The Effect of a Ketogenic Diet and Synergy with Rapamycin in a Mouse Model of Breast Cancer.

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Abstract

Background: The effects of diet in cancer, in general, and breast cancer in particular, are not well understood. Insulin inhibition in ketogenic, high fat diets, modulate downstream signaling molecules and are postulated to have therapeutic benefits. Obesity and diabetes have been associated with higher incidence of breast cancer. Addition of anti-cancer drugs together with diet is also not well studied.

Methods: Two diets, one ketogenic, the other standard mouse chow, were tested in a spontaneous breast cancer model in mice. The diets were implemented either with or without added rapamycin, an mTOR inhibitor and potential anti-cancer drug.

Results: Blood glucose and insulin concentrations in mice ingesting the ketogenic diet (KD) were significantly lower, whereas beta hydroxybutyrate (BHB) levels were significantly higher, respectively, than in mice on the standard diet (SD). Growth of primary breast tumors and lung metastases were inhibited, and lifespans were longer in the KD mice compared to mice on the SD ($p<0.005$). Rapamycin improved survival in both mouse diet groups, but when combined with the KD was more effective than when combined with the SD.

Conclusions: The study provides proof of principle that a ketogenic diet a) results in serum insulin reduction and ketosis in a spontaneous breast cancer mouse model; b) can serve as a therapeutic anti-cancer agent; and c) can enhance the effects of rapamycin, an anti-cancer drug, permitting dose reduction for comparable effect. Further, the ketogenic diet in this model produces superior cancer control than standard mouse chow whether with or without added rapamycin.

Introduction

Insulin inhibition by a ketogenic diet has been shown to slow cancer growth and prolong survival in animal models and has shown safety and feasibility in small pilot studies in humans [1], [2] [3] [4] . We previously demonstrated in a pilot study of ten people with diverse, metastatic PET positive cancers that higher levels of ketosis correlated with stability vs. disease progression [5]. The full potential of ketosis in cancer therapy, however, may reside in its potential to synergize with anti-cancer drugs and other modalities of treatment. Increased overall efficacy may permit lower drug doses, thereby reducing their toxicities and side effects. Accordingly, it may be possible that the overall improvement in therapy will result in extended survival with a better quality of life.

An understanding of ketogenic diets (KD) in cancer is limited at this point but it seems unlikely that KDs by themselves can control all the features of the oncogenic state. There is much interest, therefore, in the possibility of synergy with other drugs or other therapies. Hsieh, et al., for example, demonstrated that a squamous cell carcinoma that over expressed GLUT1 receptors showed attenuated growth when animals were on a KD [6]. There was, however, little regression of tumors. The addition of the cytotoxic agent cisplatin to mice on a KD led to regression to a greater extent than cisplatin alone. An interesting variation of this principle was recently demonstrated in mice bearing a Kras-Tp53-Pdx-Cre (KPC) mutation by coupling a ketogenic diet with a PI3K inhibitor [7]. The specific benefit in this latter case was shown to arise from ketogenic diet attenuation of hyperglycemia induced by the PI3K inhibitor. Control of the hyperglycemia resulted in reduced glucose-driven cancer growth and proliferation.

Hyperglycemia is also a well-known side effect of rapamycin in humans. Rapamycin, an antifungal compound known also as sirolimus, and congeners such as temsirolimus have been proposed and studied as anti-cancer drugs in triple negative breast cancers [8], but their usefulness may be limited by hyperglycemic side effects.

Rapamycin, in further analogy with PI3K inhibitors, has the potential to be an anti-cancer drug via its inhibition of mTOR, a signaling molecule downstream of PI3K which promotes cell growth and inhibits apoptosis. It has not achieved much clinical use due to hyperglycemic effects in humans[9]. Rapamycin causes diabetes in mice [10], although only at very high doses and extended duration of treatment. Rapamycin was selected for the current animal study because it a) has the potential to be a successful anti-cancer drug when administered at doses known to be normo-glycemic in mice b) it may yet have utility in humans and c) has been an FDA approved drug since 1999 [11]. The principle of combining a ketogenic diet with other forms of anti-cancer chemotherapies for widely metastatic disease has been reported to have additive effects in mice [12] [13] [14], as well as in limited human studies [4] [15] [16] [17].As above, rapamycin is not expected to induce hyperglycemia in our mouse model and we wished to determine if a KD would exert its cytotoxic effects even in the absence of hyperglycemia.

