tissues, it is less critical for the survival of perfused tissues. In particular, the availability of circulating oxygen in perfused tumor regions allows some cancers to undergo the Pasteur Effect. Accordingly, the increase in serum free fatty acids and triacylglycerol levels resulting from a ketogenic diet provides copious substrate for cancer cells capable of the Pasteur Effect. In this scenario, fatty acid metabolism is the primary mechanism of ATP generation. This is particularly relevant for prostate cancer which often exhibits the simultaneous loss of a key rate limiting enzyme in fatty acid

synthesis, stearyl-CoA desaturase [34], and over-expression of a key enzyme in fatty acid breakdown, α-methyl CoA racemase [35].

Lactate may also be used to generate ATP in cancer cells, albiet at less than 10% the full ATP generating potential of glucose [33]. In this scenario, release of lactate by cancer cells transmembrane monocarboxylate transporters provides a paracrine fuel for neighboring cancer cells. The activity of lactate dehydrogenase in such cells regenerates pyruvate for eventual incorporation into the citric acid cycle [33].

Endogenous or dietary amino acids also generate fuel metabolites in cancer cells. In particular, alanine, glutamic acid, and aspartic acid can be converted to pyruvate, α-ketoglutarate, and oxaloacetate, respectively. While pyruvate and α-ketoglutarate are key substrates in the citric acid cycle, oxaloacetate (as well as α-ketoglutarate) may be converted to pyruvate via the enzyme phosphoenolpyruvate carboxykinase, which further stimulates the citric acid cycle.

Lastly, hepatic ketogenesis generates acetoacetate and β-hydroxybutyrate, which can ultimately be used to produce 24 and 27 ATP per molecule, respectively, by any cell undergoing oxidative phosphorylation [33]. Under conditions of dietary ketosis, the mean serum concentrations of acetoacetate and β-hydroxybutyrate are typically 0.5 and 1.5 mM, respectively. In comparison, mean serum concentration of acetoacetate and β-hydroxybutyrate resulting from a balanced macronutrient diet are typically less than 0.1 mM for both ketone bodies [36]**,** whereas mean serum concentrations of

β-hydroxybutyrate alone can be elevated to 8.5 mM during diabetic ketoacidosis [33]. In addition, metabolism of excess ketone bodies leads to over-production and subsequent efflux of intracellular citrate due to an abundance of regenerated acetyl-CoA secondary to ketone body catabolism. Citrate efflux from such cells increases circulating citrate levels which promotes influx into citrate-deficient cells leading to ATP production via regeneration of acetyl-CoA by ATP-citrate lyase.

In summary, despite potentially limiting glucose availability by severely restricting dietary carbohydrate, a ketogenic diet may nonetheless furnish non-glucose substrates to cancer cells for ATP generation. This possibility should not, however, be viewed as a drawback of a ketongenic diet but rather as a testament to the remarkable versatility of cancer metabolism to generate ATP under a variety of physiologic conditions. While numerous substrates may indeed lead to ATP production, we will later discuss why our studies show compensatory metabolic mechanisms of prostate cancer in response to a ketogenic diet do not seem to fulfill the rapid demands of growing tumors relative to diets balanced in carbohydrate and fat content. Nevertheless, it is clear that cancer cells are armed with an array of potential metabolic strategies to promote viability even under the harshest conditions, where normal tissues would not likely survive.

1.4 Non-Dietary Strategies Targeting The Glycolytic Phenotype

Circulating glucose must be transported into cells by facilitated diffusion via specialized transmembrane transporter proteins called GLUTs [33]. These glucose

transporters exist in several isoforms throughout the body, nearly all of which deliver glucose to the intracellular compartment. Within a cell, glucose is enzymatically converted to glucose-6 phosphate (G6P), a rapid modification necessary to prevent loss of glucose back into the bloodstream. Subsequent enzymatic conversion of G6P to fructose-6 phosphate followed by further phosphorylation to fructose-1,6 bisphosphate signals the commitment of a glucose molecule to glycolysis.

Given their dependence on glucose metabolism for growth and survival, cancer cells predictably express higher levels of GLUT than normal cells [37,38]. This is particularly true for a hypoxic cancer cell, which does not utilize oxidative phosphorylation and therefore generates excess lactate. Therapeutic strategies compromising glucose delivery and/or utilization should therefore, in theory, preferentially target hypoxic cancer cells.

One strategy based on this hypothesis involves the use of a chemically modified form of glucose, 2-deoxyglucose (2DG), which is also transported into cells by GLUT. Inside a cell, 2DG, like glucose, is trapped via phosphorylation but, unlike glucose, cannot undergo further metabolism to generate ATP. Rather, phosphorylated 2DG serves to competitively inhibit the further conversion of incoming glucose to G6P, thereby reducing overall glucose metabolism, a potentially lethal event to hypoxic cancer cells. Indeed, studies show 2DG reduces rates of proliferation in some types of cancers both *in vitro* [39] and *in vivo* [40] but concern remains regarding neurotoxicity observed in some pre-clinical models. Nevertheless, 2DG has been used safely overseas in tolerability

studies combining 2DG with conventional radiation therapy in patients with advanced brain cancer [41,42]. These studies, however, have not shown any added benefit for 2DG to overall patient outcomes, although they were not specifically designed to do so. Presently, within the US, ongoing phase I dose-escalation trials are assessing tolerability to 2DG when used as first-line therapy in a variety of advanced cancers [43].

A second non-dietary strategy devised to exploit the glycolytic phenotype is based upon two mechanisms of action [44]: (1.) blocking the conversion of glucose to G6P (similar to 2DG) and (2.) promoting acidification of solid tumors. As mentioned previously, some cancers overproduce lactate to abnormally high levels. Without a means to export lactate, a cancer cell would not remain viable as the resultant decrease in intracellular pH would hamper enzyme function, ultimately resulting in cell death. Specialized transport proteins called monocarboxylate transporters (MCTs) mediate the export of intracellular lactate in a diffusion limited manner [45]. Pharmacologic blockade of MCTs, via a so-called MCT inhibitor, may therefore result in the accumulation of intracellular lactate, leading ultimately to cell death. Presently, lonidamine is the only clinically tested MCT-inhibitor in cancer. Studies reveal lonidamine does not lead to an increase in survival in women with metastatic breast cancer [46] or in patients with high grade brain cancer [47]. Nevertheless, development of novel small molecule MCT-inhibitors continues and clinical trials testing this class of agents are ongoing in the treatment of cancer.

In summary, non-dietary strategies targeting the glycolytic phenotype remain explored areas as possible therapies for cancer despite lack of success in initial trials. While clinical investigations of these and other non-dietary strategies continue to evolve, exploration of the potential utility of dietary strategies used either as alternatives or in combination to pharmacotherapy should also be explored to arm physicians with further strategies against cancer.

1.5 The Nutritional Epidemiology Of Prostate Cancer

Given the importance of glucose metabolism in cancer biology, it is perhaps not surprising that numerous epidemiologic studies have focused on the relationship between diet and cancer. In particular, the relationship between diet and prostate cancer has been extensively investigated. Early prospective studies revealed that increased fat intake may increase the risk of being diagnosed with prostate cancer [48,49], although subsequent studies failed to confirm this finding [50,51]. Studies assessing intake of protein-rich foods have also proved inconclusive. While overall meat intake has been positively correlated with prostate cancer risk [52], this finding is not universal among all men [53] but rather is observed in African American males [54]. Alternatively, a growing body of evidence reveals that over-cooked or charred meat containing thermally generated heterocyclic amines (PhIPs) causes prostate cancer in an animal model [55]. Indeed some researchers argue PhIPs, and neither protein nor fat, may ultimately explain the positive correlation between meat consumption and prostate cancer risk [56]. The role of

carbohydrate consumption is a recent topic of interest and some studies now suggest that carbohydrate intake is positively associated with risk of developing prostate cancer and may even be more important than intake of fat [57,58]. While future studies are necessary to confirm this finding, scientific evidence supports a potential role for key carbohydrate-responsive hormones of the insulin-like growth factor (IGF) axis in prostate cancer risk that may reveal how greater intake of carbohydrate may increase prostate cancer risk.

1.6 Insulin And Insulin-like Growth Factor In Prostate Cancer

Insulin is a potent mitogen and growth factor for prostate cancer *in vitro* [59,60]. Insulin is thought to exert these effects through receptor mediated activation of the mitogenic PI3-kinase/Akt signaling pathway and concurrent inactivation of the counter-regulatory PTEN signaling pathway [61]. In addition, previous studies reveal a positive correlation between diagnosis of prostate cancer and fasting serum insulin [62] and glucose levels [63]. Confirmatory evidence is also found from epidemiological studies of men with diabetes. Two prospective cohort studies [64,65] and a meta-analysis [66] reveal that men with end-stage diabetes have a decreased risk of prostate cancer, findings consistent with the occurrence of hypo-insulinemia in late-stage disease [67]. As blood glucose is the primary stimulant of insulin secretion, we recently hypothesized that increased carbohydrate consumption would elevate levels of circulating insulin to increase the growth rate of prostate cancer [68].

The insulin homologue, insulin-like growth factor-1 (IGF-1), has also been shown to increase the proliferation rate of prostate cancer *in vitro* [69,70]. IGF-1 also promotes the progression of prostate cancer cells to androgen independence [69], protects such cells from apoptosis [71], and may increase angiogenesis via over-expression of vascular endothelial growth factor (VEGF) [72]. IGF-1 also stimulates the migration of prostate cancer cells, a critical step in metastasis, via the PI3 kinase/Akt pathway [73]. These effects are diminished primarily by the serum binding protein, insulin-like growth factor binding protein-3 (IGFBP-3), which reduces the levels of "free" or bioactive IGF-1 in the circulation [74]. In addition, IGFBP-3 exerts direct receptor-mediated effects in prostate cancer such as reducing the rate of angiogenesis [75] and inducing apoptosis [76]. Epidemiologic studies reveal elevated serum IGF-1 levels are positively correlated to risk of prostate cancer [77,78] and that increased intake of carbohydrate increases serum IGF-1 while decreasing serum IGFBP-3 levels [79].

Overall, the data for insulin and IGF-1 suggest a biochemical basis for a positive correlation between carbohydrate intake and growth of prostate cancer. In light of these findings, we hypothesized that a marked reduction, or even total elimination, of dietary carbohydrate would lead to a reduction in insulin and IGF-1 levels and slow the growth rate of human prostate cancer in a controlled pre-clinical trial [68]. Given that such a regimen would be considered extreme by present day dietary standards we must first address the issues of safety and potential clinical applicability.

1.7 Carbohydrate: A Non-Essential Nutrient

The human body cannot synthesize, either absolutely or in sufficient quantities, nine out of twenty standard amino acids [33]. These so-called essential amino acids must be ingested at adequate levels to sustain protein synthesis necessary for human growth and survival. Humans must also consume a minimum amount of linolenic (ω-3) and linoleic (ω-6) fatty acids to promote the proper development of the nervous system as well as prostaglandin synthesis [33]. In contrast, there is no minimal requirement for carbohydrate intake as the carbon skeletons of amino acids and the glycerol backbone of triglycerides can be converted to glucose via gluconeogenesis [33].

These facts are not merely an extrapolation of findings from feeding studies with animals. Compelling anthropological evidence for the long-term safety of diets containing little to no carbohydrate was first documented in the early 20th century. The Canadian anthropologist, Vilhjalmur Stefansson, chronicled the lifestyle of the Inuit people of Northern Canada and Alaska in his published memoirs "My Life With The Eskimo" [80] and "Not By Bread Alone" [81]. Stefansson noted the Eskimo (which translates as "eater of raw meat") consumed a diet primarily composed of meat and blubber from captured whale, walrus, caribou, seal, polar bear, musk oxen, and fox [80]. Meat was often consumed raw although some preparations required cooking or dipping in oil from whale fat [80]. As the Arctic produced very little edible vegetation, Eskimo consumption of carbohydrate was minimal at best, consisting primarily of seaweed and berries during peak growing seasons [81]. Stefansson noted, however, that some tribes

avoided plant-based foods entirely and consumed them only to avoid the direst of circumstances such as the slaughter of sleigh dogs or cannibalism [81]. During his three expeditions to the Arctic between 1906 and 1918, Stefansson learned about the millennial history of Eskimo dietary practices and observed a healthy semi-nomadic society with a very low prevalence of cancer and heart disease, the so-called "diseases of civilization" [81]. Although epidemiologic studies of Eskimo populations would later confirm these findings [82, 83], the recent encroachment of Western lifestyle influences on Eskimo culture has been blamed for the gradual diminishment of these differences [84].

1.8 Clinical Research On Ketogenic Diets Provides Evidence Of Safety

Shortly after Stefansson's travels, ketogenic diets were tested as an alternative therapy to starvation for epilepsy [85]. Since the time of Hippocrates, patients suffering from severe seizures would often undergo voluntary starvation to reduce the intensity and frequency of seizures [86]. While effective, such therapy was intrinsically limited as whole-body wasting would ultimately ensue. Improvements in the scientific understanding of human metabolism would lead to the discovery that ketogenic diets induced a biochemical state that mimicked starvation without fatal long-term effects. Both starvation and ketogenic diets induced ketosis while only ketogenic diets spared tissue proteins and promoted growth [87]. Patients on a ketogenic diet often experienced a significant reduction in frequency of seizures by over 75%, on average, according to one early study [88]. More recent follow-up studies revealed similar effectiveness that, in

some cases, lasted for up to six years of diet therapy [89, 90]. However, the eventual discovery of anti-convulsant medications relegated ketogenic diets as a secondary option for epileptic patients failing pharmacotherapy [86]. Only recently, in the past five years, have ketogenic diets undergone a resurgence as primary therapy for epilepsy, particularly in pediatric patients [91, 92].

In addition to treating epilepsy, ketogenic diets also served as popular weight loss vehicles since the mid-19th century [93], a use that continues to this day. Numerous renditions of a ketogenic diet for weight loss appeared throughout the middle and late 20th century with arguably the most famous devised by the late Dr. Robert C. Atkins. His best-selling book, *The Atkins Diet Revolution*©, spanned four editions from 1972 to 2003 [94-97] and ignited concerns about potential health hazards resulting from excessive fat consumption. Such attention would ultimately catalyze a new field of research investigating the safety and efficacy of ketogenic diets in physician-supervised settings.

Randomized clinical trials of up to six months in duration revealed a ketogenic diet led to significant weight loss, improvements in glycemic control, and lower cholesterol levels comparable to or better than low fat diets [98-104]. With regard to glycemic control, some studies revealed a ketogenic diet led to a reduction in serum insulin and glucose levels relative to a low-fat diet [98,101,104]. In contrast, 12-month studies revealed that some benefits of a ketogenic diet observed at 6 months, such as reductions in weight loss, total cholesterol, LDL, glucose, and insulin were not observed relative to comparison diets after one year [105-107]. In contrast, these studies did reveal

a sustained reduction in serum triglycerides and hemoglobin A1C as well as an increase in HDL levels in subjects on a ketogenic diet after 12 months. Common side-effects of a ketogenic diet observed in both 6- and 12-month studies included muscle-cramping, constipation, and dehydration, treated adequately by intake of bouillon, fiber supplements, and liquids, respectively [98].

While the body of literature on ketogenic diets and weight loss continues to increase, only two clinical studies using a ketogenic diet to treat cancer have been published thus far. One study involved two female pediatric patients with malignant astrocytoma [108]. The investigators utilized a ketogenic diet to improve nutritional status and quality of life by exploiting the known susceptibility of astrocytoma to carbohydrate deprivation [109]. Both patients consumed a ketogenic diet composed of 60% medium chain triglyceride oils, 10% dietary fat solids, 20% protein, and 10% carbohydrate. The choice of medium chain triglycerides as a primary source of calories was based on previous observations that such fats are more rapidly hydrolyzed than long chain triglycerides and do not require micelle formation for absorption by the digestive tract [110]. These properties would result in a stronger state of ketosis and presumably exert greater protection against cachexia [111]. Both patients were fed the ketogenic diet to provide calories at 120% of recommended daily allowances with vitamin and mineral supplementation as needed. A pre-treatment positron-emission tomography (PET) scan and fasting blood sample were taken upon admission. Within 7 days after treatment both patients became ketotic and were subsequently discharged for weekly follow-up for 8

weeks. A final PET scan and fasting blood chemistries were obtained at the end of the trial.

Fasting blood ketone levels were significantly increased in both patients whereas glucose levels decreased by an average of 15% (10% vs. 20%). Comparison of pre- and post- PET scans revealed a reduction in tumor flouro-deoxyglucose uptake by nearly 22% in each patient. Mild elevations in serum total cholesterol, HDL cholesterol, triglycerides, and free fatty acids were also noted in each patient and both patients also exhibited a significant reduction in LDL cholesterol levels (-27% vs. -17%). The diet was well tolerated and each patient gained weight consistent with growth curves for age and height. One patient exhibited improvements in mood, gait, hand coordination, and speech which led to continuation of diet therapy for one year without progression of disease. The other patient voluntarily discontinued diet therapy at the end of the trial and remained on chemotherapy. The author concluded these results, while preliminary, should merit further investigation of a ketogenic diet for brain cancer although no study has yet been performed.

A second study investigated the use of a ketogenic diet to reduce nitrogen (i.e. protein) losses in patients suffering from cancer cachexia [112]. Five subjects were recruited with histologically diagnosed cancer: two patients had lung cancer, two had gastric cancer, and one had ovarian cancer. At the time of enrollment, each patient had lost at least 30% of pre-illness body weight and the average caloric intake for the group was 1100 kcal per person per day, equivalent to 55% of normal dietary requirements.

