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Consuming a Ketogenic Diet while Receiving Radiation and Chemotherapy for Locally Advanced Lung and Pancreatic Cancer: The University of Iowa Experience of Two Phase I Clinical Trials

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Abstract

Objective—Ketogenic diets (KD) are low in carbohydrates and high in fat which force cells to rely more heavily upon mitochondrial oxidation of fatty acids for energy. Cancer cells, relative to normal cells, are believed to exist under a condition of chronic mitochondrial oxidative stress that is compensated for by increases in glucose metabolism to generate reducing equivalents. The current study tests the hypothesis that consuming a KD while receiving concurrent radiation and chemotherapy would be clinically tolerable in locally advanced non-small cell lung (NSCLC) and pancreatic cancer and could potentially exploit cancer cell oxidative metabolism to improve therapeutic outcomes.

Methods—Mice bearing MIA PaCa-2 pancreatic cancer xenografts were fed either a KD or standard rodent chow, treated with conventionally fractionated radiation (2 Gy / fx), and tumor growth rates were assessed daily. Tumors were assessed for immuno-reactive 4-hydroxy-2-nonenal-(4HNE) modified proteins as a marker of oxidative stress. Based on this and another previously published pre-clinical study, phase I clinical trials in locally advanced NSCLC and pancreatic cancer were initiated combining standard radiation and chemotherapy with a KD (lung 6 weeks duration; pancreas 5 weeks duration).

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Results—Xenograft experiments demonstrated prolonged survival and increased 4HNE modified proteins in animals consuming a KD combined with radiation compared to radiation alone. In the phase I clinical trial, over a period of three years, seven NSCLC subjects enrolled in the study. Of these, four were unable to comply with the diet and withdrew, two completed the study, and one was withdrawn due to a dose limiting toxicity. Over the same time period, two pancreatic cancer patients enrolled in the trial. Of these, one completed the study and the other was withdrawn due to a dose limiting toxicity.

Conclusion—The pre-clinical experiments demonstrate that a KD increases radiation sensitivity in a pancreatic cancer xenograft model. However, subjects with locally advanced NSCLC and pancreatic cancer receiving concurrent radiation and chemotherapy had suboptimal oral KD compliance and hence poor tolerance.

Keywords

Ketogenic diet; non-small cell lung cancer; NSCLC; pancreatic cancer; phase I clinical trial

Introduction

In 2016, there will be an estimated 224,390 new cases of lung and 49,000 new cases of pancreatic cancer diagnosed in the United States (1). Both of these diseases have a poor prognosis with an estimated 158,000 deaths due to lung and 40,500 deaths due to pancreatic cancer in 2015 (2). The current standard of care for these cancer sites includes a combination of surgery, radiation and chemotherapy. Given the dismal prognosis of these cancers, new treatment approaches that selectively enhance tumor cell responses to radiation and chemotherapy are needed. One approach to improve therapeutic outcome is to increase tumor cell metabolic oxidative stress.

Oxidative stress is defined as a disruption in the balance between cellular anti-oxidants (for example glutathione metabolism, thioredoxin metabolism, superoxide dismutase, catalase) and pro-oxidants (for example) hydrogen peroxide, superoxide, hydroxyl radicals, lipid hydroperoxides) resulting in damage to DNA, proteins, and lipid bilayers (reviewed in (3)). Relative to normal cells, it is hypothesized that cancer cells have increased steady-state levels of mitochondrial $O_2^{\bullet-}$ and H_2O_2 leading to a chronic condition of metabolic oxidative stress that can be further enhanced by the addition of mitochondrial electron transport chain blockers (4). To counteract this increase in mitochondrial $O_2^{\bullet-}$ and H_2O_2 , cancer cells are thought to increase glucose metabolism to generate reducing equivalents, including NADPH, necessary to detoxify pro-oxidants (4). Furthermore, radiation and chemotherapy induce various levels of oxidative stress causing cell damage (5, 6). Thus, we hypothesized that dietary reductions in glucose with increasing reliance on the oxidative metabolism of fatty acids would selectively enhance tumor cell versus normal cell responses to standard-of-care radiation and chemotherapy due to the enhancement in metabolic oxidative stress.

Ketogenic diets (KD) are high in fat and low in carbohydrate (7) and are currently used clinically as an alternative therapy for childhood epilepsy (8). Recently, KD's have been studied as an adjuvant to cancer therapy in a variety of animal cancer models and in humans (9–22). We have previously shown that KDs increase non-small cell lung cancer (NSCLC)

radiation and chemo-radiation sensitivity in xenograft models via a mechanism that increases cancer cell oxidative stress (9, 11). In the current study we extended these observations to a pancreatic cancer xenograft model and then opened two phase-I clinical trials assessing the tolerability of a KD combined with radiation and chemotherapy in locally advanced NSCLC and pancreatic cancer.