Material and Methods

Diets

Diets were purchased from Research Diets Inc. The ketogenic diet (KD) composition in calorie percent ratio of carbohydrate/fat/protein was 0.1/89.9/10.0. The standard diet's (SD) distribution was 80/10/10. Both diets contain the same quality and quantity of mineral and vitamins and other necessary components. The KD and SD contain 6.71 and 3.85 calories/g of energy, respectively. In general, a mouse needs 13.7 to 14.6 calories from their food [18].

Cancer model and treatment.

Four-week old, female FVB/N-Tg(MMTV-PyVT)634Mul/J mice were purchased from Jax Lab. These mice (100%) develop breast tumors spontaneously during their lifetime. The tumors can be seen as early as 5 weeks of age. At four months, 80-94% of these mice will have developed lung metastasis [19] [20].

Mice were randomly divided into 6 groups of 4-10 mice each, numbering 34 animals in total. One week after arrival at the animal facility, mice were assigned to the SD, SD plus rapamycin at 0.4 mg/kg (SDr0.4), SD plus rapamycin at 4 mg/kg (SDr4), KD, KD plus rapamycin at 0.4 mg/kg (KDr0.4), and KD plus rapamycin at 4 mg/kg (KDr4). At the third week of the special diets, rapamycin was given to mice by oral gavage with 22 gage feeding needle at a dose 0.4 mg/kg or 4 mg/kg daily for 2 weeks.

Housing and husbandry

Our mice were housed and husbanded in the institution's barrier animal facility. This was not secondary to intrinsic immunocompromise, as in nude mice. It was based, rather, on a requirement specific to the IACUC of our institution: that all cancer mice,

particularly those receiving chemotherapy (which may compromise the immune system secondarily), must be housed in our institutional barrier to reduce the infection rate. As we employed a spontaneous cancer mouse model in which all mice developed breast cancer after 5 weeks of age and some received chemotherapy, the institutional IACUC required barrier housing. Special training of the first author (YZ) was provided in animal handling, anesthesia, tumor measurement, moribund determination, oral gavage, and cardiocentesis.

Blood sampling and analysis

At designated time points, mice were bled from the tail vein with a 21 G injection needle puncture. The peripheral blood drops were used to measure glucose and Beta-hydroxybutyrate (BHB) separately using Keto Mojo, a blood glucose and ketone monitoring system. Each glucose data point is a daily average of three measurements at 3 different time points 9 am, 1 pm, and 5 pm. Values of BHB and glucose by Keto Mojo were validated against the Beckman Coulter AU480 Chemistry Analyzer at Biomarker Analytic Research Core of Albert Einstein College of Medicine (data not shown).

The serum obtained after cardiocentesis at the time of euthanasia (see below) was used to measure insulin level with an ELISA based on chemiluminescence. Blood chemistry included urea nitrogen, potassium, chloride, sodium etc. These (as well as BHB and glucose for validation, as above) were determined with the Beckman Coulter AU480 Chemistry Analyzer.

Determination of moribund status and humane endpoints.

Animals were examined daily for signs of moribund behavior, and weekly to measure tumor volume. Mice were determined to have reached moribund status when they could no longer reach their food and/or when the sum of tumor volume within a mouse exceeded 4 cm³. Once mice reached this condition, they were euthanized within four hours which then constituted the duration of the experiment. All (n = 34) animals were euthanized; none were euthanized prior to reaching this point. Moribund mice were euthanized in accordance with IACUC recommendations as well as with ARRIVE guidelines for humane endpoints. All animal welfare considerations were taken, including minimization of suffering and distress, including special barrier housing, as described above. They were anesthetized with 2.5% isoflurane, and blood (≥1 ml) was taken by cardiocentesis resulting in immediate death.

Tumor size and survival measurement

Tumor size was measured weekly with calipers. Volume was calculated from the longest (L) and shortest (S) dimensions according to $Volume (mm^3) = \frac{1}{2} L \times S^2$. In this spontaneous breast cancer model, each mouse develops multiple tumors. The combined tumor volume represented by the sum of all visible tumor volumes was used as a surrogate measure of the overall tumor growth rate. (This measure does not include additional growth due to metastases). Longevity was determined when a mouse attained a morbid state, characterized by the inability to reach food and water normally, or when the sum of its tumor volume exceeded 4 cm³. Moribund mice were euthanized

based on these IACUC approved criteria of our institution. The lung and other major organs were resected and weighed, and the lung-to-body weight ratio was calculated. The tissues were fixed with 10% formalin and prepared for later pathological evaluations.