Throughout the study, all patients were fed at a constant rate over a 24-hour period via a nasogastric tube for an average caloric infusion of 1700 kcal per day. During days 1-6, patients were administered a standard diet composed of 31% fat, 55% carbohydrate, 14% protein, and balanced for vitamin and mineral content. From days 7-13, patients were administered a ketogenic diet composed of 70% medium chain triglycerides that supplied calories, vitamins, minerals, and protein equivalent to the standard diet. Both diets were well tolerated and all patients became ketotic by day 13. Collection of 24-hour urine was used to estimate nitrogen balance and fasting blood samples were acquired at days 0, 6, 8, 10, and 13 to assess serum chemistries and hormone levels.

Mean group body weights at distinct time points in the study were as follows (mean ± SEM): Day 0 (38.6 kg ± 3.8 kg), Day 6 (38.2 kg ± 3.4 kg), Day 13 (40.5 kg ± 3.4). These data revealed a significant increase of 2.3 kg in average body weight from day 6 to 13 although the extent to which weight gain was due to the ketogenic diet alone could not be determined as the order in which patients were placed on diets was not randomized. Significant reductions in serum glucose (-14%) and lactate (-46%) levels were also noted from days 6 to 13, although no significant differences in insulin levels were observed during this timeframe. A positive nitrogen balance was maintained and whole-body protein turnover was not changed throughout the study (days 1-13), results the authors suggested were likely due to an increase in total calorie intake and not type of diet.

The key strengths of this study included matching of diets for calories, protein, and micronutrients as well as for manner and rate of delivery of feeding. However, the study was insufficiently powered to adequately investigate a ketogenic diet in three types of cancer. Ideally, as in the brain cancer study, the authors would have selected one type of cancer and patients would be randomized to start on either a ketogenic or standard diet. In this manner, the effect of diet sequence on changes in body weight would be removed as a possible confounder.

In conclusion, both clinical studies investigating a ketogenic diet in cancer revealed preliminary evidence for safety and potential efficacy. However, as neither study was sufficiently powered, larger studies are indicated to investigate whether favorable long-term outcomes would occur in a significant percentage of patients. To date there are no published randomized controlled trials examining the effects of a ketogenic diet in the management of cancer.

1.9 The Challenge Of Compliance

Long-term compliance is a significant barrier to achieving sustained effectiveness for most therapies. This is particularly true for a ketogenic diet which represents an extreme diet by most nutritional standards. Therefore, while it is important to demonstrate the theoretical benefits of a ketogenic diet in an experimental setting, it is also important to address the practical issue of dietary compliance in cancer patients, as non-compliance may otherwise mitigate potential clinical benefits.

Most individuals change dietary habits in order to achieve rapid weight loss, a motivation often ensuring short-term but not long-term compliance. In a randomized prospective study of four dietary approaches to achieve weight loss, one-year discontinuation rates were 35-50% with the highest rates noted in the most extreme diet groups (i.e. ketogenic and very low-fat diets) [107]. Not surprisingly, compliance was the best predictor of weight loss for all diets.

With regard to clinical trials utilizing ketogenic diets for weight loss, an accurate assessment of long-term dietary compliance is difficult to obtain. In these studies [98-107], participants were typically monitored for compliance via detection of urinary ketones. While useful as an immediate and short-term measure, urinary ketones do not indicate long-term compliance as subjects may adopt a ketogenic diet for only seven days and still exhibit significant urinary ketosis [97,112]. It is therefore unclear whether differences in outcomes between 6- and 12-month weight loss trials were influenced, at least partially, by poor dietary compliance. More frequent monitoring of study subjects, ideally on a weekly basis, would likely clarify this issue although, admittedly, such aggressive monitoring may itself lead to non-compliance or loss to follow-up.

One arena in which the use of ketogenic diets has been associated with relatively good compliance is the treatment of epilepsy. Indeed, some patients with epilepsy have remained on a ketogenic diet for up to six years and experienced sustained therapeutic benefits throughout this time [90]. Clearly, these patients are motivated to maintain dietary compliance in order to minimize the frequency and intensity of debilitating

seizures. Analogously, it may be argued that men with prostate cancer would view the potential to mitigate disease as sufficient motivation to maintain compliance with a treatment protocol. Powerful evidence for this claim is offered by the extended use of small molecule chemotherapeutic agents which commonly induce severe nausea, vomiting, lethargy, and hair-loss, side-effects not associated with ketogenic diets. While such an argument seems reasonable, caution must nonetheless be exercised as it remains unclear whether diet, in general, provides challenges that render compliance in cancer patients more difficult than chemotherapy despite reductions in side-effect severity. However, as no studies have been published on the issue of dietary compliance in men with prostate cancer, and given the success of 6-month trials for weight loss, we assume that a 6-month timeframe represents a conservative goal for dietary compliance for most men with prostate cancer. The rationale for such a timeframe will be discussed further in a later section of this thesis.

1.10 Pre-Clinical Studies On Ketogenic Diets And Cancer

Ketogenic diets have been used in early pre-clinical studies to relieve cancer-induced cachexia in rodents. Tisdale et al. transplanted pure strain NMR1 mice with tumor fragments of the murine colon cancer (MAC 16) cell line and utilized a ketogenic diet composed of 80% medium chain triglycerides by calorie to successfully reduce the rate of cachexia [113]. In this study, the ketogenic diet was compared to an isocaloric standard chow diet and average body weights of both groups were similar throughout the

study period. After tumor implantation, all mice were fed their respective diets for 25 days and then sacrificed to alleviate suffering as average body weight reduction was greater than 25%. At the time of sacrifice, mice fed the ketogenic diet had greater fat and non-fat carcass mass by 14% and 68%, respectively, relative to the standard chow group. In addition, mice fed the ketogenic diet were more ketotic and had 33% smaller tumors at time of sacrifice than mice fed a standard chow diet. The ketogenic diet group also exhibited a 20% reduction in serum insulin levels relative to the standard chow group although no significant differences in serum glucose levels were noted. These results provided the first evidence that a ketogenic diet may slow the rate of cancer-induced cachexia without promoting tumor growth relative to a standard diet.

A second study by Beck et al. utilized the same tumor model to assess the effects of daily insulin injections (20 units/kg/day) to treat cancer cachexia [114]. In this study, mice were transplanted with cancer and initially fed standard chow diets isocalorically until the occurrence of tumor palpability and loss of body weight indicative of cachexia (within 14-21 days after tumor injection). Mice were then randomized to one of three groups: (1.) mice receiving daily insulin injections and fed a standard chow diet; (2.) mice receiving daily saline injections and fed a standard chow diet; (3.) mice fed an 80% medium chain triglyceride ketogenic diet (as described previously). Groups were fed isocalorically for nine days at which point all mice were sacrificed. In this study, the insulin-treated and ketogenic diet groups possessed greater fat and non-fat carcass mass, by 85% and 15%, respectively, than saline-treated controls. In addition, the insulin-

treated mice grew tumors that were 50% and 100% greater, by dry-weight mass, than tumors from saline-treated and ketogenic diet mice, respectively. There were no differences in serum glucose levels among the groups. These results demonstrated the potent effect of insulin to stimulate an increase in tumor mass and body weight. In addition, this study replicated the key observation, observed previously [113], that a ketogenic diet may reduce cachexia-induced weight loss without promoting tumor growth.

A third study by Fearon et al. utilized a ketogenic diet composed of 70% medium chain triglycerides by calorie to treat cachexia induced by the Walker 256 carcinosarcoma tumor line [115]. Female Wistar rats fed the ketogenic diet did not experience significantly greater weight gain or differences in weight distribution (fat vs. non-fat mass) relative to rats on a standard diet. In addition, tumor size was similar in both groups and rats fed the ketogenic diet had *higher* blood glucose levels despite being ketotic, an observation the authors labeled as "atypical ketosis," without adequate explanation.

Together, these studies re-enforced the importance of insulin on tumor growth rate and the potential effectiveness of a ketogenic diet to counteract cachexia without promoting tumor growth in some cancer models. It remained unclear, however, why Fearon observed an increase in glucose levels in rats fed a ketogenic diet whereas Tisdale noted a decrease in glucose and insulin in a similar group. Whether differences in tumor metabolism (Walker 256 vs. MAC16) or composition of diet (70 % vs. 80 % medium

chain triglycerides) or experimental animal (mouse vs. rat) were responsible for the difference in outcomes is unknown. Further studies investigating the effects of ketogenic diets in cancer cachexia have not been performed.

Pre-clinical studies have also utilized ketogenic diets to control the growth rate of experimental brain cancer. Seyfried et al. utilized a syngeneic orthotopic tumor model in C57BL/6 mice to compare the effects of a ketogenic diet to a low-fat standard diet (LFSD) on the growth rate of CT-2A brain cancer, a highly malignant astrocytoma derived from mouse [116]. The macronutrient content per kilogram of LFSD was 55g fat, 520g carbohydrate, 225g protein, and 45g fiber which delivered 4.4 kcal per gram of intake. In contrast, the ketogenic diet contained 700g fat, 0g carbohydrate, 128g protein, and 109g fiber per kilogram and delivered 7.8 kcal per gram of intake. At day 0 of the experiment, each mouse was housed separately and received a $1mm^3$ tumor fragment via surgical implantation into the cerebral cortex. After implantation, each mouse was randomized to one of four diet groups: LFSD *ad lib* (LFSD-AL), ketogenic diet *ad lib* (KD-AL), LFSD calorie-restricted by 40% relative to LFSD-AL (LFSD-40R), or KD calorie-restricted by 40% relative to LFSD-AL (KD-40R). Mice were fed for 13 days and then sacrificed for tumor harvesting. Fasting blood was collected before tumor implantation and immediately prior to sacrifice.

In this study, both LFSD-AL and KD-AL groups consumed similar amounts of calories although body weight at time of sacrifice was approximately 7% higher for the KD-AL group (it was not reported whether this difference was statistically significant). In

contrast, average body weights for LFSD-40R and KD-40R groups were statistically similar and both groups consumed similar total calories per day. Measurement of tumor dry mass, which avoids variation from edema or hemorrhage, revealed the KD-40R and LFSD-40R groups had reductions in average tumor weight by 80% and 86% relative to KD-AL and LFSD-AL groups, respectively. There were no significant differences in tumor dry mass between *ad lib* groups or between calorie-restricted groups. Linear regression analysis revealed serum glucose and IGF-1 levels at time of sacrifice predicted tumor size.

Overall, this study demonstrated a ketogenic diet did not accelerate the growth of CT-2A tumors in mice relative to a LFSD and that calorie restriction, regardless of diet, slowed the growth rate of CT-2A tumors in mice. Furthermore, calorie restriction, regardless of diet, was associated with decreases in serum glucose and IGF-1 levels. It should be noted that reductions in serum IGF-1 have been shown in previous xenograft models of human prostate [73] and brain cancers [117] although these studies did not investigate a ketogenic diet. Overall, these findings, while intriguing, did not reveal a specific benefit for a ketogenic diet relative to a standard diet.

A major drawback of this study is that diets were not matched for protein or fiber content. Rather, the LFSD contained nearly two times more protein and less than one-half the amount of fiber than the ketogenic diet. In addition, the fat content of the ketogenic diet was derived solely from lard while the fat content in the LFSD was derived from rodent chow, composed of various sources of fat, including lard. Given these facts, it

remains unclear whether the ketogenic diet may have led to alternative outcomes if diets were matched maximally in macronutrient content and composition. As a ketogenic diet must differ from any comparison diet only in carbohydrate and fat content it is unclear why the authors did not minimize compositional differences in order to more accurately compare diets.

A second drawback concerns the short duration of the study. As will be discuss in a later section, our studies indicate at least five weeks are required to detect a statistical difference in tumor volume between a ketogenic diet and comparison diet in a prostate cancer tumor model. While it is uncertain whether such a timeframe would lead to similar results in brain cancer, it is possible that a longer study may have revealed differences in tumor growth rates.

A more recent study by Zhou et al. also compared the effects of a ketogenic diet to standard low fat diet (SLFD) on experimental human brain cancer. In this study, SCID mice were injected subcutaneously with the U87-MG cell line [118], a highly malignant human glioma. After injection, mice were housed singly and fed a SLFD *ad lib* (SLFD-AL) for seven days. Each mouse was then randomized to one of three diet groups: SLFD-AL, ketogenic diet *ad lib* (KD-AL), or a ketogenic diet restricted to 65-70% of calories consumed by KD-AL (KD-R). Mouse survival was tracked until tumor size was greater than 2.5 cm^3 at which time a mouse was fasted for blood collection and sacrificed for tumor harvesting.

Analysis of diet composition in this study revealed the SLFD had a macronutrient content of 55g fat, 520g carbohydrate, 225g protein, and 45g fiber per kilogram of food which delivered 4.4 kcal per gram. In contrast, the ketogenic diet was a commercially prepared formula (KetoCal®) containing 720g fat, 30g carbohydrate, 150g protein, and 0g fiber per kilogram of food and delivered 7.2 kcal per gram. The fat content in KetoCal® was derived exclusively from soybean oil [118]. In contrast, the composition of fat in the SLFD was a mixture of fats derived from standard mouse chow.

The authors observed that KD-R mice lived nearly 14 days longer, on average, than either SLFD-AL or KD-AL mice. In contrast, there was no significant difference in survival between SLFD-AL or KD-AL mice. In addition, KD-R mice had significantly lower glucose levels by nearly 50%, on average, than either SLFD-AL or KD-AL mice, whereas blood glucose levels were statistically similar between SLFD-AL and KD-AL mice. Visualization of micro-vessel density by Factor VIII antibody staining revealed tumors in KD-R mice had fewer blood vessels per high powered field than either SLFD-AL or KD-AL mice, whereas micro-vessel density was statistically similar between SLFD-AL and KD-AL mice.

Overall, this study provided evidence that an *ad lib* ketogenic diet did not accelerate the growth of U87-MG tumors relative to an *ad lib* SLFD. It was unclear, however, whether calorie-restriction in the context of a ketogenic diet or whether calorie-restriction regardless of diet composition was responsible for the diminished micro-vessel density and/or growth rate of U87-MG tumors. In order to appropriately address these

uncertainties, this study required a calorie restricted standard low-fat diet control group to determine whether the ketogenic diet *per se* under conditions of calorie restriction truly accounts for the increase in mouse survival. As mentioned previously, Seyfried et al. observed calorie restriction is sufficient to slow the growth rate of murine experimental brain cancer [116] and thus, without evidence to the contrary, it seems likely this reason would account for tumor growth delay in this study. In addition, diets in this study were not matched for macronutrient content or composition, precluding an optimal comparison of diets.

Overall, pre-clinical studies investigating ketogenic diets as therapy in experimental cancer reveal intriguing although incomplete results. While some studies clearly indicate a role for insulin [114] and IGF-1 [116] in tumor growth, other studies reveal a ketogenic diet may be useful in reducing levels of these potent hormones and thereby slow the growth rate of certain tumors [113,116]. A ketogenic diet seems to selectively promote an increase in body weight while simultaneously slowing tumor growth in certain cancers. It remains to be seen whether this remains true for prostate cancer. Caution must be exercised when extrapolating these results to other tumor models, however, as most of the studies discussed previously suffer from correctable errors in design, particularly in regard to diet composition.

1.11 Research Objectives

The goals of the thesis are to examine the effects of dietary carbohydrate restriction in a murine model of prostate cancer. In particular, we examined the effects of a no carbohydrate ketogenic diet (NCKD) on tumor growth and overall animal survival and whether these parameters are correlated with changes in endocrine parameters and/or intracellular protein and gene expression. The effects of dietary carbohydrate restriction were compared to two separate diets: (1.) a Western-type diet (WD) modeling typical consumption patterns in US males diagnosed with prostate cancer and (2.) a low-fat diet (LFD) representing the present day standard of clinical care. All diets were optimally constructed to minimize confounders in nutrient composition or content. In pursuit of these goals our experimental approach was the following:

Objective 1. Conduct the first ever dietary carbohydrate restriction comparison study comparing the effects of an NCKD to a WD and LFD in a murine xenograft model of human prostate cancer using an androgen-sensitive human prostate cancer cell-line (LAPC-4).

Objective 2. Conduct a second study utilizing an identical experimental protocol and dietary interventions with a different androgen-sensitive human prostate cancer cell line (LNCaP) in order to observe whether total dietary carbohydrate restriction exerts a robust effect in more than one type of hormone–responsive prostate cancer cell line. This

follow-up study would also provide the opportunity to further assess molecular mediators that may account for our observations.

2. Experimental Procedures

2.1 Cell-Based Methods

2.1.1 LAPC-4 Study (Discussed In Chapter 3)

LAPC-4 human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Iscove's modified medium with 10% Fetal Bovine Serum and supplemented with the synthetic androgen R1881 at 1nM. Cells were grown in 5% CO_2 at 37^{o}C and harvested on day 0 by trypsinization at 70-80% confluence in log phase growth.

2.1.2 LNCaP Study (Discussed In Chapter 4)

LNCaP human prostate cancer cells were obtained from the American Type Tissue Culture (Rockville, MD). Cells were maintained in RPMI-1640 modified medium with 10% Fetal Bovine Serum. Cells were incubated in 5% CO_2 at 37^{o}C and harvested by trypsinization at 70-80% confluence in log phase growth on the day of tumor injection (Day 0).

2.2 Animals-Based Methods

2.2.1 LAPC-4 Study (Discussed In Chapter 3)

After obtaining approval from the Johns Hopkins Institutional Animal Care and Use Committees, 3–4-week-old Severe Combined Immuno-deficient (SCID) (CB.17

scid/scid) mice were obtained from Jackson Laboratories (Bar Harbor, ME). We chose a xenograft tumor model utilizing SCID mice based upon previous diet/tumor growth studies demonstrating LAPC-4 as a viable xenograft in such mice [119,120]. We reasoned this choice may yield the additional benefit of permitting comparison of our results with previous studies using the same model to yield a more comprehensive picture of the effects of diet on LAPC-4 tumor biology *in vivo*.