Methods

Tumor xenografts

Mia Paca-2 cells (pancreatic cancer cell line) were obtained from the American Type Culture Collection (ATCC). Mia Paca-2 cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% FBS (Hyclone). Female 4 to 6 week old athymic nu/nu mice were purchased from Harlan Laboratories. Mice were housed in the Animal Care Facility at our institution and all procedures were approved by our Institutional Animal Care and Use Committee and conformed to NIH guidelines. Mice were subcutaneously injected with 2.5×10^6 Mia-Paca-2 pancreatic cancer cells into the right flanks. When tumors reached approximately 4 mm in diameter, mice were treated with ionizing radiation (12 Gy in 6×2 Gy fractions) or ionizing radiation combined with KD for 25 days as previously described (9). The 4:1 KetoCal® diet for the animal studies was purchased from Nutricia North America, Inc. (Gaithersburg, MD) and prepared as per the manufacturer's instructions while the controls were fed standard mouse chow. Daily weighing of the unfinished food pellets monitored food intake. To ensure mice fed with KetoCal® were in ketosis, blood HbA1c was calculated using Crystal Chem mouse assay kit (Downers Grove, IL) and blood ketone levels were measured with the Precision Xtra ketone monitoring system (Abbott; Alameda, CA).

4-Hydroxy-2-nonenal-(4HNE)-modified protein immuno-blotting assay

4HNE dot blot analysis was performed as previously described (9). Briefly, approximately 20 mg of mouse tumor protein was harvested from each treatment group with protein concentration determined by Lowry Assay. 25 µg of protein was blotted onto Sequi-Blot PVDF membrane (BIO-Rad) and incubated in 250 mM sodium borohydride + 100 mM MOPS, pH 8.0, for 15 min to chemically reduce the Schiff base adduct to reveal the Michael addition product for antibody recognition. The blot was then incubated with the primary antibody recognizing the Michael addition product of 4HNE-modified cellular proteins (23) followed by 2 hours with a secondary antibody with a horseradish peroxidase-conjugate. Chemiluminescence detection (ECL Plus Western Blotting Detection System, GE Healthcare) with X-ray film and analysis of integrated densities using Image J software.

Clinical Trials

Investigator-initiated protocols combining a KD in non-small cell lung cancer (NSCLC; ketolung) and pancreatic cancer (ketopan) were approved by our Iowa Institutional Review Board (IRB-01) and were listed on clinicaltrials.gov (NCT01419483, ketopan; NCT01419587, ketolung).

Patients with histological diagnosis of pancreatic cancer (AJCC stage IIA, IIB, or III) or NSCLC (inoperable stage III or oligometastatic stage IV) were invited to participate in these clinical trials. Required hematologic values for both trials included leukocyte $3000/\text{mm}^3$, absolute neutrophil count (ANC) of $1,500/\text{mm}^3$, and platelets of $100,000/\text{mm}^3$. Required chemistries for pancreas patients included a total bilirubin 3.0mg/dl , AST $5\times$ institutional upper limit normal (ULN), creatinine $1.5\times$ ULN or clearance $60\text{mL/min}/1.73\text{m}^2$ and Hgb A1C 8% . Required chemistries for lung patients included a total bilirubin 1.5mg/dl , AST $2\times$ ULN, creatinine $1.5\times$ ULN or clearance $60\text{mL/min}/1.73\text{m}^2$ and Hgb A1C 8% . Exclusion criteria included co-morbidities such as a second malignancy, uncontrolled intercurrent illnesses, pregnancy, or active use of other investigational agents (see supplementary information for trial protocol).

The treatment schemas are provided (Figures 1A and 1B). Prior to beginning the KD, baseline glucose, ketones, BUN, serum, uric acid, and fasting whole blood lipid panels were obtained. Participants met with a Registered Dietitian prior to beginning the KD to obtain food preferences, determine calorie needs, taste sample foods, and discuss dietary procedures. KD meals were designed to provide a 4:1 ratio of fat grams to grams protein + carbohydrate (90% of calories from fat, 8% from protein, 2% from carbohydrate). All meals were prepared in the Metabolic Kitchen (MK) at our institution, and foods were weighed to the nearest gram. Nutrient composition was calculated using the USDA Standard Reference Database and manufacturer's data. Breakfast usually consisted of a beverage prepared with 4:1 KetoCal® powder and carbohydrate-free flavorings. Lunches and dinners were composed of conventional foods and recipes modified to be low in carbohydrate. Water and carbohydrate-free beverages were allowed ad lib and discretionary foods were provided that could be consumed if the participant was hungry. To enhance compliance, menus were individualized for each subject taking into account their food preferences and any changes in ability to eat as a result of radio-chemotherapy. Table 4 shows two sample menus. Participants ate lunch at the MK weekdays and took the remaining meals as well as all weekend meals home to eat. Participants kept a daily food checklist to document consumption of food sent home and were queried daily about any foods or beverages consumed that were not part of the meal plan. If subjects were unable to consume the entire meal, they were asked to eat a proportional amount of all foods and beverages so as to maintain the 4:1 ratio as closely as possible. Any uneaten food was returned to the MK for weighing and documentation. Participants continued taking their usual dietary supplements during the study. Supplements and medications were checked for carbohydrate content to maintain the 4:1 dietary ratio.