Comparisons between SD vs. KD groups with respect to tumor size, and lung metastasis weight were performed using non-parametric Mann Whitney U tests. Serum measurement of insulin, BHB, and glucose were compared between groups using unpaired Student t-tests, also used for body weight comparisons. Longevity between groups was compared using log-rank testing.

Results

KD and SD had similar effects on body weight of mice

Four-week old, female mice were randomly divided into 6 groups of 4-10 mice each. Three groups were assigned to a standard diet (SD) and three to a ketogenic diet. Within each diet group, after 2 weeks, rapamycin was administered by oral gavage with 22 gauge feeding needle at a dose 0.4 mg/kg or 4 mg/kg daily for 2 weeks.

The mice in all SD groups and KD groups (with or without rapamycin) were given the same caloric energy from start point to the end (< 8 weeks). As shown in Fig 1A, the body weights of the mice in KD groups were not significantly different from those in SD groups.

KD reduced the blood glucose level in mice

Blood serum glucose concentrations in all mouse after one week in all KD groups (KD, KD r0.4, and KD r4), decreasing further after 3 weeks of KD feeding. Blood glucose was significantly lower than that of mice in all SD groups at all time points. Rapamycin at a low dose did not have a clear effect on the glucose level. Higher dose of rapamycin (4 mg/kg) enhanced the glucose levels slightly in both SD r4 and KD r4. At day 57 the mildly elevated glucose ratios of KD r4/KD and SD r4/SD were 158/127 ($p = 0.0042$) and 227/199 ($p = 0.0223$), respectively (Figure 1B).

KD increased the blood Beta-hydroxybutyrate level in mice

After 3 weeks, mice in all KD groups showed at least a four-fold elevation of serum BHB concentrations compared with SD mice. These elevations all reached statistical significance ($p < 0.005$). Rapamycin did not affect the BHB level (Figure 1C).

KD reduced the blood insulin concentration in mice

At study termination (see Methods), we collected the blood from each mouse and measured insulin levels. As Figure 1D demonstrates, the insulin serum concentrations in all SD mice groups were 8 to 20-fold higher than the levels in the respective KD mice groups ($p < 0.0005$). The paired comparison is shown in Table 1.

Serum bicarbonate, sodium, calcium, potassium, creatinine, BUN, were also measured with no significant differences found between KD and SD groups (data not shown). See **Fig 1**.

Fig 1. The blood glucose (A), beta hydroxybutyrate (B), and insulin (C) levels. Each glucose data point is a daily average of 3-9 mice, and each mouse was measured 3

times in that particular day at 3 different time points (9 am, 1 pm, and 5 pm). Each beta hydroxybutyrate data point is a daily average of 3-9 mice with single measurement per mouse. The insulin levels were measured when the mice moribund. The serum was used to measure insulin level with an ELISA method. The blue lines or bars represent the data from SD groups. The red lines or bars are the data from KD groups. r0.4 and r4 means rapamycin at the dose 0.4 mg/kg and 4 mg/kg for 2 weeks, respectively.

Table 1. Blood Insulin Level and Comparison

Compared Groups	Mean Insulin Levels (ng/ml)	P Value
SD vs KD	11.55: 1.08	0.0001
SD r0.4 vs KD r0.4	12.55: 0.96	0.0039
SD r4 vs KD r4	8.90: 0.62	<0.0001

KD inhibited tumor growth and prolonged mouse longevity.

Mice were fed SD or KD from 6 weeks of age until they reached a moribund condition. All tumor sizes were measured in each mouse weekly from day 1 to day 56, the time period during which no mice had yet reached moribundity. The combined tumor volumes (sum of all measurable tumor volumes in each mouse) of KD mice were smaller than that of SD mice (mean KD 506 mm³ vs, mean SD 1262 mm³, p <0.0001, 2way ANOVA; Fig 2A). Rapamycin further enhanced the tumor growth inhibition: the mean combined tumor volume of KD r0.4 and KD r4 versus KD was 359 mm³ vs. 506 mm³ (p = 0.0049) and 195 vs. 506 (p < 0.0001), respectively.