Given the importance of energy balance in modulating tumor growth [121], all animals were housed one mouse per cage to permit the maintenance of isocaloric intake among groups. The diets were prepared by TestDiet (Indianapolis, IN) (Table 1). Assessment of caloric intake, body weight, and feeding of all groups was performed thrice weekly. Initially, a two-week palatability study (without tumor injection) comparing ad libitum intake of all three diets was performed. There were six animals per diet group in this pilot study. Animals fed the low-fat diet (LFD) consumed the least amount of calories. During the xenograft experiment, the LFD mice were fed ad libitum and the other arms were given food in a modified paired feeding protocol to match the average caloric intake per gram of body weight of the LFD group. This approach has been used successfully to provide equivalent energy intake in previous feeding studies [119,120].

On day -24, 25 mice each (total of 75 mice) were randomized to one of three diets (Table 1): LFD (kcals: 12% fat, 15% protein, 73% carbohydrate), WD (kcals: 40% fat, 15% protein, 45% carbohydrate) or NCKD (kcals: 85% fat, 15% protein, 0%

carbohydrate). On day 0, all mice were injected subcutaneously in the flank with 1 X 10^5 LAPC-4 tumor cells in 0.1ml of Matrigel (Becton Dickinson, Franklin Lakes, NJ). When tumors became palpable, the tumor dimensions (in units of mm) were measured using calipers. Tumor volumes were calculated using the formula: (width) x (height) x (length) x 0.5236 [120]. At day -2 and day 35, mice were bled via the facial vein to measure blood glucose using a hand-held Ascensia Contour glucometer (Bayer Healthcare, Tarrytown, NY) and urine was obtained via gentle suprapubic pressure to measure urinary acetoacetate using semi-quantitative urine ketone strips (Ketostix, Bayer Healthcare). When tumors approached 1,000 mm^3 or when the health of mice appeared compromised, mice were sacrificed by CO_2 asphyxiation, serum was obtained via a cardiac puncture, and sections were taken from the liver and fixed overnight in 10% neutral-buffered formalin, embedded in paraffin, and stained with Hematoxylin and Eosin.

Liver sections from a random sample of 11 mice per group were independently scored for fatty infiltration by two board-certified pathologists blinded to dietary assignment using a clinical scoring system based on a standardized ordinal scale: 0 = none; 1 = minimal (sparse, spotty macrovesicular and/or microvesicular steatosis involving <5% of the acinar tissue); 2 = mild (macrovesicular and/or microvesicular steatosis involving 5% to <33% of the acinar tissue); 3 = moderate (macrovesicular and/or microvesicular steatosis involving 33–66% of the acinar tissue); 4 = marked (macrovesicular and/or microvesicular steatosis involving >66% of the acinar tissue) [122].

Serum from the median surviving 11 mice from each group (total 33 mice) was assayed for hormone levels. The levels of murine IGF-I and IGFBP-3 were measured using mouse-specific in-house enzyme-linked immunoassays (ELISA) that have been developed at University of California Los Angeles (UCLA) and described previously [123,124]. Murine insulin, leptin, and glucagon levels were assayed using a multiplex ELISA (Linco Research Inc., St. Louis, MO).

2.2.2 LNCaP Study (Discussed In Chapter 4)

After obtaining approval from the Duke University Institutional Animal Care and Use Committees, 130 eight-week-old, male, Fox Chase SCID mice (strain#: CB17SCRF-M) were obtained from Taconic Farms (Hudson, NY). Given the importance of energy balance in modulating tumor growth [121], mice were housed individually to permit maintenance of precise feeding schedules. All diets were prepared by TestDiet (Indianapolis, IN) (Table 1). Assessment of caloric intake and feeding of all groups was performed thrice weekly. Measurement of body weight and tumor volume was performed twice per week. We previously found that mice on a NCKD have a proclivity to overeat and gain weight when fed ad libitum relative to mice fed either a WD or LFD. Similarly, isocaloric feeding for all three diet groups leads to weight loss in the NCKD group [125]. Based on these observations, we performed an initial eight-week pilot feeding study (without tumor injection) in order to determine the precise amount of excess calories mice fed an NCKD must consume in order to maintain body weights similar to ad libitum

fed LFD mice. We found that allowing NCKD mice to consume 12.5% extra calories relative to ad libitum fed LFD mice resulted in similar body weights. Of note, this degree of excess caloric intake is less than the natural proclivity of these mice to overeat and thus forced feeding was not necessary in order to attain this level of excess caloric intake.

For the xenograft experiment, 41/41/48 mice were randomized to LFD/WD/NCKD groups, respectively. All mice were initially fed ad libitum for eight days on their respective diets to ensure diet tolerability. After this time, LFD mice were fed ad libitum and the other groups were fed by a modified paired-feeding protocol to maintain isocaloric feeding conditions between LFD and WD groups and 12.5% extra calorie intake for the NCKD group relative to the LFD group. Such a protocol has been used in previous studies to implement precise feeding schedules [119,120]. The overall macronutrient composition of each diet was as follows (Table 1): LFD (kcals: 12% fat, 15% protein, 73% carbohydrate), WD (kcals: 40% fat, 15% protein, 45% carbohydrate), and NCKD (kcals: 85% fat, 15% protein, 0% carbohydrate).

After 34 days of modified paired feeding, all mice were injected subcutaneously in the right flank with $1x10^6$ LNCaP tumor cells in 0.1ml of Matrigel™ (Becton Dickinson, Franklin Lakes, NJ). When tumors became palpable, tumor dimensions were measured with a digital caliper and volume was calculated using the formula: width x height x length x 0.5236 [120]. Fifteen days before and 13 days after tumor injection, all mice were bled via the facial vein to measure fasting glucose in whole blood using a hand-held Ascensia Contour glucometer (Bayer Healthcare, Tarrytown, NY) and urine

was obtained via gentle suprapubic pressure to measure urinary acetoacetate using semi-quantitative urine ketone strips (Ketostix, Bayer Healthcare). When tumors reached 1,000mm^3 or greater or when the health of the mouse appeared compromised, mice were sacrificed by CO_2 asphyxiation, serum was obtained via a cardiac puncture, and sections were taken from the liver, prostate, and kidney for either snap freezing or overnight fixation in 10% neutral-buffered formalin, embedded in paraffin, and stained with Hematoxylin and Eosin. Serum samples were snap frozen at -80^oC for further analysis. Immediately prior to sacrifice, all mice were assessed for urine ketone and fasting glucose levels.

Liver sections from a random sample of 11 mice per group were independently scored for fatty infiltration by a board certified pathologist blinded to dietary assignment using a previously validated 0-4 scoring system [122] with severity of fatty infiltration increasing with higher score. All surviving mice were sacrificed 92 days after tumor injection and mice with tumor volumes <1,000mm^3 were censored as alive at last follow-up. Prior to tumor injection, two mice died from dehydration (1 LFD and 1 NCKD), two mice died a result of blood aspiration (1 LFD and 1 NCKD) and one mouse died due to unexplained causes (1 WD). Therefore, 125 mice were used for survival analyses.

Serum from the median surviving 11 mice per group (total 33 mice) was assayed for hormone levels. The levels of fasting IGF-I and IGFBP-1,-2,-3 were measured using a mouse-specific in-house ELISA developed at the University of California Los Angeles and described previously [123,124]. Fasting serum levels of insulin, RANKL, leptin,

ACTH, IL-6, and TNF-α were assayed using a murine multiplex ELISA kit from Millipore Life Sciences Research Inc. (St. Charles, MO).

2.3 Tumor Assays (conducted in LNCaP study only)

2.3.1 Akt Analysis

Tumor samples from the median surviving five mice per diet group were analyzed for intracellular content of serine 473 phospho-Akt (ser473 p-Akt), total Akt (t-Akt), and β-actin. LNCaP tumor lysates were prepared as follows: 1mL of lysis buffer [2% Triton X-100, 300mM sodium chloride, 20mM Tris (pH7.4), 2mM EDTA, 0.5mM sodium orthovanadate, 1% Nonidet P40, Protease inhibitor cocktail (Complete tablets, Roche Applied Sciences, Indianapolis, IN)] was added to thawed tissue samples (~10mg). Samples were then homogenized for 10 seconds in ice and centrifuged at 14,000 *g* for 50 minutes at 4°C. Protein concentration in the supernatant fraction was determined and extracts were stored at -80°C. Protein bands were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblots were developed with enhanced chemiluminescence (ECL) Plus reagent (Amersham Pharmacia, Piscataway, NJ). Antibodies to ser473 p-Akt, t-Akt, and β-actin were obtained from Cell Signaling Technology (Danvers, MA). Protein bands were quantified by densitometric analysis.

2.3.2 RNA Isolation

RNA was extracted from 0.05 µg of tumor tissue from the median surviving 11 mice per group using the mirVana mRNA Isolation Kit (Ambion) and assessed for integrity (RNA 6000 Nano assay Kit, Agilent Technologies) and quantified (NanoDrop, Thermal Fisher Scientific). The targets for Affymetrix DNA microarray analysis were prepared according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was produced by *in vitro* transcription, fragmented and subsequently hybridized to the Affymetrix High Throughput Human Genome U133A array. All subsequent analyses were performed in a MIAME (minimal information about a microarray experiment)-compliant fashion, as defined in the guidelines established by MGED (www.mged.org). Tumors from two mice in the LFD and one mouse in the NCKD group had unusable RNA resulting in thirty examined samples (LDF=9, WD=11, NKCD=10).

2.3.3 Gene Set Enrichment Analysis

GSEA v2.0 (http://www.broad.mit.edu/gsea) was performed for each xenograft sample based on pre-defined diet phenotype and previously published methods [13]. Gene sets were preprocessed to exclude gene sets with <10 and >500 genes and one thousand iterations were performed per analysis with a signal to noise metric used to rank genes based upon their differential expression across groups.

2.4 Statistical Methods

Identical statistical methods were used for both tumor-growth studies. Comparisons among diet groups were performed using the non-parametric Kruskal-Wallis test for continuous variables and chi-square (χ^2) test for categorical variables. Two-way comparisons were performed using the non-parametric Mann-Whitney test for continuous variables and χ^2 test for categorical variables. Survival was compared among the groups using the log-rank test and the Cox proportional hazards model. All statistical analyses were performed using STATA 9.2 (Stata Corp., College Station, TX) with an α level equal to 0.05. The α level was not adjusted for multiple comparisons.

3. Dietary Carbohydrate Restriction Extends Overall Survival In A LAPC-4 Tumor Murine Xenograft Model

3.1 Introduction

Prostate cancer is the most common non-cutaneous cancer diagnosed in men in Western society and is the second leading cause of cancer-related death in men over 50 years of age [126]. It is thought that lifestyle plays a central role in these trends given the comparatively reduced rates of incidence and mortality in Asian men in native nations relative to Asian men in Western nations. Diet is one aspect of lifestyle that clearly distinguishes Western from traditional Asian societies and has been explored as a potential risk factor for prostate cancer. In particular, the role of dietary fat in prostate cancer risk has been extensively investigated although epidemiologic studies have yielded unclear results thus far [48-51]. In contrast, the role of dietary carbohydrate in overall prostate cancer risk remains largely unexplored. Given the potent stimulatory effects exerted by insulin [59-63] and IGF axis hormones [69-74] on prostate cancer growth, and given the well established mechanism of carbohydrate-stimulated insulin secretion by pancreatic β cells, as well as evidence that carbohydrate restriction may reduce IGF-1 levels [79], we hypothesized that a diet devoid of carbohydrate would slow prostate tumor growth and thereby prolong overall survival in mice consuming such a diet. We tested this hypothesis in a pre-clinical trial comparing the effects of a no-carbohydrate ketogenic diet (NCKD) to two other diets: (1.) a typical high-fat/high-

carbohydrate diet reflecting consumption patterns of men in Western society (WD) and (2.) a low-fat/high-carbohydrate diet (LFD) previously shown in pre-clinical studies to reduce prostate tumor growth and increase overall survival [119,120].

3.2 Results

3.2.1 Diet Composition & Comparison

Inspection of Table 1 reveals the overall components of each diet. A comparison of diets reveals equivalence with regard to every intake variable with the exception of fat and carbohydrate. In particular, all diets are equivalent in weight per calorie density for protein, vitamins, minerals, fiber (including cellulose), and essential nutrients (choline, DL-methionine, cholesterol, and amino acids). Thus one-calorie of food from each diet contains an equivalent amount of protein, micronutrients, and essential nutrients. In contrast, the total amount of fat per calorie of food is different for each diet. However, the relative composition of total fat is equivalent in all diets at a ratio of 10:10:1 for lard:milk:corn-oil fat, respectively. Similarly, total carbohydrate, while different in absolute amount for both the WD and LFD, is composed in a 7:3.5:1 ratio of sucrose:maltodextrin-10:dextrin in both diets. By constructing the diets in this manner, we assured that the relative contribution to total caloric intake for a given type of fat or carbohydrate was identical across all diets (with the exception, of course, of carbohydrate in the NCKD). All diets were synthesized from purified ingredients in order to optimize homogeneity between food batches thereby maximizing accurate measurement of

nutrient and overall calorie intake. In summary, these diets were carefully constructed to minimize confounding variables in nutrient composition in order to accurately assess the effects of carbohydrate deprivation on tumor growth in our experimental models.

3.2.2 Pilot Feeding Study

Prior to conducting our first xenograft study, we conducted a pilot feeding study to assess safety and tolerability of the NCDK. Given that "standard" mouse chow primarily consists of solid grains, we were initially concerned whether mice would even tolerate an NCKD having a pasty texture and composed of nearly 85% of calories from fat. Such a pilot study would also provide preliminary data on ad libitum consumption rates that would be used a benchmark to anticipate feeding behaviors in subsequent xenograft studies. A sixteen-day pilot study utilizing six individually housed mice per diet group revealed mice consuming an NCKD consumed approximately 15% more calories per day and experienced more weight gain relative to mice consuming either a LFD or WD. The NCKD mice thrived and did not exhibit any behavioral abnormalities, confirming the safety and tolerability of the diet. As mice fed the LFD consumed the fewest calories, the LFD group served as the control group in a modified paired feeding protocol in our xenograft study.

3.2.3 Calorie Intake & Body Weight

In our first xenograft study, we housed one mouse per cage and fed and weighed all mice three times per week using a modified paired feeding protocol. In this feeding protocol, all LFD mice (N=25) consumed *ad lib* and both WD (N=25) and NCKD (N=25) mice were fed an equal amount of calories as the LFD mice on a per unit body weight basis. All mice were started on their respective diets on the first day of the study (day -25). The day of tumor injection was considered day 0.

Although similar average body weights were maintained initially, the NCKD mice began to decrease their rate of weight gain after 14 days of feeding (Figure 1A). In retrospect, weight loss resulting from an NCKD is not surprising as this effect has been observed in numerous clinical trials [98-104]. However, prior to our study, we did not know whether a similar effect would be observed in mice. Given that we could not simultaneously control for calorie intake and body weight, we decided to control for body weight for the remainder of the study as excess body weight has been positively correlated with risk of aggressive prostate cancer [127]. We thus began to over-feed the NCKD group during the two weeks prior to scheduled tumor injection (days -14 to 0) (Figure 1B). Despite these initial attempts at overfeeding, NCKD mice remained approximately two grams lighter (nearly 10% of average body weight) than either LFD (Mann-Whitney, p=0.002) or WD (Mann-Whitney, p=0.02) mice at time of tumor injection (day 0). We therefore continued to aggressively over-feed NCKD mice (in some cases, by as much as 50% more calories) for another 4 weeks in order to re-establish

statistically similar body weights among all groups. By day 25, average body weights across all groups were statistically similar (Kruskall-Wallis, p=0.96). Following the feeding adjustment period, average body weights remained statistically similar and relatively stable among all groups for the remainder of the study (Figure 1A). Overall, the NCKD mice consumed a median 9% more calories throughout the study than either LFD or WD mice.

3.2.4 Serum Glucose & Urine Acetoacetate

We measured non-fasting glucose in whole blood and urinary ketones two days prior to tumor injection (day -2) and five weeks after tumor injection (day 35). Surprisingly, non-fasting glucose for the NCKD group was higher than both the LFD and WD groups on both days -2 (Kruskall-Wallis, p=0.03) (Figure 2A) and 35 (Kruskall-Wallis, p=0.03) (Figure 2B). Although consistent with an increase in calorie intake, an elevated blood glucose level in the NCKD group was nonetheless surprising given the complete absence of carbohydrate consumption. Ultimately, this observation likely reflects the remarkable potency of gluconeogenic mechanisms to compensate for the most extreme reductions in dietary carbohydrate intake. However, as blood was not collected under imposed fasting conditions for any group, it is not known whether comparisons between groups are entirely valid in this regard.

A significant elevation in urinary ketones was observed in the NCKD group relative to the LFD and WD groups on day -2 (Kruskall-Wallis, $p<0.001$) (Figure 2C)

and on day 35 (Kruskall-Wallis, $p<0.001$) (Figure 2D). This ketotic response was consistent with observations in previous pre-clinical studies [113-116,118]. In light of the initial weight loss and the occurrence of ketosis in the NCKD group, we were confident that our mice were responding appropriately to the NCKD and that our study would test the effect(s), if any, of dietary carbohydrate restriction and/or ketosis on LAPC-4 cell tumor growth.