Subjects began the KD at least 2 calendar days prior to beginning concurrent radiation and chemotherapy. Fingerstick glucose and ketone measurements were obtained prior to the day's radiation therapy treatment in addition to weekly serum ketones and glucose measurements from the in-house clinical pathology lab. Fingerstick glucose measurements were obtained using Accu-chek Aviva or Aviva Plus (Roche Diagnostics; Indianapolis, IN). Daily ketone measurements were performed on capillary blood samples utilizing a portable and calibrated electrometer (Precision Xtra Blood Glucose and Ketone Monitoring System, Abbott; Alameda, CA). Weekly ketone values were performed on venous blood samples utilizing a beta-hydroxybutyrate assay (Stanbio Laboratory; Boerne, TX) and

spectrophotometer (Roche Diagnostics; Indianapolis, IN). Subjects were assessed weekly with physical examinations and standard of care laboratory assessments. A repeat fasting lipid panel was obtained 3 weeks into radiation treatment. Subjects consumed a KD for a period of approximately 5 weeks (pancreas) to 6 weeks (NSCLC) while receiving concurrent chemoradiotherapy (gemcitabine 600 mg/m² weekly along with concurrent radiation - 50.4 Gy/28 fractions for pancreatic cancer or carboplatin AUC = 2 per Calvert formula and paclitaxel 50 mg/m² weekly along with concurrent radiation - 66 Gy/33 fractions. for NSCLC) Following completion of the combined therapy, subjects were followed every 3 months for 1 year for adverse events related to therapy.

For the purpose of these studies, adverse events were defined and graded utilizing the Common Terminology Criteria for Adverse Events v.4.0 (CTCAE). Dose limiting toxicities (DLTs) were adverse events that the research team deemed unacceptable in severity that occurred during the first 5 weeks of combined therapy. Identified DLTs included symptomatic grade 2 hypoglycemia, grade 3 hypoglycemia or constipation, and grade 4 diarrhea, nausea, or vomiting. Serious adverse events (defined by 21CFR§312.32) with reasonable attribution to KD were also considered DLTs. If a subject experienced a DLT, the KD was withheld for up to 1 calendar week to determine if the adverse event diminished or resolved. Depending upon the nature and severity of the event, the KD could be resumed to test causality. The treating physician initially determined attribution to the KD, this was then reviewed by the study PIs and the medical monitor.

These phase I clinical trials were designed to assess the safety and tolerability of the KD combined with concurrent chemoradiation therapy. Enrollment followed a 3 × 3 study design. If while recruiting subjects, 3 out of 6 subjects experienced a DLT, accrual was stopped and the diet, combined with therapy was not tolerable and required revision. If fewer than 3 subjects experienced a DLT, the combined therapy would be considered tolerable and investigated in a phase 2 trial powered for efficacy.

Oxidative stress biomarker: protein carbonyls

Protein carbonyl content in subject's plasma was measured according to a protocol adapted from the Protein Carbonyl Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI). Protein carbonyl content is a commonly used marker of general systemic protein oxidation. Briefly, 200 µL of subject plasma from weekly blood draws was mixed with 800µL of 2,4 dinitrophenylhydrazine to allow protein carbonyls to form a Schiff base and corresponding hydrazone. The amount of protein hydrazone produced was quantified spectrophotometrically at an absorbance of 360–385, and the carbonyl content was standardized to protein concentration. The protein carbonyl content of each subject was normalized to pre-diet levels and a standard curve provided by the manufacturer was used to establish that the samples were in the linear range of the assay. Data were expressed as nmol carbonyl/mg protein.