3.2.5 Tumor Growth Rate & Overall Mouse Survival

After twenty-five days of controlled feeding, each mouse received a subcutaneous injection of $1x10^6$ LAPC-4 cells in Matrigel™ in the upper right flank. All mice developed tumors and growth was monitored three times per week. Pre-specified criteria for animal sacrifice were the following: (1.) tumor volume of $1cm^3$ or greater or (2.) clear behavioral signs of suffering (e.g. persistent lethargy, cachexia, failure to groom, or ruffled fur). Ultimately, no mouse exhibited signs of suffering during the experiment and therefore all mice were sacrificed on the basis of tumor size. Tumor growth kinetics were similar for all groups during the early phases of the study (Figure 3). In particular, both the average time to develop a palpable tumor (day 27) and the initial volume of a palpable tumor were similar for all three groups. However, median tumor volume at day 41 and day 51 was smaller in NCKD mice relative to WD mice by 18% (Mann-Whitney, $p=0.05$) and 33% (Mann-Whitney, $p=0.009$), respectively. In contrast, there were no

differences in median tumor volume between NCKD and LFD or between LFD and WD mice at any time point during the study.

Overall survival data were consistent with the tumor growth rate data. When comparing survival curves for all groups (Figure 4), we observed a significant association between diet and overall survival (Kruskall-Wallis, p=0.03). In particular, the NCKD group was associated with longer survival, by an average of two weeks, when compared to the WD group. This increase equated to a 23% extension in median survival from the time of tumor injection for NCKD mice relative to WD mice. This association was sustained even after adjusting for differences in body weight during the time of tumor injection (Hazard Ratio=0.48, 95% CI [0.27-0.86], p=0.01). In contrast, comparisons between the NCKD and LFD groups and the LFD and WD groups revealed no association between diet and overall survival even after adjustments for body weight.

3.2.6 Insulin & IGF

We evaluated the levels of the following hormones in fasting serum collected at the time of sacrifice: insulin, IGF-1, IGFBP-3, leptin, glucagon, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6). Overall serum insulin levels differed among diet groups (Kruskall-Wallis, p=0.01) (Figure 5A). In particular, the NCKD group exhibited 75% lower median levels of insulin relative to the WD group. IGF-1 levels also differed among diet groups (Kruskall-Wallis, p=0.03) (Figure 5B) with both NCKD and LFD groups exhibiting ~20% lower IGF-1 levels than the WD group. In addition, IGFBP-3

levels differed among diet groups (Kruskall-Wallis, p=0.02) (Figure 5C) and with higher levels in the WD and NCKD groups by 40% relative to the LFD group. Lastly, we evaluated the IGF-1:IGFBP-3 ratio, a measure of the "free" or "bioactive" fraction of circulating IGF-1. We found the IGF-1:IGFBP-3 ratio also differed significantly among groups (Kruskall-Wallis, p=0.03) (Figure 5D) and was lowest for the NCKD group by 35% and 36% relative to the WD and LFD groups, respectively. This finding, in conjunction with low serum insulin levels, is consistent with the slowest rate of tumor growth observed in the NCKD group. There were no statistically significant differences among groups with regard to serum levels of leptin, glucagon, TNF-α, or IL-6.

3.2.7 Hepatic Steatosis

The extremely high fat content of the NCKD raised concerns about induction of hepatic steatosis, i.e. fatty liver, in mice fed this diet. We therefore commissioned two board certified pathologists, each blinded to dietary group, to independently examine histologic sections of livers from a random sample of 12 mice per group and score them according to a standard 0 to 4 point clinical scale with increased severity of disease corresponding to a higher score. The average of both scores for each liver was calculated and used to determine a group average.

This analysis revealed the NCKD group exhibited the least amount of fatty liver (Kruskal-Wallis, p=0.01) (Figure 5E) on average by a full clinical grade lower than the WD group and one-half clinical grade less than the LFD group. These results, while

initially surprising, are consistent with a previous study showing rats fed high-saturated fat diets did not develop fatty liver [128]. In addition, a six month pilot study examining the use of a low-carbohydrate ketogenic diet ($\leq$ 20 g/day) to treat patients with non-alcoholic fatty liver disease [129] also revealed significant, biopsy-confirmed improvements in hepatic steatosis and inflammation. In particular, liver biopsies obtained before and after dietary treatment revealed significant improvements in fatty liver scores in 4 out of 5 subjects.

3.2.8 Urine Acetoacetate Predicts Overall Survival In The NCKD Group

As the NCKD group experienced the longest overall survival time, we analyzed whether levels of urinary ketones correlated with longer survival times in mice within the NCKD group. Using a conservative cutoff value of greater than or equal to 40 mg/dL of urine acetoacetate to denote a high level of urinary ketosis and controlling for serum glucose and body weight, we found that mice with high urinary ketone levels at day 35 experienced the longest survival relative to mice with low urinary ketone levels at the same time point. In particular, a mouse with low urinary ketone levels was more than five times as likely to die on any given day than a mouse with high urinary ketone levels (Cox hazard ratio = 0.19). Despite the occurrence of ketosis in a minority of mice from the WD and LFD groups at the same time point (Figure 2D), we did not observe a similar correlation between survival and urinary ketone levels within these two groups.

3.3 Discussion

We conducted this initial xenograft study to test the hypothesis that an NCKD would slow prostate tumor growth relative to a WD and consequently lead to an increase in overall animal survival. In addition, we included a LFD group to assess whether an NCKD would be comparable to, if not better, than the clinical standard of care. Our results show that mice fed the NCKD had slower growing tumors and longer survival times than mice fed a WD. In addition, mice fed an NCKD experienced similar survival times relative to mice fed a LFD, although a trend in favor of longer survival for the NCKD group was noted (log-rank, p=0.2). Lastly, subgroup analysis of the NCKD group revealed mice with a greater level of urinary ketosis experienced the longest survival times. We believe these data provide strong provisional evidence for a direct relationship between dietary carbohydrate restriction and prolonged survival in mice bearing prostate cancer tumors.

Our results, while intriguing, do not explain whether an NCKD slows tumor growth primarily via induction of ketosis or through hormonal changes. In our study, NCKD mice had the lowest levels of serum insulin and bioactive IGF-1. These findings would be predicted to slow tumor growth and extend survival as insulin and IGF-1 are potent prostate cancer growth factors [59-63,69-74] and have been linked epidemiologically with prostate cancer risk [64-67,77,78].

One possible mechanism accounting for the growth kinetics of tumors in our study involves a decrease in glucose delivery to tumor cells. In this scenario, elevated

levels of circulating insulin, as observed in the WD group, would promote a greater degree of glucose flux within cancer cells thereby stimulating tumor viability and growth. In contrast, lower levels of circulating insulin, as observed in the LFD and NCKD groups, would reduce glucose flux into tumor cells. Such a mechanism may explain, at least partly, why the NKCD mice had slower growing tumors despite paradoxically exhibiting the highest levels of circulating glucose. In essence, the excess endogenous glucose in NCKD mice may have been sequestered in the circulation by low levels of insulin. Further study is needed to confirm or disprove this hypothesis.

A major challenge in our study was to maintain similar average body weights for all diet groups. Ideally, during a feeding study, calorie intake and body weight should exhibit a positive linear relationship. While this is true for most diets, ketogenic diets can induce an osmotic diuresis resulting from urinary ketosis. Such water loss, in conjunction with calorie loss in the form of urinary ketones, would result in an underestimation of calories necessary to maintain average body weight, invalidating the assumption of linearity between calorie intake and body weight. A further complication stems from our surprising observation that ketosis and diuresis are not universal responses to a KD. In fact, within a group of genetically identical animals the majority will develop ketosis readily although some will be either refractory to ketosis or undergo ketosis only after extended fasting. Ultimately, continuous daily monitoring would be necessary to correct for individual responses to diet. However, with over 70 singly housed animals in a barrier facility, such an effort would be difficult at best, if not impossible, without implementing

cost-prohibitive measures such as metabolic cages. As a result, we were ultimately faced with the choice of equating calorie intake or body weight. We chose body weight based on the strength of a previous clinical study correlating total body weight and aggressiveness of prostate cancer [127].

In our xenograft study, despite the consumption of identical calories among all groups during the pre-tumor injection phase, the NCKD group gradually lost body weight relative to the LFD and WD groups. This weight loss required a modification in feeding protocol. NCKD mice were subsequently overfed in an effort to re-establish statistically similar body weights among all groups. Ultimately, these modifications resulted in an average increase of 9% calorie consumption per day during the entire experimental timeframe for the NCKD group relative to the LFD group. At the time of tumor injection (day 0) NCKD mice had the lowest average body weight and were consuming more calories than LFD and WD mice. In light of this fact, it may be argued that a lower body weight exerted protective effects against tumor growth in NCKD mice thereby biasing the study in favor of the NCKD. Alternatively, tumor growth may have been stimulated by the increase in calorie intake for nearly the first month after tumor injection as NCKD mice experienced a rapid rate of weight gain during early stages of tumor growth. Whether either effect occurred in this study is ultimately unknown but justification for either scenario seems plausible. In our statistical analyses, we controlled for body weight differences at the time of tumor injection and yet still observed a statistically significant extension in animal survival in mice consuming the NCKD.

Our observation that both LFD and NCKD groups experienced similar survival deserves comment. Both groups exhibited marked reductions in serum insulin while only the NCKD group also had a significantly lower IGF-1:IGFBP-3 ratio. This latter finding may reflect an additive effect on improved survival mediated by insulin and bioactive IGF-1. In particular, the additional reduction in IGF-1 may account for the observed trend toward longer survival by the NCKD group relative to the LFD group (log-rank, $p=0.2$).

Lastly, our results seem to indicate that the effect of diet to slow prostate tumor growth may follow a bimodal distribution. In particular, at a fixed intake level of calorie and protein, the amount of dietary carbohydrate must vary inversely with the amount of dietary fat. Our study shows that very low or very high levels of dietary carbohydrate intake may slow prostate tumor growth whereas intermediate levels of dietary carbohydrate may promote tumor growth. One possible explanation for this observation is that diets balanced in carbohydrate and fat (in particular, saturated fat in our study) may fuel intermediary metabolism primarily with carbohydrate thereby sparing saturated fats to mediate pro-carcinogenic effects, e.g. membrane anchoring of inositol phosphates critical to PI3-kinase activation and mitogenic Akt signaling. In contrast, unbalanced diets provide either small amounts of fats (LFD) thereby limiting their role in mitogenic signaling or no carbohydrate (NCKD) thereby committing the majority of dietary fat to ATP synthesis.

In conclusion, we found that an NCKD significantly extends survival in mice bearing human prostate cancer tumors relative to a WD. We further observed an NCKD

is equivalent in this regard to a LFD. While encouraged by these results, we were concerned whether they reflected an inherent characteristic of LAPC-4 tumors or whether an NKCD would exert such effects in other experimental models of human prostate cancer. We therefore conducted a larger follow-up study to assess whether an NKCD would lead to similar results in a second human prostate cancer cell line. In addition, this study would provide the opportunity to further examine the molecular underpinnings of how an NCKD exerts a protective effect against prostate cancer.

4. Carbohydrate Restriction Extends Overall Survival In A LNCaP Xenograft Model

4.1 Introduction

Insulin and insulin-like growth factor-1 (IGF-1) are potent mitogens for prostate cancer that exert pleiotropic effects including increasing proliferation rate, promoting androgen independence, and preventing apoptosis [59,69]. Epidemiologic studies have supported these observations by showing elevated serum levels of either insulin [62] or IGF-1[77] increase prostate cancer risk. Therefore strategies aimed at reducing serum levels of these hormones may be of therapeutic benefit and merit critical investigation.

We previously showed dietary carbohydrate restriction is one possible approach to reduce serum insulin and IGF-1 levels [125]. In particular, we tested whether a diet devoid of carbohydrates, that is, a no-carbohydrate ketogenic diet (NCKD), would extend survival in a prostate cancer xenograft model when compared to a diet reflecting consumption patterns of males in Western society, i.e. a Western diet (WD) [125]. We also included a low-fat diet (LFD) in our prior study to compare our intervention to the clinical standard-of-care for cancer patients. Ultimately we found an NCKD led to slower prostate tumor growth and prolonged overall survival relative to a WD. In addition, an NCKD was at least equal to a LFD in these parameters. In particular, our study showed an NCKD was associated with reductions in serum insulin and the IGF-1:IGFBP-3 ratio, results predictive of increased survival. However, mice fed the NCKD in our prior study

lost body weight relative to comparison groups prior to tumor injection and thus whether prolongation of survival was driven by energy restriction or carbohydrate restriction remained unclear.

Similar results have been reported by others, including a recent study investigating carbohydrate restriction in prostate cancer xenografts that found a positive association between carbohydrate intake and serum insulin levels [130]. In that prior study, mice on a low-carbohydrate diet experienced slower tumor growth, although these mice also consumed fewer calories and had lower average body weights than mice fed a high-carbohydrate/high-fat diet. Carbohydrate restriction has also been used in xenograft models of experimental brain cancer and was shown to reduce serum insulin and IGF-1 levels, although, again, in the setting of calorie restriction and/or weight loss [116].

To date, no study has shown whether carbohydrate restriction, in the absence of weight loss, reduces serum insulin and IGF-1 levels and whether such changes slow tumor growth and extend overall survival relative to a high-fat/high-carbohydrate diet and/or a low-fat diet. We therefore sought to test the robustness of an NCKD to slow tumor growth in another experimental prostate cancer model and to also assess a broader array of molecular mediators that may underlie our initial observations. We conducted a second xenograft study using identical diets as described previously [125] and the canonical hormone-sensitive prostate cancer cell line, LNCaP, to test the effect of an NCKD on tumor growth rate and overall animal survival.

4.2 Results

4.2.1 Calorie Intake & Body Weight

Results from a prior pilot feeding study indicated NCKD mice should consume 12.5% extra calories relative to ad libitum fed LFD mice in order to maintain equal body weights among all diet groups prior to the day of tumor injection (day 0 in our study). However, after 12 days of initial feeding (day -30), NCKD mice became heavier than LFD mice (Mann-Whitney, $p<0.03$) (Figure 6). Therefore, on day -24, modified paired feeding was adjusted to supply NCKD mice with only 10% extra calories relative to LFD mice. This feeding schedule was maintained throughout the remainder of the study. At two days prior to tumor injection, the average body weight of the NCKD group was 2.3g (9.3%) and 1.7g (6.7%) greater than the LFD and WD groups, respectively (Kruskal-Wallis, $p<0.0001$). After day 19, despite being fed 10% extra calories, NCKD mice lost weight such that by day 36 (first day of sacrifice), body weights were similar between NCKD and LFD groups, although both groups were heavier than the WD group by 1.5g (6.4%) (Kruskal-Wallis, $p=0.04$). Body weights subsequently normalized and were similar among groups at the conclusion of the study.

4.2.2 Glucose & Ketone Measurements

There were no significant differences in fasting blood glucose levels among diet groups prior to tumor injection (day -15) (Kruskal-Wallis, $p=0.70$) (Figure 7A) or after tumor injection (day 13) (Kruskal-Wallis, $p=0.14$) (Figure 7B). Non-fasting urine

acetoacetate levels at day 19 were greater than 3-fold higher in the NCKD group relative to LFD and WD groups (Figure 7C) (Kruskal-Wallis, $p<0.0001$).

4.2.3 Tumor Growth & Survival

At 92 days after tumor injection, overall tumor take was 94% with no significant differences among groups. Seven mice failed to develop tumors (3 LFD, 1 WD, and 3 NCKD). Time to tumor palpability (median 20 days) (log-rank, p=0.93) and tumor volumes at the time of initial palpability (Kruskal-Wallis, p=0.98) were similar among groups. By day 35, median tumor volumes for LFD/WD/NCKD groups were $0mm^3/530mm^3/146mm^3$, respectively (LFD vs. WD, p=0.001; NCKD vs. WD, p=0.04) (Figure 8). Tumor volumes in the WD remained significantly larger than LFD and NCKD tumors for the remainder of the study. With the exception of day 39, when tumor volumes of the LFD group were significantly smaller than the NCKD group (Mann-Whitney, p=0.03), there were no significant differences in tumor volumes between these groups during the study.

Diet group was significantly associated with overall survival (log-rank, p=0.004) (Figure 9). Relative to the WD, survival was significantly prolonged for both the LFD (hazard ratio=0.50, 95% CI 0.29-0.79, p=0.005) and NCKD (hazard ratio=0.59, 95% CI 0.37-0.93, p=0.02). There was no significant difference in survival between LFD and NCKD groups (Mann-Whitney, p=0.46). At the time of sacrifice, tumor volumes were similar across all groups (Kruskal-Wallis, p=0.74).

4.2.4 Serum Insulin & IGF Axis Hormone Levels

Overall, serum insulin levels differed significantly among diet groups (Kruskal-Wallis, p=0.002) (Figure 10A) with the WD group having median levels that were 85% and 52% higher than the LFD and NCKD groups, respectively. Serum IGF-1 levels also differed significantly among diet groups (Kruskal-Wallis, p=0.02) (Figure 10B) with the highest median levels in the LFD group by 25% and 67% relative to WD and NCKD groups, respectively. Although median serum IGFBP-3 levels did not differ significantly among groups (Kruskal-Wallis, p=0.06) (Figure 10C), the IGF-1:IGFBP-3 ratio, a measure of "free" or bioactive IGF-1, was significantly different among groups (Kruskal-Wallis, p=0.02) (Figure 10D). The NCKD group had the lowest median IGF-1:IGFBP-3 ratio, which was nearly 25% lower than either LFD or WD groups. Serum IGFBP-1 levels also differed significantly among groups (Kruskal-Wallis, p=0.004) (Figure 10E) with the NKCD group having the highest median levels by 16- and 4-fold relative to LFD and WD groups, respectively. In addition, the IGF-1:IGFBP-1 ratio, a measure of "free" IGF-1 levels under fasting conditions, differed significantly among groups (Kruskal-Wallis, p=0.004), with the LFD group having the highest median value by 17-fold (Mann-Whitney, p=0.01) and 3-fold (Mann-Whitney, p=0.004) relative to the NCKD and WD groups, respectively (Figure 10F). We also observed a trend, which did not reach statistical significance, for the NCDK group to have a lower IGF-1:IGFBP-1 ratio than the WD group (Mann-Whitney, p=0.08). Similarly, serum IGFBP-2 levels differed significantly among groups (Mann-Whitney, p=0.004) (Figure 10G) with the NCKD

group having median levels that were 25% and 14% higher than the LFD and WD groups, respectively. There were no significant differences among groups in serum levels of RANKL, leptin, ACTH, IL-6, and TNF-α.

4.2.5 Tumor Protein Analysis

Western blot analysis of individual tumor extracts revealed significant differences in the ser473 p-Akt:t-Akt ratio (Kruskal-Wallis, p=0.04),a fractional measure of activated Akt protein. Paired comparisons revealed the NCKD group had a significantly lower average ser473 p-Akt:t-Akt value than the WD group (Mann-Whitney, p=0.009), but was similar to the LFD group (Mann-Whitney, p=0.60). There was a trend, which did not reach statistical significance, for the LFD group to have a lower average ser473 p-Akt:t-Akt level than WD mice (Mann-Whitney, p=0.08). Data from pooled tumor extracts (N=5 per group) are shown in Figure 11 for illustrative purposes.

4.2.6 Gene Expression Analysis

Gene set enrichment analysis revealed a diverse group of pathways significantly enriched across different diet phenotypes. A comparison between WD and NCKD groups revealed 26 gene sets were significantly enriched in the WD group and 67 in the NCKD group. Eleven gene sets were significantly enriched in the WD group relative to the LFD group and 74 pathways were enriched in the LFD group. Comparison of the LFD and NCKD groups revealed 35 pathways significantly enriched in the LFD group and 14 in

the NCKD group. Pathway analysis revealed greater inflammatory pathway activation with increasing carbohydrate intake. In addition, gene pathways implicated in obesity, insulin resistance and inflammation were significantly enriched in the WD group relative to the NCKD group, whereas pathways implicated in the MAP kinase cascade and NF-κB activation were enriched in the WD group relative to the LFD group.

4.2.7 Hepatic Steatosis

Given concerns that intake of a high-fat NCKD diet may lead to the development of hepatic steatosis, i.e. fatty liver, we assessed for evidence of hepatic steatosis in histologic sections of livers from the median 11 surviving mice from all groups. Overall, diet was significantly associated with extent of hepatic steatosis (Figure 12), with mice in the NCKD group having the *least* amount of fatty infiltration by a median grade of 1.5 points lower than LFD or WD groups (Kruskal-Wallis, p=0.0006).

4.3 Discussion

We previously showed a NCKD slows tumor growth relative to a WD in a murine xenograft model using the hormone-sensitive prostate cancer cell line, LAPC-4 [125]. In that study, slowing of prostate cancer tumor growth by an NCKD was associated with a marked reduction in serum levels of insulin and the IGF-1:IGFBP-3 ratio. However, in that study, NCKD mice lost weight prior to tumor injection and thus whether these results were due to overall energy restriction or specifically due to carbohydrate restriction was

unclear. Similar results have been reported by other laboratories, although always in the context of calorie restriction and/or weight loss [116,130].

While intriguing, our findings required further testing in the setting of no energy restriction given concerns that the observed benefits may have been due to energy restriction and not carbohydrate restriction alone. Moreover, the very high fat content of a NCKD may nonetheless stimulate growth in other prostate cancer models [119,120,131]. This concern was particularly relevant to our formulation of an NCKD, which is primarily composed of saturated fats derived from milk and lard for an overall composition of 84% fat by calorie. Therefore we sought to further examine the effects of the same three diets used previously (i.e. NCKD, LFD, and WD) on the growth of a more commonly used prostate cancer cell line, LNCaP. Furthermore, we sought to better elucidate the molecular mechanisms through which diet influences prostate tumor growth.

The LNCaP cell line used in this study offers numerous advantages when testing the role of an NCKD on prostate tumor growth. First, the LNCaP cell line is a canonical model of hormone responsive prostate cancer, potentially broadening the applicability of our results to other investigations using this cell line. Second, the LNCaP cell line represents an ideal control to the LAPC-4 cell line used in our prior study as both cell lines share key characteristics: (1) LAPC-4 is also utilized as a model for hormone responsive prostate cancer; (2) both tumors undergo stimulated growth in response to increased dietary fat intake [14]; (3) both cell lines have increased *in vitro* cell

proliferation in response to insulin [132] and IGF-1 [69]. In light of these similarities, we hypothesized the current study would generalize our results and confirm the benefits of carbohydrate restriction in the absence of energy restriction, both of which we felt were necessary prior to proceeding with human clinical trials investigating dietary carbohydrate restriction and prostate cancer.

Maintaining similar average body weights among all groups posed a challenge. We previously observed an isocaloric feeding schedule for all groups led to weight loss in the NCKD group relative to LFD and WD groups [125]. In contrast, the LFD and WD groups maintained similar average body weights when fed isocalorically. In light of these prior observations, we conducted a pilot feeding study (without tumor injection) prior to the onset of this study to determine the percentage of excess calories necessary to maintain similar average body weights between the LFD and NCKD groups. Given the proclivity of mice fed an NCKD to overeat relative to mice fed an LFD, our pilot study revealed the NCKD group required an excess of 12.5% calories per day relative to the LFD group in order to maintain similar body weight between both groups. While using this information to guide our feeding protocol, we nonetheless observed the NCKD group remained consistently heavier on average than the other two groups, even after reducing the daily calorie supplement early in the study from 12.5% to 10%. Our experience highlights the complexities of replicating animal feeding behaviors, which may be dependent on genetic and/or environmental factors difficult to control in successive

experiments. This is particularly challenging when testing more than two diets as the potential for variation between groups is greater than in paired feeding settings.

Diet group was significantly associated with overall survival with both LFD and NCKD groups experiencing significantly longer survival and reduced risk of death than the WD group. The survival benefit in NCKD mice was accompanied by favorable changes in both serum levels of key IGF axis hormones and in IGF axis signaling within the tumor as measured by the downstream marker p-Akt. This suggests reduced IGF axis signaling may underscore the observed survival benefit of an NCKD. Although survival was similarly prolonged in the LFD group, we saw fewer significant changes in the serum IGF axis and less reduction in p-Akt levels, suggesting the LFD may have growth inhibitory affects which are less strongly mediated via IGF signaling. Alternatively, IGF mediated growth inhibition by an NCKD may be, in part, neutralized by the very high fat content of the diet or by the excess body weight observed in NCKD mice in this study.

In our prior study comparing these three diets, there was a suggestion that an NCKD prolonged survival relative to a LFD, although this did not reach statistical significance (HR=0.71, p=0.24). Therefore, we powered the current study to detect a 29% prolongation of survival, which required 50% more mice per group than in our prior study. Despite these efforts we did not observe a significant difference in survival between the LFD and NCKD groups in the present study. It is noteworthy that NCKD mice were significantly heavier, on average, than LFD mice during the majority of the experimental timeframe. Given that a reduction in body weight may slow tumor growth

[121,133], this may have biased our results against the NCKD, which may explain the lack of difference in tumor growth and/or survival between LFD and NCKD groups. Alternatively, it is possible that dietary fat may stimulate growth of LNCaP xenografts to a greater extent than LAPC-4 thereby partially negating the superior benefits of reduced IGF-1 signaling resulting from the NCKD. Regardless, in both the current and prior study, we found no evidence that a LFD, the current accepted gold standard for cancer patients, was superior to a NCKD. Whether similar results would be obtained in human studies remains unknown.

We previously noted significant reductions in serum insulin and IGF-1 in both LFD and NCKD groups relative to a WD. Similarly, in the current study, serum insulin levels were significantly reduced for LFD and NCKD groups relative to the WD group. In contrast to our prior study, however, serum IGF-1 was highest in the LFD mice, which approached significance relative to the WD (Mann-Whitney, $p=0.07$) and was significantly higher than NCKD mice (Mann-Whitney, $p=0.01$). Thus, the benefits of a LFD on the IGF axis appears to be less pronounced and mixed in the current study compared to clearer benefits observed previously [125]. On the contrary, the NCKD resulted in significant reductions in insulin, IGF-1, and the IGF-1:IGFBP3 ratio relative to the WD in both the current and prior study. Of note, in our prior study, IGFBP-1, and -2 were not measured as mice were not fasted at the time of sacrifice. In the current study, mice were fasted for a minimum of 3 hours prior to sacrifice, permitting accurate measurement of multiple components of the IGF axis. We found IGFBP-1 and -2 levels

were significantly higher in the NKCD group relative to both LFD and WD groups. Furthermore, the IGF-1:IGFBP-1 levels, a marker of IGF axis activity under fasting conditions was lowest in the NCKD, thus, further supporting the benefits of a NCKD-mediated reduction on IGF axis. Ultimately, these favorable changes in serum IGF axis hormones and insulin levels resulted in a significant reduction in p-Akt:t-Akt ratios in the NCKD mice relative to the WD mice.

To better explore the complex molecular mechanisms through which our diets may affect cancer growth, we also performed gene expression arrays in tumors from mice from each diet group. Our expression analysis showed pathways related to insulin resistance, obesity, and anti-apoptosis were upregulated in tumors from WD mice relative to NCKD and LFD mice. In particular, NF-κB and MAP-kinase pathways were up-regulated in WD tumors. One possible mechanism that may account for our observed survival outcomes involves NF-kB activity. Although NF-kB is normally expressed at low levels in LNCaP cells [134] an increase in NF-kB would activate anti-apoptotic pathways. In addition, elevations in serum insulin levels may stimulate tumor growth rate by activating MAP-kinase pathways [135]. Further increases in MAP-kinase signaling by elevated serum levels of bioactive IGF-1 found in WD mice may synergize with insulin signaling through the MAP-kinase pathway to further promote LNCaP tumor growth [135]. In contrast, as LFD and NCKD mice did not exhibit elevated serum levels of insulin nor increased expression of anti-apoptotic pathways, mice in these diet groups

would be predicted to have comparatively slower growing tumors, consistent with our observations.

Lastly, assessment of liver status in mice from each diet group revealed NCKD mice showed the least amount of fatty infiltration, results consistent with our previous study [125]. Similar benefits from carbohydrate restriction were also observed in a previous study assessing patients with non-alcoholic fatty liver disease [129]. In that study, four of five patients showed histological improvements in extent of fatty infiltration, inflammation, and fibrosis after six-months of dietary carbohydrate restriction. Further benefits for these patients included marked weight loss as well as favorable changes in key parameters such as HDL, LDL, and triglycerides, indicating trends toward reduced cardiovascular risk. Indeed, as mentioned previously, numerous clinical trials have shown carbohydrate restriction leads to sustained weight loss for up to one year and beneficial changes in serum lipoproteins such as HDL, total cholesterol, and triglycerides [98,102-106]. In looking forward toward humans studies of carbohydrate restriction in prostate cancer patients, it remains possible that, in the absence of weight gain in the NCKD group and noting the significant weight loss in humans on carbohydrate-restricted diets, clinical trials may demonstrate an even greater benefit of carbohydrate restriction than observed in our preclinical studies to date.

One primary limitation of our present study is that mice were randomized to different diets *prior* to tumor injection. Typically, lifestyle modifications, including dietary changes, are employed after cancer diagnosis. We choose to use the current model

to maximize the time mice were on the diet and thus demonstrate proof-of-concept prior to conducting a post-injection experimental model. A second limitation of this study concerns the use of a diet completely devoid of carbohydrate, which may not be feasible in a clinical setting. Alternatively, further research may determine an upper limit of carbohydrate intake that preserves tumor inhibitory effects associated with carbohydrate restriction. Lastly, our formulation of an NCKD may pose practical challenges given its high saturated fat content primarily derived from milk and lard. While we purposefully formulated the NCKD in this manner to test whether benefits in survival would be observed with a diet composed mostly of saturated fat, future studies will be aimed at testing whether other formulations of an NCKD composed of mixtures of healthier fats that may yield further benefits in survival.

In conclusion, this study demonstrated that an NCKD, in the absence of energy restriction, reduced tumor growth and significantly prolonged survival relative to a WD. In addition, survival in the NCKD group was similar to the LFD group despite greater average body weight and higher median daily caloric intake. These changes in the NCKD were associated with favorable changes in IGF signaling, p-Akt:t-Akt ratio, and reduced expression of inflammatory pathway genes. Despite its very high fat content, the NCKD was well-tolerated and did not cause toxicity. These results provide further support for pursuing clinical trials to test whether carbohydrate restricted diet slows human prostate cancer growth.

5. Perspectives

This thesis provides evidence that restriction of dietary carbohydrate slows prostate tumor growth. While such a dietary strategy has been applied experimentally in other types of cancer, our studies represent the first therapeutic application of dietary carbohydrate restriction in prostate cancer.

While we are greatly encouraged by these results, we must also address the broad limitations of our studies as well as suggest future directions. An open question stemming from our work is whether complete avoidance of dietary carbohydrate is necessary to slow prostate tumor growth. Stated another way, will moderate consumption of carbohydrate also slow prostate tumor growth? While it seems unlikely that *complete* avoidance of dietary carbohydrate would be necessary to slow tumor growth, future studies should examine carbohydrate restricted diets systematically by increasing amounts of carbohydrate, for example at 5 grams increments (5g vs 10g vs 15g or higher), and evaluate the comparative effects of such diets on overall prostate tumor growth. Furthermore, does choice of carbohydrate matter? For example, would simple sugars, such as glucose or fructose, diminish the effect of diet relative to starches and/or fibers by providing a more readily usable form of fuel to cancer cells?

Another issue raised by our studies concerns optimizing the composition of fat contained in an NCKD. As mentioned previously, our version of an NCKD was intentionally constructed to consist of the worst kinds of non-trans fat (e.g. saturated fats

from lard, butter, and milk fat) in order to assess the competing influences of carbohydrate restriction and dietary fat intake to overall prostate tumor growth. Given that we observed a reduction in tumor growth rate despite the intake of significant amounts of harmful dietary fats, a logical next step would be to re-construct the NCKD to include healthier fats that may possibly exert more potent effects on slowing prostate tumor growth. In particular, inclusion of a greater amount of essential poly-unsaturated fats, specifically at a high ω-3 to ω-6 ratio [136] may slow prostate tumor growth to a greater extent than observed in our studies.

In addition to exploring changes in overall composition of diet, future studies should also adopt experimental models of tumor invasion and metastasis as our studies exclusively utilized models of early pre-invasive stages of prostate cancer. In particular, our studies utilized cell lines (LAPC-4 and LNCaP) that grow partly in response to levels of circulating androgens. In contrast, cell lines that have undergone the transition to an androgen-independent state (PC-3, DU145, and 1-LN) are viewed as models of advanced invasive and/or metastatic disease and therefore should be used in future studies.

Given that we did not examine metastasis models of prostate cancer, we must proceed with caution when extrapolating our results to scenarios of advanced stages of disease. In particular, our studies show that dietary carbohydrate restriction alone ***is not a cure*** for prostate cancer but rather slows tumor growth. In light of these observations, we speculate that patients most likely to benefit from carbohydrate restriction therapy may be men with early stage disease who are also candidates for more conventional therapeutic

modalities. For example, men with prostate cancer who are scheduled for surgery could be placed on a carbohydrate restricted diet in order to slow tumor growth during the interim waiting period, which typically spans less than 6 months (and therefore minimizes difficulties in long-term compliance as mentioned previously). A similar scenario is also possible for men scheduled for brachytherapy or cryotherapy. In these instances, the goal is to utilize a carbohydrate restricted diet to slow continued growth and possible invasion of a primary tumor and thereby maximize the chance for a curative outcome by conventional therapies. A carbohydrate restricted diet would be preferred over a low fat diet in these settings because (1.) carbohydrate restricted diets lead to a greater degree of weight than low-fat diets, particularly during the first 6 months of use [98,102-106], which may improve the overall health of the patient as well as improve outcomes of treatment; (2.) carbohydrate restricted diets lead to more favorable changes in critical lipid parameters (HDL, total cholesterol, LDL) than low-fat diets [98, 102-106] which may ultimately reduce the overall risk of future cardiovascular events, presently the most common cause of mortality of men with prostate cancer [137]; (3.) carbohydrate restricted diets lead to greater reductions in serum insulin than low-fat diets [21,138,139], a fact that, as our data suggests, may ultimately lead to comparatively slower tumor growth.

In addition to such benefits, the inclusion of diet to an overall cancer treatment program may improve patient care in ways that may be underappreciated or even forgotten in modern medical practice. Diet, in general, provides a rare opportunity for

patients to assume an active and continuous role in their own therapy. Although the use of diet as therapy has a dubious reputation in the medical and scientific community, likely stemming from the abuse and misuse of information (particularly by the popular media), the existence of rigorous and reproducible data revealing potential therapeutic applications of diet should be examined without bias. With regard to prostate cancer, the use of diet requires active participation by the patient that may be particularly important psychologically by providing attainable goals on a daily and long-term basis. Success, i.e. compliance, in these arenas may improve self-confidence and overall outlook in preparation for more conventional therapies. Such psychological benefits are important and should not be undermined as a minor aspect of overall therapy for any disease, particularly cancer.

Overall, this thesis provides evidence that dietary carbohydrate restriction slows the rate of tumor growth in xenograft models of prostate cancer and therefore may represent a novel form of adjunctive therapy in clinical settings against this devastating disease. Future studies should focus on further unraveling the mechanistic details of our observations as well as optimizing carbohydrate and fat composition to obtain a diet formulation that will, firstly, minimize prostate tumor growth and, secondly, maximize taste.