Results

KD enhances radiation therapy response in a pancreas xenograft model

Weekly blood tests confirmed mice were in ketosis (Figure 2A). Hemoglobin A1c was significantly lower in mice eating standard mouse chow compared to those on the KD (3.9 vs 4.2 respectively $p < 0.05$, Figure 2B). Mice treated with KD and radiation survived significantly longer than mice treated with radiation alone (Figure 3A, $p < 0.05$). In addition, mice treated with KD and radiation had a slower tumor growth rate than mice treated with radiation alone (Figure 3B, $p < 0.05$). Similar to previous reports, the KD was well tolerated as mice maintained their weight through the diet and maintained a level of activity similar to mice receiving standard mouse chow (Figure 3C) (9, 11). Interestingly, the KD also appeared to prevent the weight loss seen in mice treated with IR and standard chow (Figure 3C). These data support the conclusion that 4:1 KDs enhance radiation response in mouse xenograft models without causing increased toxicity.

KD increase immuno-reactive 4HNE-modified proteins in pancreas xenograft tumor tissue

As a marker of *in vivo* oxidative protein damage caused by lipid peroxidation, immunoreactive 4-hydroxy-2-nonenal (4HNE) modified protein was analyzed from in tumors harvested from control, KD, radiation, and KD + radiation treatment groups. Dot blot analysis of tumor samples harvested at the end of two weeks of ketogenic diet and fractionated radiation therapy (Figure 4 A and B) demonstrated increased 4HNE modified proteins in pancreas xenografts that consumed a KD relative to standard mouse chow or IR alone ($p < 0.05$). These data support the hypothesis a ketogenic diet increases lipid peroxidation derived aldehydes that oxidatively damage proteins in tumor tissue.

Patient Characteristics and Treatment Parameters

Patients were screened and enrolled from July 2011 to June 2014. Eleven subjects were screened for the ketolung study with seven enrolling in the trial. Five subjects were screened for the ketopan trial with two enrolling in the trial. Reasons for screen failures included biopsy proven metastatic disease, subject self-withdrawal prior to starting therapy, and choosing to participate in alternative clinical trials. Subject characteristics of those enrolled are provided (Table 1).

Subject Experience

On average, subjects consuming the KD entered ketosis (> 0.6 mg/dL beta hydroxybutyrate) at day three with ketones continuing to rise throughout treatment (Figure 5A–F). Finger stick ketone assessment of beta-hydroxybutyrate (Figure 5BDF) correlated closely with weekly clinical laboratory ketone values (Figure 5ACE). Both methodologies displayed ketosis at the same time point; however, venous blood sample testing exhibited less variation about separately constructed R-squared regression lines (Figures 5A–F). Dietary compliant subjects also showed no consistent reductions in blood glucose levels (Figure 6ABC). None of the subjects developed ketoacidosis.

Of the seven patients enrolled, two ketolung subjects completed the clinical trial. Four ketolung subjects discontinued the KD during therapy because of difficulty complying with

the KD, constipation, fatigue, bloating, and nausea. One ketolung subject experienced a DLT (asymptomatic hyperuricemia, grade 4; > 10 mg/dL) and was removed from the study. One of the two ketopan subjects completed the study. The second ketopan subject experienced a DLT (dehydration, grade 3) and was removed from the study. Table 2 provides the distribution of graded adverse events. The average weight loss of all ketolung subjects was 5.6 kg (6.0% of beginning body weight; Grade 1) over the 6-week course of radiation and chemotherapy with the two subjects who completed the study losing 8.6 kg and 5.4 kg respectively (6.2% of pre-treatment body weight; Grade 1). The ketopan subject who prematurely stopped the KD lost 9.4 kg (9.5% of pre-treatment body weight; Grade 1) over the 5-week course of their radiation and chemotherapy while the subject who completed the KD lost 6.9 kg (10% of pre-treatment body weight) over the course of their radiation and chemotherapy.

Protein carbonyl content is a commonly used general marker for assessing oxidative damage to proteins during biologically relevant stress (24). Carbonyl content was measured in plasma samples obtained from lung cancer subjects Lung-5, Lung-6, and Panc-1 (successfully completing the diet) along with three weeks of Lung-2 (while subject was in ketosis as confirmed by daily fingerstick β -hydroxybutyrate). The data in Figure 7 demonstrate that subjects in ketosis during therapy demonstrated significant increases in plasma protein carbonyl content suggesting that treatment with radiation, chemotherapy, and the KD resulted in an increase in the steady-state levels of oxidatively damaged proteins.

Subjects were followed for long-term outcome (Table 3). The limited number of subjects and lack of subjects who remained compliant with the diet prevented statistical analysis to determine if an oral KD was medically tolerable when combined with radiation and chemotherapy. Overall, ketolung subjects consumed a KD while receiving concurrent radiation and chemotherapy for an average of 16.9 days (range of 0 to 42 days) of a planned 42 days. Ketopan subjects consumed a KD for an average of 21 days (range of 8 to 34 days) of a planned 34 days while receiving concurrent radiation and chemotherapy.