Appendix A.: Tables & Figures

Table 1. Ingredients of experimental diets*

	LFD		WD		NCKD	
	Grams	% of energy	Grams	% of energy	Grams	% of energy
Fat - total	60.0	12.0	200.3	40.0	422.6	84.3
Corn oil	2.9	0.6	9.5	1.9	20.1	4.0
Milk fat	28.6	5.7	95.4	19.0	201.2	40.2
Lard	28.6	5.7	95.4	19.0	201.2	40.2
Protein	197.1	15.7	197.1	15.7	197.1	15.7
Casein	194.1	15.5	194.1	15.5	194.1	15.5
DL-Methionine	3.0	0.2	3.0	0.2	3.0	0.2
Carbohydrate	815.7	72.3	500.0	44.3	0.0	0.0
Dextrin	81.57	7.2	50.0	4.4	0.0	0.0
Maltodextrin 10	163.14	14.5	100.0	8.9	0.0	0.0
Sucrose	571	50.6	350.0	31.0	0.0	0.0
Cholesterol	1.5	0.0	1.5	0.0	1.5	0.0
AIN-76 mineral mix	35.0	0.0	35.0	0.0	35.0	0.0
AIN-76 vitamin mix	10.0	0.0	10.0	0.0	10.0	0.0
Cellulose	50.0	0.0	50.0	0.0	50.0	0.0
Calcium carbonate	4.0	0.0	4.0	0.0	4.0	0.0
Choline bitartrate	2.0	0.0	2.0	0.0	2.0	0.0
Total grams	1175.4	100.0	1000.0	100.0	722.2	100.0

* based upon amount of food needed to deliver 4509.75 kcal of energy

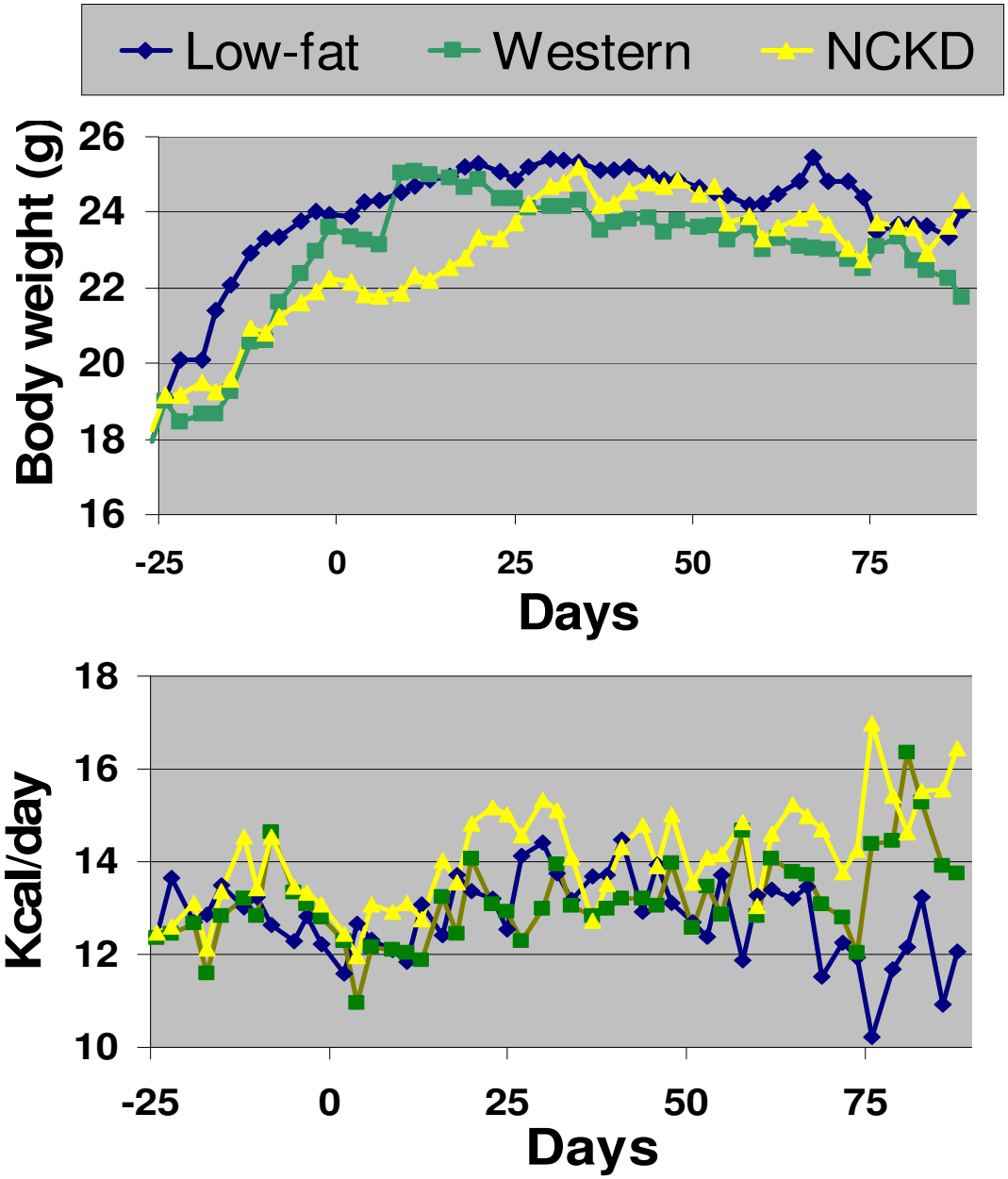

FIGURE 1. (A.) Mouse body weights. 81 eight week-old male SCID mice were fed either a low-fat, Western, or NCKD diet for 25 days and then injected subcutaneously in the flank with 1 x 10^5 LAPC-4 tumor cells in 0.1 ml of Matrigel™(Day 0). Mice were weighed twice per week from the start of the experiment. (B.) Daily energy intake. Food intake was measured for each mouse two times per week by subtracting the weight of uneaten food from the weight of the food placed into feeding receptacles at the start of each feeding period.

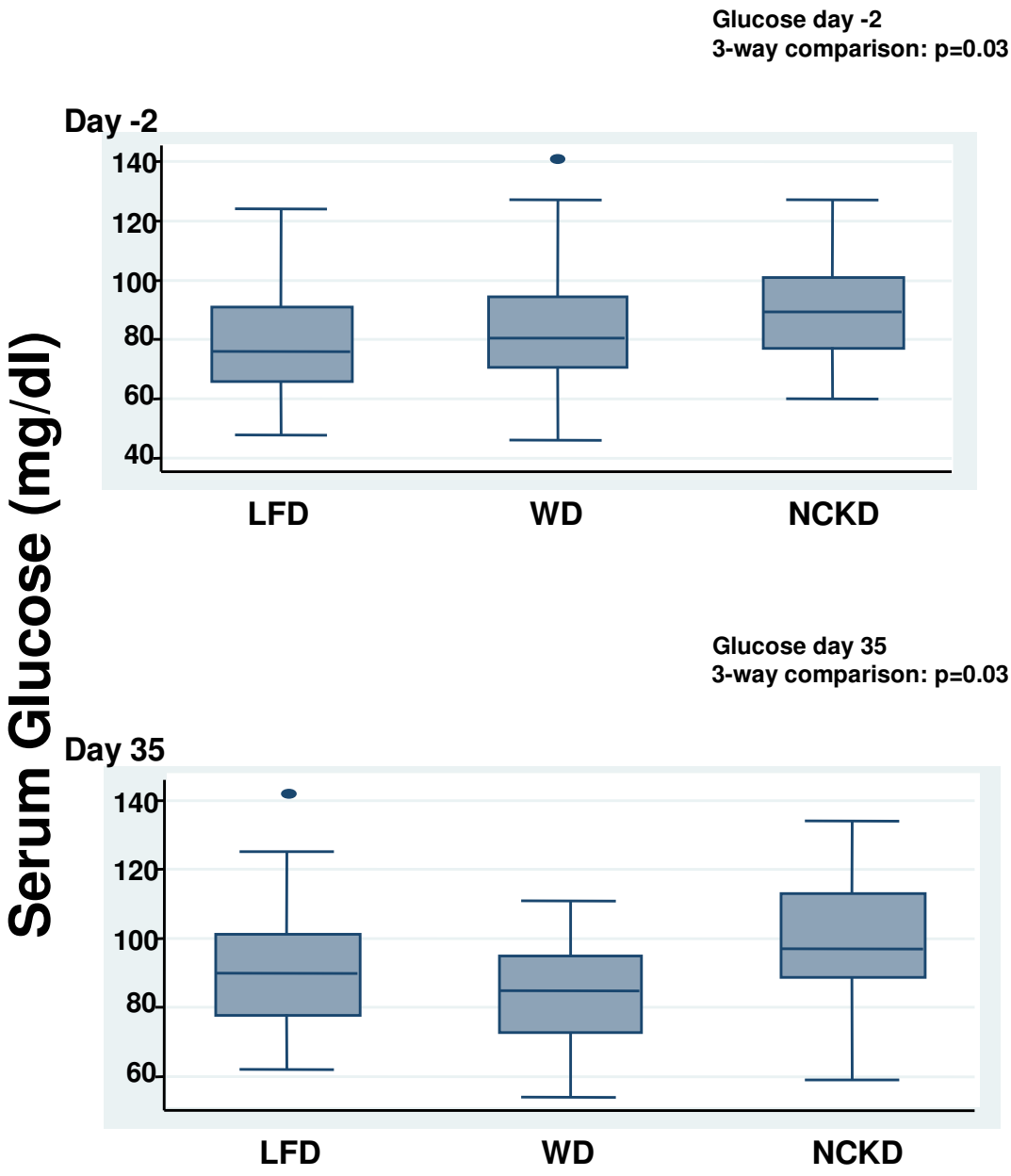

FIGURE 2. Box-plot expression of A) non-fasting serum glucose measured at day -2; B) non-fasting serum glucose measured at day 35. Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Whiskers correspond to 5th and 95th percentile. Dots represent outlier values. Three-way comparisons are performed with the Kruskal-Wallis test.

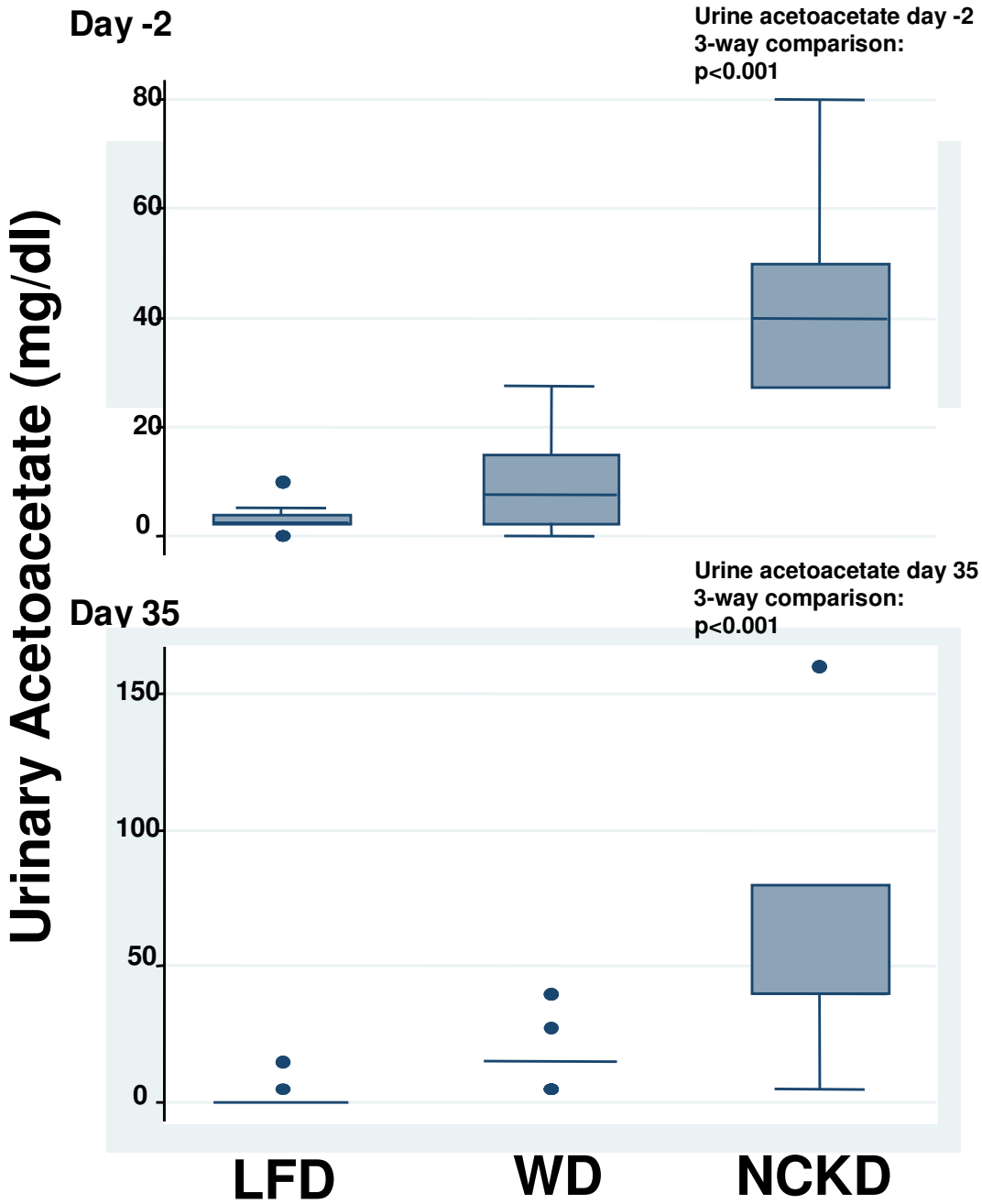

FIGURE 2. Box-plot expression of C) non-fasting urine acetoacetate measured at day -2; D) non-fasting urine acetoacetate measured at day 35. Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Whiskers correspond to 5th and 95th percentile. Dots represent outlier values. Three-way comparisons are performed with the Kruskal-Wallis test.

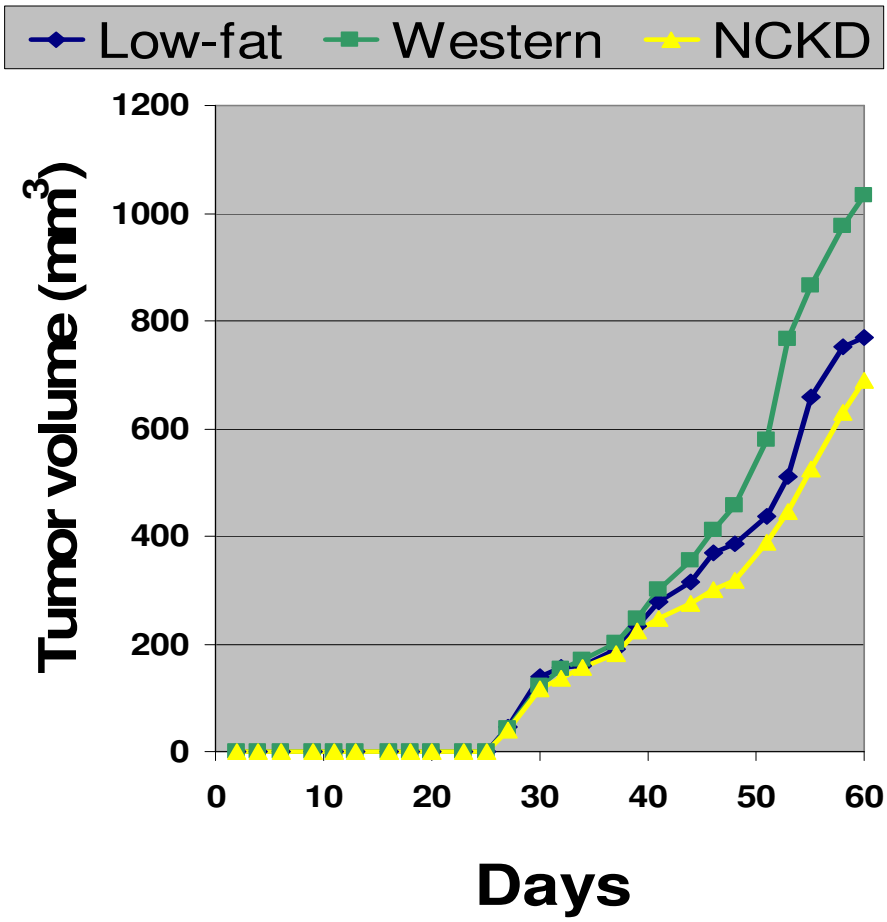

FIGURE 3. LAPC-4 xenograft tumor growth. On Day 0, mice were injected subcutaneously in the upper right flank with 1 x 10^5 LAPC-4 cells suspended in 0.1 ml of Matrigel. Once tumors became palpable, tumor volume was measured twice per week. Values are expressed as the median of each group.

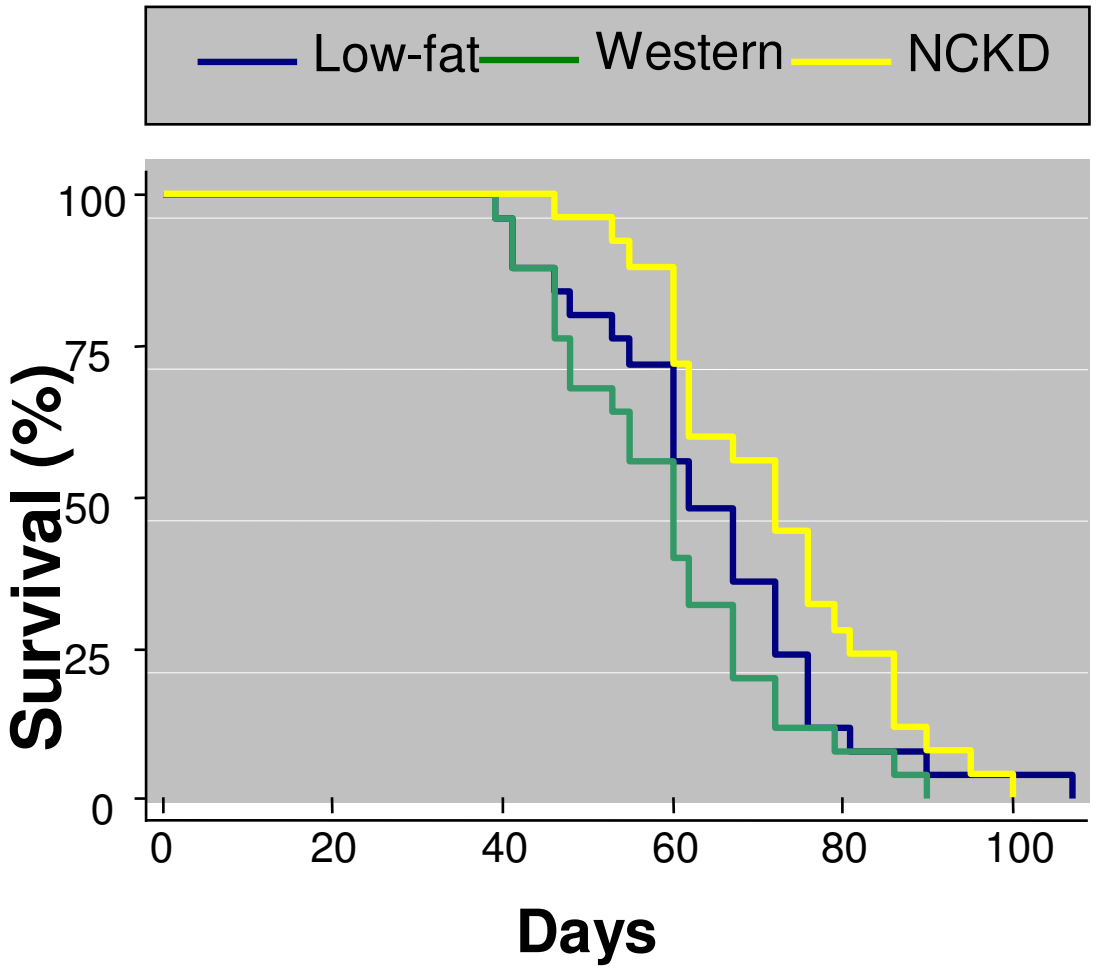

FIGURE 4. Kaplan-Meier survival plot of overall mouse survival by diet group. Vertical axis represents the overall fraction of surviving mice per group. Horizontal axis represents the number of days after tumor injection. Two- and 3-way comparisons are made via log-rank test.

3-way comparison, p=0.03
LFD vs WD, p=0.22
NCKD vs WD, p=0.006
LFD vs NCKD, p=0.20

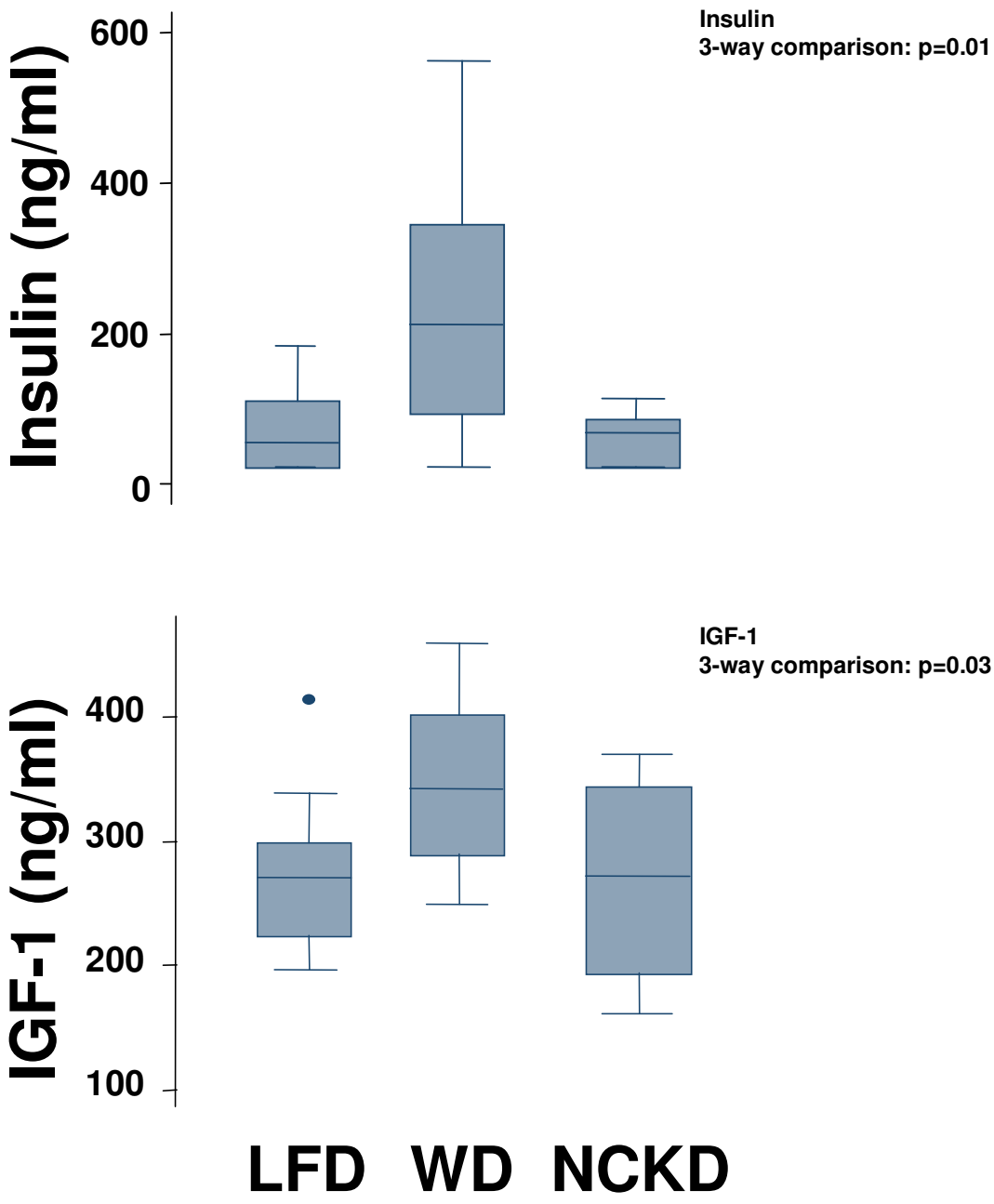

FIGURE 5. Box-plot expression of A.) insulin and B.) IGF-1. Lower and upper boundaries of each box represent 25th and 75th percentile for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 5th and 95th percentile, respectively. Dots represent outlier values. Three-way comparisons are performed with the Kruskal-Wallis test. Values derived from serum obtained from median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

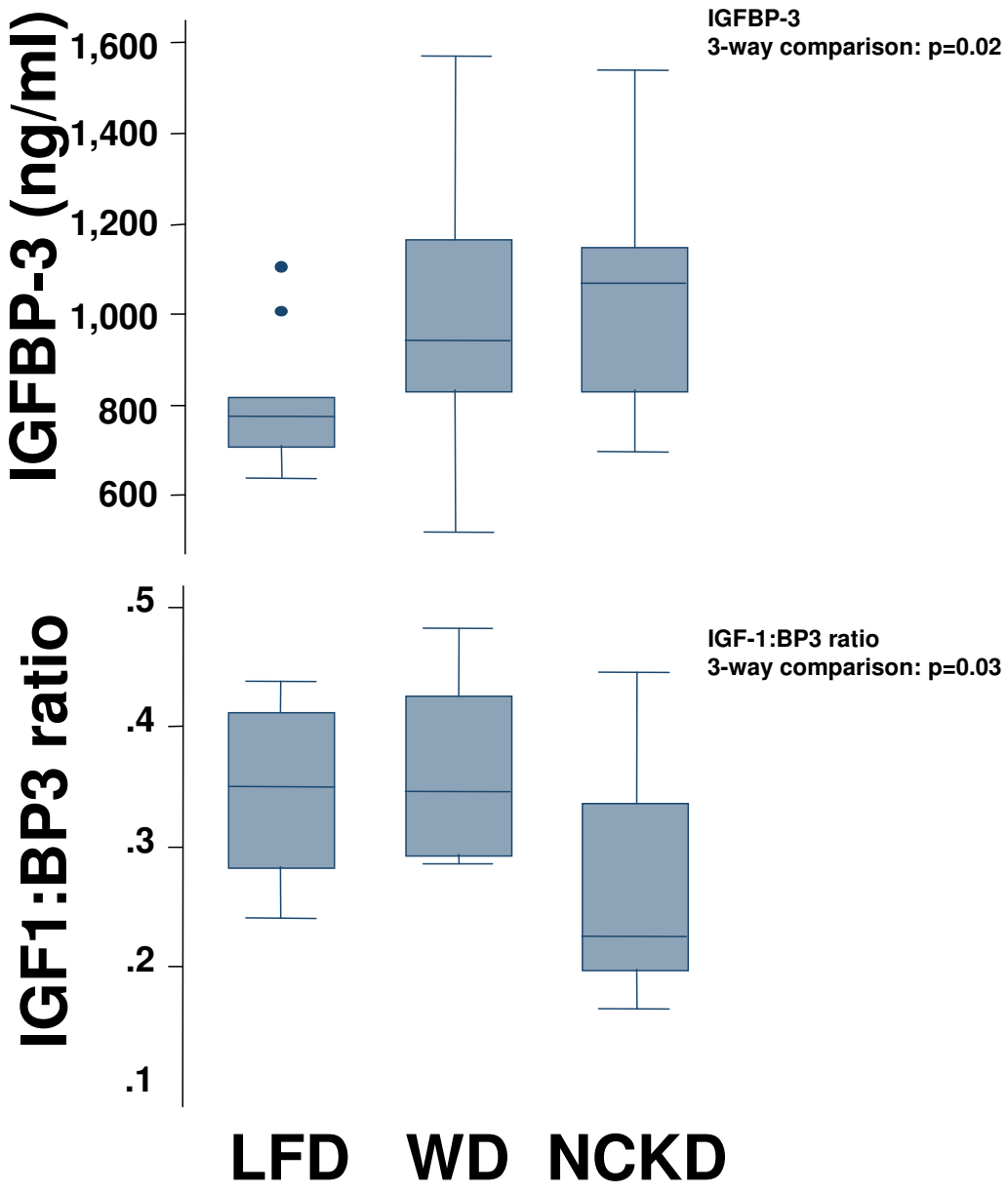

FIGURE 5. Box-plot expression of C.) IGFBP-3 and D.) IGF-1:IGFBP-3 ratio. Lower and upper boundaries of each box represent 25th and 75th percentile for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 5th and 95th percentile, respectively. Dots represent outlier values. Three-way comparisons are performed with the Kruskal-Wallis test. Values derived from serum obtained from median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

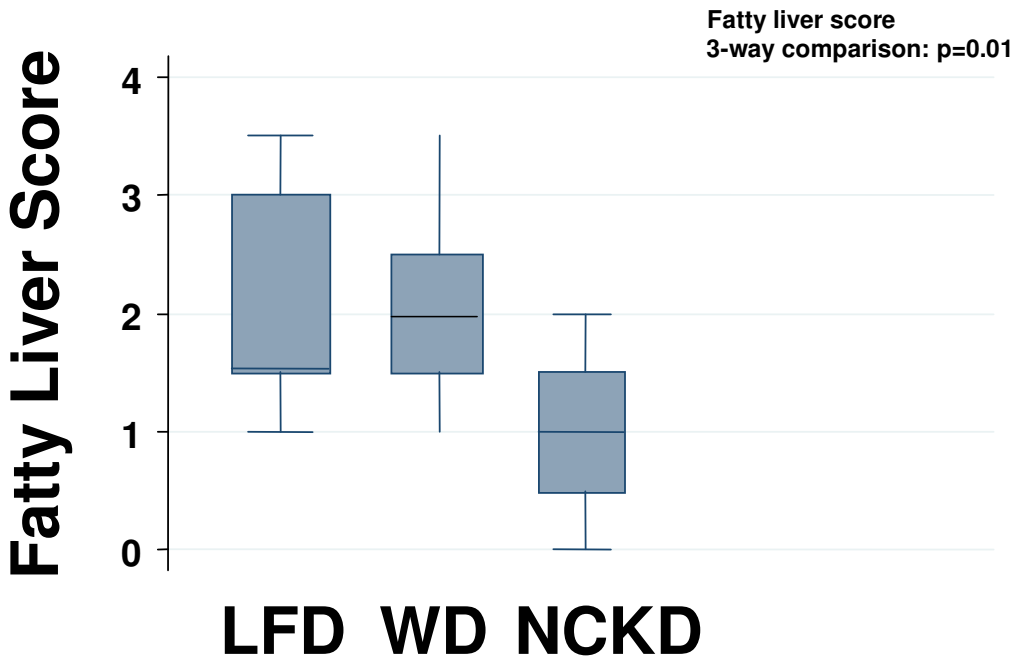

FIGURE 5E. Box-plot expressions of fatty liver score. Lower and upper boundaries of each box represent 25th and 75th percentile for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 5th and 95th percentile, respectively. Three-way comparisons are performed with the Kruskal-Wallis test. Values derived from livers obtained under fasting conditions from median 11 surviving mice in each diet group.

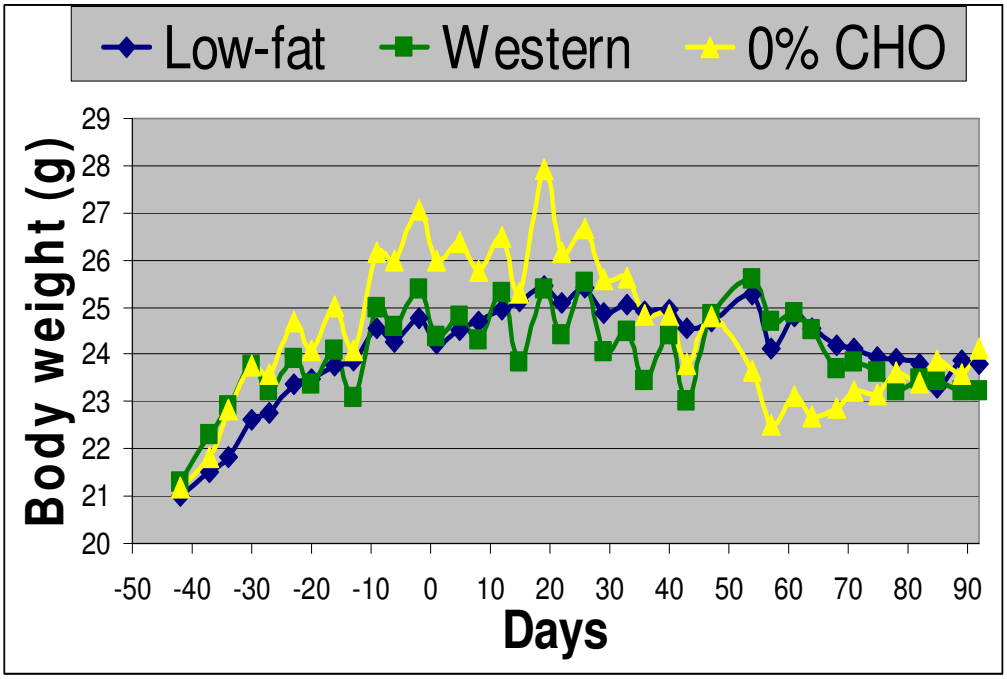

FIGURE 6. Mouse body weights. 130 eight week-old male SCID mice were fed either a low-fat, Western, or NCKD diet for 42 days and then injected subcutaneously in the flank with 1 x 10^6 LNCaP tumor cells in 0.1 ml of Matrigel™(Day 0). Mice were weighed twice per week from the start of the experiment.

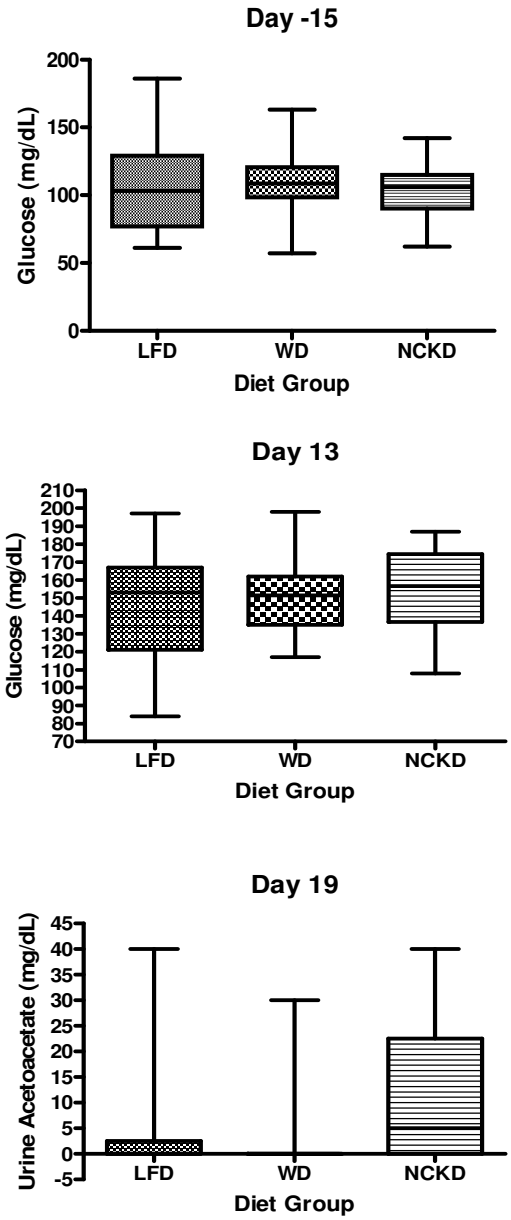

FIGURE 7.