These phase-1 clinical studies were not powered to detect differences in progression free survival (PFS) and overall survival (OS). In the ketolung study, the median PFS for those who prematurely stopped consuming the KD was 7.5 months (range: 3.2 months to 33 months) while the PFS in the one known subject who completed the KD with concurrent radiation and chemotherapy was 4.6 months. The other ketolung subject who completed the KD while receiving concurrent radiation and chemotherapy was lost to follow-up but a date of death was established from the public record. After tumor progression was determined, subjects went on to receive additional therapies including stereotactic radiosurgery for brain metastasis or different chemotherapy regimens. The median OS in the ketolung subjects who prematurely stopped consuming the KD was 22 months (range: 3.7 to 33.3 months) while the median OS in those who completed the KD while receiving concurrent radiation and chemotherapy was 17.7 months (range: 9.4 months to 26 months). Two of five ketolung subjects who stopped the KD prematurely are still alive (OS of 33.3 and 18.4 months) while one of two subjects who completed the KD is still alive (OS of 26 months). The PFS in the ketopan subject who prematurely stopped the KD was 5.3 months and OS of 10 months. The

ketopan subject who completed the KD developed a biliary tract obstruction and sepsis resulting in death 2 months post-therapy.

Discussion

Despite advances in developing new chemotherapies and radiation targeting techniques, locally advanced NSCLC and pancreatic cancer continue to have a poor prognosis with median overall survivorship less than 2.5 years (25, 26). Targeting fundamental differences between cancer and normal cells mitochondrial oxidative metabolism with manipulations such as the KD could represent a rapidly implementable strategy for improving outcomes to standard of care radiation and chemotherapy.

Fatty acid oxidation occurs primarily in the mitochondria and is dependent upon efficient and well-integrated mitochondrial electron transport chain activity. Cancer cell mitochondria are thought have inefficiencies in mitochondrial electron transport chain activities leading to increased steady-state levels of $O_2^{\bullet-}/H_2O_2$ and they increase glucose metabolism to generate reducing equivalents to compensate for excess H_2O_2 (3, 4). From these basic science observations we hypothesized that limiting consumption of glucose and increasing dependence on oxidation of fatty acids during consumption of a KD would selectively enhance tumor cell versus normal cell sensitivity to radiation and chemotherapy by a mechanism involving oxidative stress.

The classical KD is typically designed to deliver 90% of calories from fat, 8% from protein and 2% from carbohydrate with a 4:1 ratio of fat to protein + carbohydrate. We have also previously shown in preclinical NSCLC xenograft models that feeding a KD increases radiation and chemotherapy efficacy that is accompanied by increased tumor levels of a marker for lipid oxidation and protein damage (4HNE-modified proteins, (9). In the current study, we extend these observations to show that a KD increases sensitivity to radiation in a pancreatic cancer xenograft model as well as extend these observations to initial results obtained from two phase-I clinical trials assessing the tolerability of consuming a KD while receiving concurrent radiation and chemotherapy in locally advanced pancreatic and NSCLC subjects.

The phase- I clinical trial results demonstrate the significant difficulty achieving dietary compliance that has been encountered in previous studies with adult epilepsy subjects consuming an oral ketogenic diet (27). In addition, our study was extremely rigorous in assuring compliance and entry into ketosis as we were seeking a therapeutic response. In our studies, two out of seven NSCLC subjects and one of two pancreatic subjects were able to complete the KD (~33%) while receiving concurrent radiation and chemotherapy. On average, Ketolung study subjects were able to consume a KD for 16.9 of a planned 42 days while receiving concurrent therapy. Similarly, Ketopan study subjects were able to consume a KD for an average of 21 days out of a planned 34 days while receiving concurrent therapy. Subjects met with a Registered Dietitian and kitchen staff prior to beginning the clinical trial to discuss their food preferences and sample the diet. In addition, subjects could modify their food preferences throughout the course of the trial. Efforts were made to include low carbohydrate versions of milk, bread and desserts as well as modified versions of familiar

foods such as cream soups, tacos, and pizza. In addition, all meals were provided for the subjects so they did not have to calculate and prepare their food at home as was done with some clinical studies. However, despite having prepared meals and the ability to choose menu options, most subjects were unable to consume the KD for the duration of their chemo-radiation treatment. The majority of subjects, despite expressing their initial motivation for the study, reported difficulty complying with the KD while receiving concurrent radiation and chemotherapy because of the oiliness of the diet, restriction/elimination of carbohydrate-containing foods such as fruits, vegetables, milk, breads, and sweets and their substitution with less palatable low carbohydrate versions, constipation, fatigue, bloating, and/or nausea.

When considering our experiences in the context of previous studies in which the KD diet has been used in epilepsy patients the factors contributing to unexpected poor compliance with the diet are multifactorial. First, it is unclear that stringent compliance with daily biochemical verification of ketosis has been attempted with epilepsy patients. Secondly, most epilepsy patients are children and may demonstrate a better compliance with the diet because they do not have as many preformed dietary preferences and habits as adults. Third, epilepsy patients are not receiving cancer therapy combined with the KD, which may increase the likelihood of noncompliance because of the toxicities associated with chemotherapy and radiation, which also include loss of appetite, nausea, and fatigue. Fourth, the classical KD with a 4:1 ratio is very restrictive and requires consuming a high proportion of fat. Another form of the KD such as 3:1, medium chain triglyceride, or modified Atkins would liberalize dietary intake and may enhance subject compliance. Regardless of the underlying etiology, it is clear from our limited clinical experience that the current approach was not successful in attaining the verifiable dietary compliance with a high enough percentage of subjects necessary for the continued testing of KDs in larger trials for pancreatic and NSCLC.

Subjects on the KD were typically in ketosis after the third day on the diet. In the Ketolung study, one subject experienced hyperuricemia (possibly diet related, grade 4) and two subjects developed grade 3 hypokalemia (unlikely related to the diet). In the Ketopan study, one subject experienced grade 3 esophagitis (unlikely related to the diet). While these studies were not powered to detect a difference in progression free survival and overall survival, there was no observed difference in PFS or OS between subjects who prematurely stopped the KD *versus* those who consumed the KD while receiving concurrent radiation and chemotherapy. These results are consistent with previous reports in glioblastoma subjects (28).

While there is robust pre-clinical data in rodents demonstrating that a KD is safe and effective, adhering to the diet has been shown to be difficult for adult human subjects. In a pilot trial examining 16 subjects with advanced disease and no further established therapeutic options, subjects were offered a low carbohydrate, high fat diet of no more than 70 g of carbs. Even with these less stringent carbohydrate requirements, only 5 of 16 patients completed the study, with 3 patients reaching stable ketonuria of greater than 0.5 mmol/L (17). Similar to our study, subjects on this previous trial reported mild constipation, nausea, fatigue, as well as significant weight loss of 2 kg. The relative small weight loss in

this study may be attributed to the fact that patients were not undergoing concurrent chemoradiation.

In another retrospective study examining the safety of a KD during concurrent chemoradiotherapy for glioblastoma multiforme, six patients who consumed a KD tolerated the diet well and had no grade 3 toxicity, demonstrating the potential for the KD's safety profile (28–30). Patients in this study also reported weight loss, and interestingly, had significant reductions in their serum glucose level on the diet despite concurrent treatment with high dose steroids. In the ERGO trial studying KD's in recurrent glioblastoma, the authors reported higher compliance rate to the diet, with 12 out of 17 patients completing the trial (28). However, ketosis was only confirmed weekly by urine ketones at greater than 0.5 mmol/L. In addition, in this trial ketosis was not maintained in 5/12 patients, as their urine was positive for ketones less than 60% of the time, indicating a possible difficulty with adherence to the KD.

Overall, the current study continues to support the hypothesis that KDs are capable of increasing tumor responses to radiation in rodent xenograft models. Our studies in humans also continue to demonstrate the difficulty for adults to comply with a KD while receiving concurrent radiation and chemotherapy in locally advanced NSCLC and pancreatic cancer. One possible solution to difficulties with KD compliance is to deliver the KD in a clinical setting where PEG tube usage is high during therapy. To test this hypothesis additional studies assessing the delivery of a KD *via* PEG tube in head and neck cancer subjects are ongoing (NCT01975766).

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Figure 1. Schematic overview of the ketolung (1A) and ketopan (1B) clinical protocols
 Subjects consumed a 4:1 ketogenic diet for a period of 5.5 – 7 weeks while receiving standard of care radiation and chemotherapy for their respective cancers. Subjects were followed for up to 1 year after completing the ketogenic diet assessing for subsequent adverse events.

Figure 2. Mice fed KDs had lower blood glucose and higher blood ketone levels than mice fed std chow

Mice (N=3 per group) were fed 4:1 KetoCal® or standard (std) chow for 25 days. A) β -hydroxybutyrate (β HB) levels were taken via tail vein stick at various times in the first two weeks of diet via Precision Xtra ketone monitoring system. B) Mouse blood taken via cardiac puncture at sacrifice was assayed for HbA1c using Crystal Chem mouse assay kit. Analysis using one way ANOVA with Newman Keul's posttest yielded * $p < 0.05$ vs control.