Box-plot expressions.

Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 0-25th and 75-100th percentile ranges, respectively. Three-way comparisons are performed with the Kruskal-Wallis test and paired comparisons are performed with the Mann-Whitney test. Glucose values reflect whole blood obtained from all surviving mice in each diet group under fasting conditions. Urine acetoacetate values obtained under non-fasting conditions.

A) Glucose levels on day -15
3-way comparison: p=0.70
LFD vs WD: p=0.64
NCKD vs WD: p=0.35
LFD vs NCKD: p=0.98

B) Glucose levels on day 13
3-way comparison: p=0.14
LFD vs WD: p=0.70
NCKD vs WD: p=0.11
LFD vs NCKD: p=0.08

C) Urine acetoacetate on day 19
3-way comparison: p<0.0001
LFD vs WD: p=0.72
NCKD vs WD: p<0.0001
LFD vs NCKD: p=0.0002

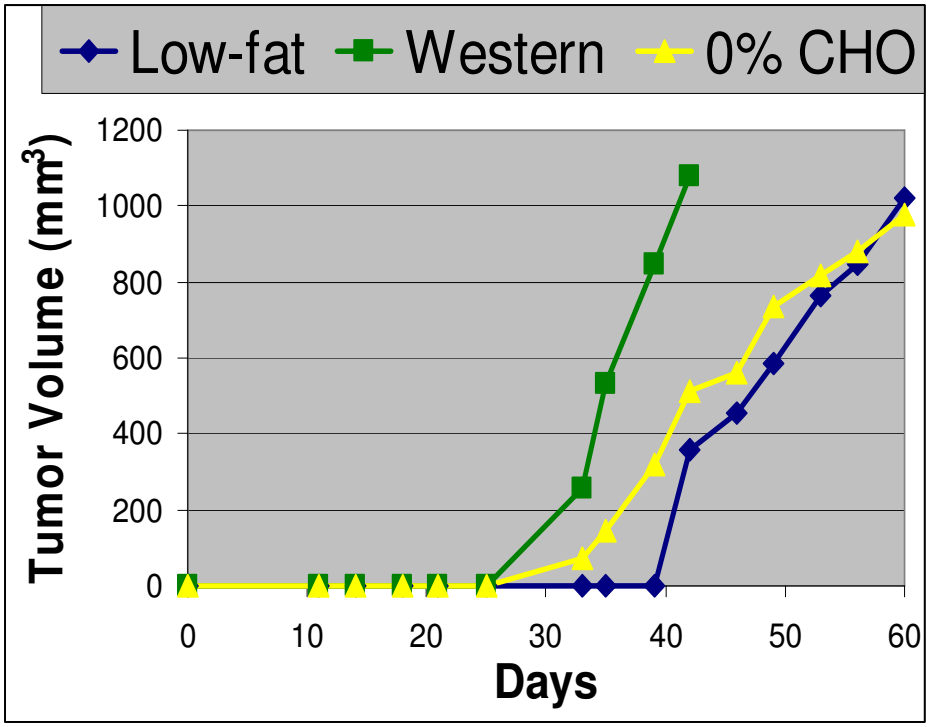

FIGURE 8. LNCaP xenograft tumor growth. On Day 0, mice were injected subcutaneously in the upper right flank with 1 x 10^6 LNCaP cells suspended in 0.1 ml of Matrigel. Once tumors became palpable, tumor volume was measured twice per week. Values are expressed as the median of each group.

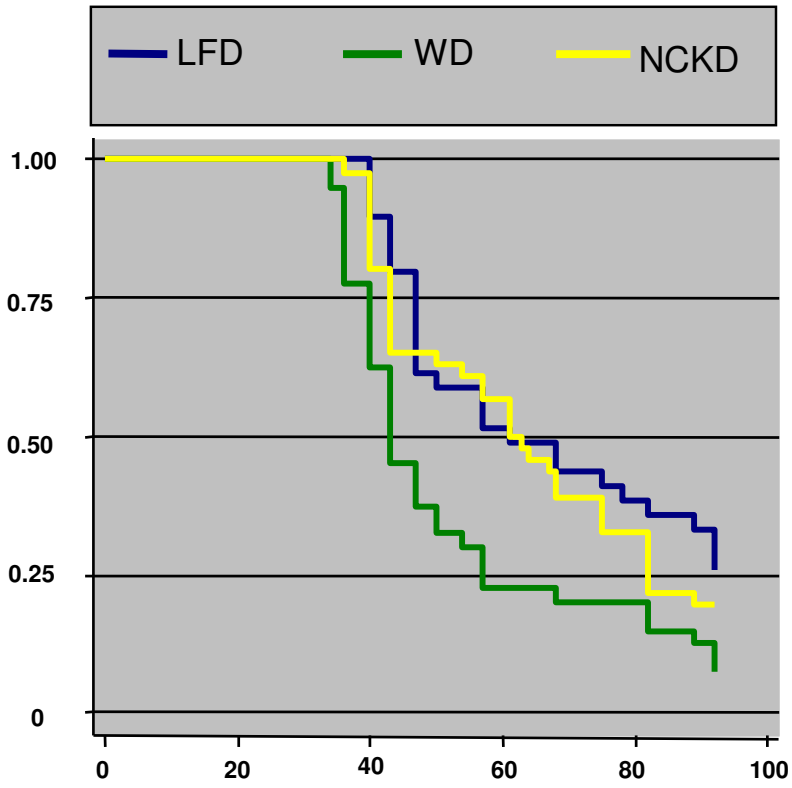

FIGURE 9. Kaplan-Meier survival plot of overall mouse survival by diet group. Vertical axis represents the overall fraction of surviving mice per group. Horizontal axis represents the number of days after tumor injection. Two- and 3-way comparisons are made via log-rank test. Day of tumor injection is day 0.

3-way comparison, p=0.004
LFD vs WD, p=0.002
NCKD vs WD, p=0.007
LFD vs NCKD, p=0.67

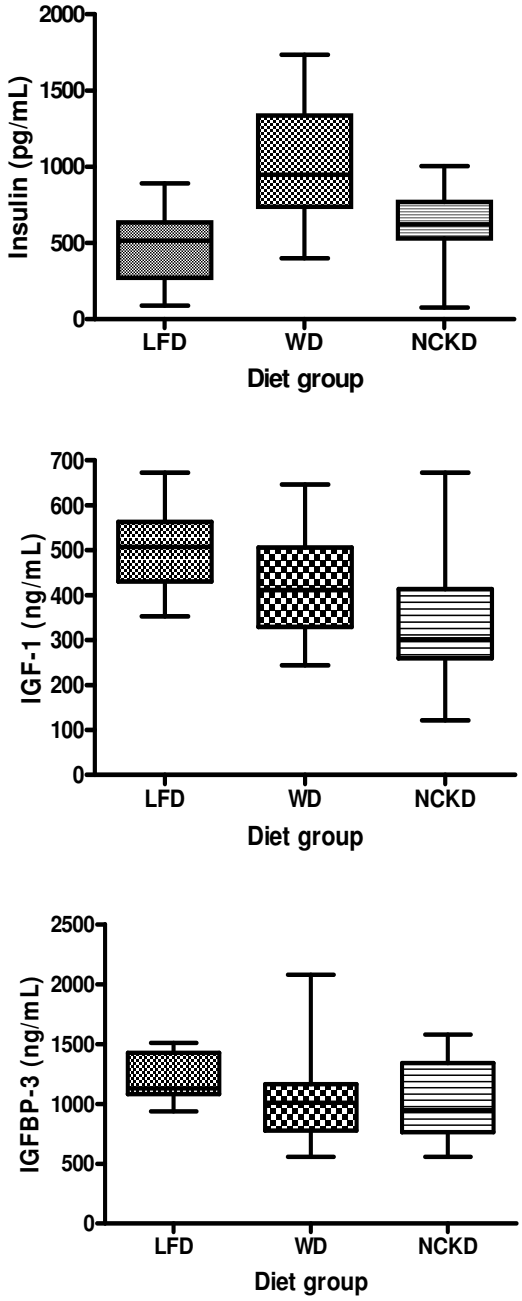

FIGURE 10.

Box-plot expressions.

Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 0-25th and 75-100th percentile ranges, respectively. Three-way comparisons are performed with the Kruskal-Wallis test and paired comparisons are performed with the Mann-Whitney test. Values reflect serum obtained from median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

A) Insulin
3-way comparison: p=0.002
LFD vs WD: p=0.001
NCKD vs WD: p=0.01
LFD vs NCKD: p=0.26

B) IGF-1
3-way comparison: p=0.02
LFD vs WD: p=0.07
NCKD vs WD: p=0.15
LFD vs NCKD: p=0.01

C) IGFBP-3
3-way comparison: p=0.06
LFD vs WD: p=0.03
NCKD vs WD: p=0.97
LFD vs NCKD: p=0.07

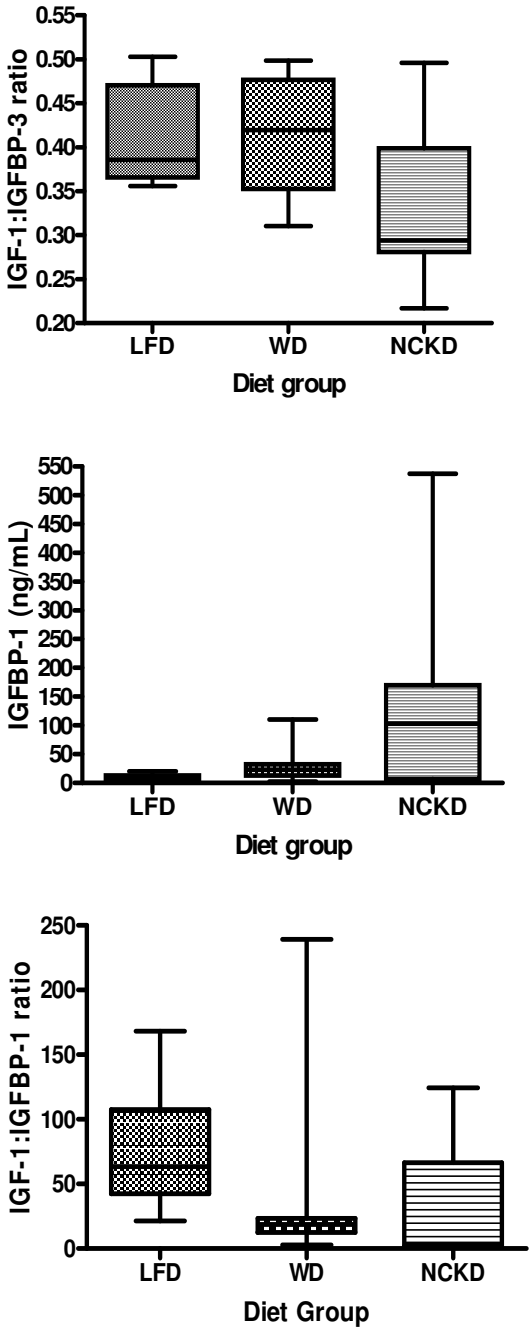

FIGURE 10.

Box-plot expressions.

Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 0-25th and 75-100th percentile ranges, respectively. Three-way comparisons are performed with the Kruskal-Wallis test and paired comparisons are performed with the Mann-Whitney test. Values reflect serum obtained from median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

D) IGF1:IGFBP-3 ratio
3-way comparison: p=0.02
LFD vs WD: p=0.65
NCKD vs WD: p=0.02
LFD vs NCKD: p=0.03

E) IGFBP-1
3-way comparison: p=0.004
LFD vs WD: p=0.009
NCKD vs WD: p=0.08
LFD vs NCKD: p=0.007

F) IGF-1:IGFBP-1 ratio
3-way comparison: p=0.004
LFD vs WD: p=0.004
NCKD vs WD: p=0.08
LFD vs NCKD: p=0.01

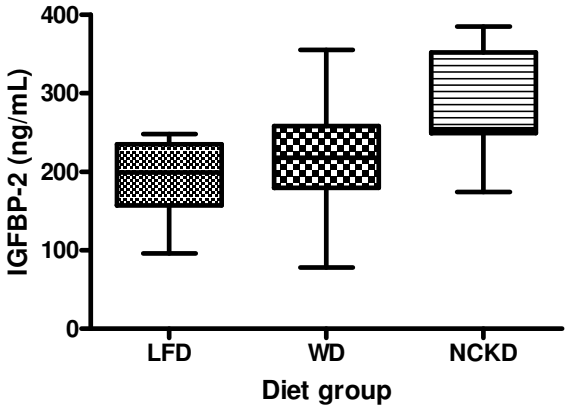

FIGURE 10.

Box-plot expressions.

Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 0-25th and 75-100th percentile ranges, respectively. Three-way comparisons are performed with the Kruskal-Wallis test and paired comparisons are performed with the Mann-Whitney test. Values reflect serum obtained from median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

G) IGFBP-2
3-way comparison: p=0.02
LFD vs WD: p=0.65
NCKD vs WD: p=0.02
LFD vs NCKD: p=0.03

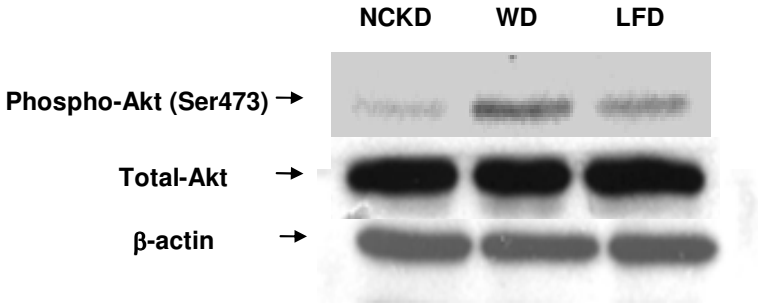

FIGURE 11. Western blot analysis of Phospho-Akt (Ser473), Total-Akt, and Beta-actin in pooled tumor lysates for each diet group (N=5 per group)

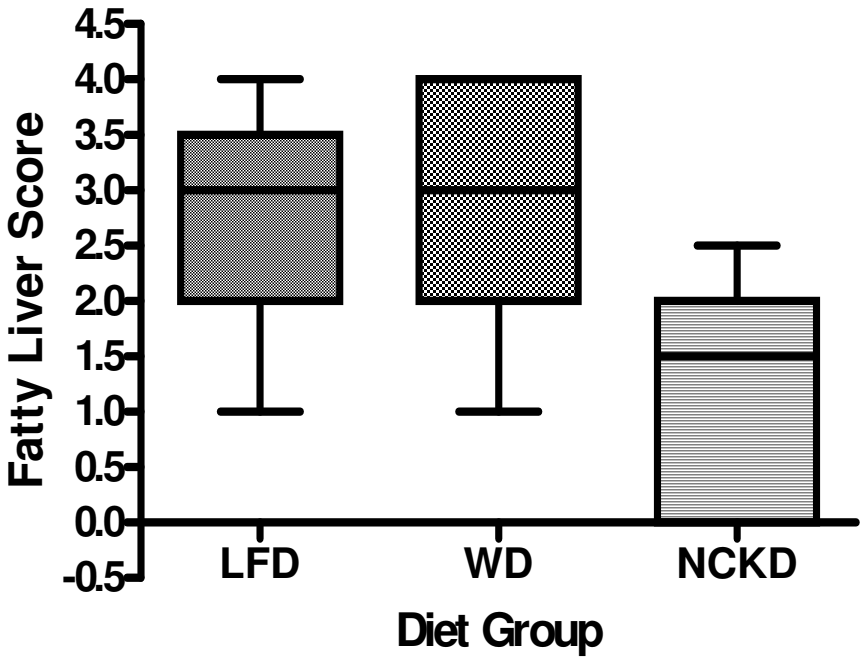

FIGURE 12.

Box-plot expressions for fatty liver score.

Lower and upper boundaries of each box represent 25th and 75th percentile expression for each group, respectively. The middle bar represents the median. Lower and upper whiskers correspond to 0-25th and 75-100th percentile ranges, respectively. Three-way comparisons are performed with the Kruskal-Wallis test and paired comparisons are performed with the Mann-Whitney test. Values reflect the median 11 surviving mice in each diet group under fasting conditions on day of sacrifice.

3-way comparison, p=0.0006
LFD vs. WD, p=0.72
NCKD vs. WD, p=0.001
LFD vs. NCKD, p=0.001

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Biography

John C. Mavropoulos was born in the Northeastern state of New Jersey. He went to college in the Midwest at the University of Chicago (where he obtained a BS in Chemistry), and, after a few years of conducting biomedical research, finally landed in the South for an extended stint in the Medical Scientist Training Program at Duke University School of Medicine. While in pursuit of the MD and PhD degrees, he also obtained an MPH degree from the University of North Carolina at Chapel Hill School of Public Health in August 2005. He looks forward to continuing his research interests and hopes to discover novel ways to help treat cancer patients. For the most current listing of his publications, please visit Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/), type his name in reverse order (*without* the middle initial) in the search bar, and then click "GO". Thank you for reading his thesis and he hopes you've learned something interesting and useful from it.

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