Figure 3. Feeding a Ketogenic Diet Sensitizes Mia PaCa-2 Human Pancreatic Cancer Xenografts to Radiation

Nude mice (5–6 animals per group) were injected with Mia PaCa-2 cells in their right flank and tumors were allowed to grow to approximately 4 mm in maximum diameter. Mice were irradiated (IR) with 6 fractions of 2 Gy over a period of 2 weeks. Control mice with Mia PaCa-2 xenografts were fed ad libitum standard rodent chow (Control) or 4:1 KetoCal® ketogenic diet (Keto) 2 days prior to first radiation dose and continued until two days following last radiation dose for a total of 16 days. A) Kaplan-Meier survival plots for Control, Keto, IR and IR + Keto treatment groups. Keto + IR resulted in significantly longer

survival than all groups alone $p < 0.05$. B) Keto + IR treatment resulted in significantly slower tumor growth rate than Control or Keto alone $p < 0.05$. C) After 15 days of diet the mice in the KD group weighed significantly more than mice treated with IR. No other treatments resulted in significant changes in weight. $*p < 0.05$ using one way ANOVA with Fisher's LSD.

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Figure 4. KD increases 4HNE-modified proteins in pancreas cancer xenografts

Equivalent protein quantities isolated from pancreas xenograft animals treated as in Figure 3 (N=3 from each treatment group) were blotted onto PVDF membrane and stained with a polyclonal antibody against 4HNE-modified proteins. (A) Negative (-) and positive (+) controls represent the immunoreactivity derived from Mia Paca-2 cell homogenates treated with and without 100 μ M 4HNE for 1 hour. (B) shows the quantification of dot blots by Image J analysis with normalization to the background. Error bars represent $\pm$ 1 SEM. One way ANOVA with Newman-Keuls Multiple Comparison Test demonstrated that homogenates from KD fed mice were significantly greater than mice fed standard mouse chow (control and IR)($*p<0.05$).

Figure 5. Circulating levels of ketones in subjects consuming a 4:1 ketogenic diet while receiving concurrent radiation and chemotherapy in NSCLC and pancreas cancer as determined by clinical lab values (A, C, E) as well as by the fingerstick method (B, D, F)

Day 0 indicates the day subjects began the ketogenic diet. Subjects were typically in ketosis by day 3 of the diet. There was not a significant difference in weight between subjects who prematurely stopped the ketogenic diet as opposed to those who consumed the ketogenic throughout the course of radiation and chemotherapy (data not shown).

Figure 6. Serum glucose levels in subjects compliant with the 4:1 ketogenic diet

Figure 7. Lung and pancreas cancer subjects on a 4:1 ketogenic diet have significantly higher plasma protein carbonyl content when compared to their baseline pre-diet value

Blood from subjects with lung cancer (Lung-2, Lung-5, Lung-6) and pancreatic cancer (Panc-1) were drawn weekly and the carbonyl content was measured in plasma and normalized to pre-diet levels. Subjects were in confirmed ketosis and data points represent weekly values. * $p < 0.05$ with unpaired *t*-test

Table 1

Baseline subject demographic and clinical characteristics

Characteristics of lung patients (N=7)	Completed KD: n=2	Did not complete KD: n=5
Age—yr		
Median	66	67
Range	55–76	51–83
Sex		
Male	0	2
Female	2	3
Karnofsky Performance Status		
90	1	2
80	1	3
Staging		
Tumor Stage		
T1	0	1
T2	0	0
T3	1	2
T4	1	2
Node Stage		
N2	1	3
N3	1	2
Metastasis Stage		
M0	1	4
M1B	1	1
Characteristics of pancreas patients (N=2)	Completed KD: n=1	Did not complete KD: n=1
Age—yr	69	67
Sex		
Male	0	1
Female	1	0
Karnofsky Performance Status		
80	1	1
Staging		
Tumor Stage		
T3	1	0
T4	0	1
Node Stage	0	0
N0	1	1
Metastasis Stage	0	0

Characteristics of pancreas patients (N=2)	Completed KD: n=1	Did not complete KD: n=1
M0	1	1

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Table 2

Treatment-Related Adverse Events by Disease Site and Completion of KD

Completed KD: N=2					Did not complete KD: N=5				
Adverse Events in lung subjects	Grades 1-2	Grade 3	Grade 4	Grade 4	Grades 1-2	Grades 3	Grade 4	Grade 4	Grade 4
Esophagitis	1				3		1		
Nausea	1				5				
Vomiting	1				4				
Constipation	1				1				
Diarrhea	1				3				
Skin	1				4				
Arthralgia	1				1				
Dizziness	1				1				
Concentration	1								
Thrombocytopenia									
Hypoglycemia									
Hypokalemia		2							
Hyperuricemia			1						

Completed KD: N=1					Did not complete KD: N=1				
Adverse Events in pancreas subjects	Grades 1-2	Grade 3	Grade 4	Grade 4	Grades 1-2	Grades 3	Grade 4	Grade 4	Grade 4
Esophagitis									
Nausea							1		
Vomiting						1			
Constipation						1			
Diarrhea									
Skin									
Arthralgia									
Dizziness									

Completed KD: N=1					Did not complete KD: N=1		
Adverse Events in pancreas subjects	Grades 1-2	Grade 3	Grade 4	Grade 1-2	Grade 3	Grade 4	
Concentration	1						
Thrombocytopenia	1			1			
Hypoglycemia							
Hypokalemia	1			1			
Hyperuricemia							
Dehydration						1	

N=7 lung cancer subjects who enrolled on the ketogenic diet (KD) protocol. One grade 4 event, hyperuricemia occurred in one subject after completion of the KD. The patient remained asymptomatic and uric acid level resolved without intervention.

N=2 pancreatic cancer subjects who enrolled on the ketogenic diet (KD) protocol.

Table 3

Subject outcome following enrollment on ketolung or ketopan phase I clinical trial.

Initial Treatment			Secondary Treatment					Overall survival (month)	Current status
Subject	Primary treatment (with KD)	Ketosis duration (days)	Tumor response	Progression Free Survival (months)	Modality	Response			
Lung-1	44Gy/22fx (of planned 60 Gy/30fx), PC. Developed brain mets. Treated with SRS followed by chemo.	0	Stable	33.3	Navelbine	Chest stable, CR in brain mets, alive with good PS	33.3	Alive	
Lung-2	66Gy/33fx, PC	22	PR	5.4	Navelbine Gemcitabine, SRS to brain mets	Malignant effusion and brain mets	22.2	Deceased	
Lung-3	60Gy/30fx, PC, changed to Carboplatin/ Navelbine	2	PR	7.5	Abraxane/ Carboplatin	Chest PR	26	Deceased	
Lung-4	50 Gy/25fx, PC	5	PR	3.2	None	Chest PR, diffuse bone mets	3.7	Deceased	
Lung-5	60Gy/30fx, and PC	42 *	PR	4.6	None	Chest PR, brain mets	26	Alive	
Lung-6	64 Gy/32fx, PC	42 *	PR	u/k	u/k	Unknown response, went to hospice	9.4	Deceased	
Lung-7	60 Gy/30fx, PC	5	Stable	18	None	Stable disease on imaging, alive with good PS	18.4	Alive	
Panc-1	50 Gy/25fx, Gemcitabine	34 *	PR	2	None	Biliary obstruction and sepsis	2	Deceased	
Panc-2	50 Gy/25fx, Gemcitabine , 5FU	8	PR	5.3	None	Ascites	10	Deceased	

KD: ketogenic diet. PR: progression. CR: complete response. OS: overall survival from date of initiation of primary treatment with radiation. PS: performance status. PC: Paclitaxel and Carboplatin. RT: radiation. SRS: stereotactic radiosurgery.

* completed prescribed KD. u/k: unknown, lost to follow up. mets: metastases. date of death established from the public record.

Table 4

Sample 4:1 Ketogenic Meals

Meal	Menu A	Menu B
Breakfast	<ul style="list-style-type: none"> • Strawberry Flavored 4:1 KetoCal® Beverage • Coffee with Cream 	<ul style="list-style-type: none"> • Low Carbohydrate Pancakes • Butter • Calorie-Free Pancake Syrup • Bacon • Low Carbohydrate Hot Cocoa • Coffee with Cream
Lunch	<ul style="list-style-type: none"> • Ground Beef Patty • Mustard/Mayo Sauce • Mushroom Soup • Celery w/Peanut Butter Dip • Flavored Water 	<ul style="list-style-type: none"> • Low Carbohydrate Taco (Cheddar Cheese Shell, Taco Meat, Lettuce, Guacamole) • Low Carbohydrate Vanilla Cupcake with Chocolate Frosting • Fruit Flavored Diet Soda w/Cream
Dinner	<ul style="list-style-type: none"> • Broccoli Cheddar Soup • Chicken • Low Carbohydrate Bread • Butter • Calorie-Free Strawberry Spread • Blueberries • Diet Pop 	<ul style="list-style-type: none"> • Sirloin Steak • Mushroom Soup • Celery with Ranch Dip • Sugar-Free Gelatin • Whipped Cream • Hot Tea