As shown in Figure 2B, the median survival of KD mice increased to 78 days as compared to 65 days for SD mice, a 20% increase (p = 0.0002, log rank test). KD, when

combined with rapamycin at dose 4 mg/kg further increased the median survival to 95 days when compared to KD diet alone (78 days, as above, $p = 0.002$); and vs. SD r4 (81 days, $p = 0.0049$). See **Fig 2**.

Figure 2. Tumor size and survival. Tumor size (A) was measured once a week. Volume (mm^3) was calculated as $0.5 L \times S^2$ (L and S is the longest and shortest dimensions). The sum of all visible tumor volumes in each mouse was used as its tumor volume, and each point represents tumor volumes from 3-9 mice. Longevity (B) was determined to the time a mouse became moribund. The blue and red lines are the data from SD groups KD groups respectively. r0.4 and r4 represents rapamycin at the dose 0.4 mg/kg and 4 mg/kg for 2 weeks, respectively.

KD reduced metastases in the lungs of mice

Moribund mice were euthanized and their major organs were resected as described in Methods. Only lungs were found to have metastatic tumors among all organ systems. Lungs were weighed before further pathological evaluation. The lung/body weight ratio of each mouse was also calculated as it is positively related to the lung tumor number (n) and/or size [21]. The data is shown in **Figure 3A** and Table 2.

Figure 3. The lung metastases. Moribund mice were weighed, and their lungs were resected and weighed after taking ≥ 1 ml blood out from cardiac puncture. (A) The lung-to-body weight ratio from each group is presented. Round dots represent KD groups. Triangle dots represent SD groups. r0.4 and r4 represent rapamycin at doses of 0.4

mg/kg and 4 mg/kg separately. (B) The lung tissue pathology images are shown. The top row demonstrates lung tissue sections from SD, SDr0.4, and SDr4 groups. The bottom row shows lung tissue sections from KD, KDr0.4, and KDr4. The arrows point out tumor nodules. The magnification is 2.5 x for all pictures. The overall mass of tumors is reduced in the KD images.

Table 2. Lung/Body Weight Ratios (mg/g)

Comparison	Average Ratio	P Value
SD/KD	26.0/15.3	< 0.0001
SD r0.4/KD r0.4	23.1/15.4	0.0542
SD r4/KD r4	16.4/10.2	0.0045
KD/KD r4	15.3/10.2	0.0163

There is a significant difference of average lung/body weight ratios between SD and KD groups: 26.0 ± 2.2 mg/g vs. 15.3 ± 3.3 mg/g, $p < 0.0001$. SD/KD with rapamycin at the lower dose of 0.4 mg/kg trended toward further inhibition of lung metastasis ($p=0.0542$), with lung/body weight ratio 23.1 ± 3.3 vs. 15.4 ± 4.5 mg/g. Rapamycin at a higher dose (4 mg/kg), when combined with KD showed more significant reduction of lung metastases: SD r4 vs. KD r4 was 16.4 ± 2.5 : 10.2 ± 1.9 mg/g, $p = 0.0045$, and KD vs. KD r4 was 15.3 ± 3.3 : 10.2 ± 1.9 , $p = 0.0163$, respectively. See **Fig 3**. (above).

Discussion

We have tested the effect of two diets, one very low in carbohydrate ($\leq 0.1\%$ of Calories); the other a standard diet (i.e. 80% calories from carbohydrate), on cancer

growth and animal survival in a spontaneous breast cancer model in mice. Strict limitation of carbohydrate was effective at inhibiting serum insulin and glucose and inducing ketosis. While there were no ‘cures’, average overall tumor mass was reduced. The ketogenic group demonstrated prolonged survival as well. We also compared the effects of two different doses of rapamycin when added to the diets. Rapamycin delayed tumor growth in a dose-dependent manner and improved survival in both dietary groups. Greatest effects were seen when KD and rapamycin were combined.

In all groups, with or without rapamycin, the KD mice demonstrated longer survival, lower serum glucose and lower insulin concentrations than the corresponding SD mice. The total lung tumor mass in the KD animals was significantly and substantially smaller than in the corresponding SD mice. In view of significantly longer survival of the KD animals, both of these results are consistent with slower tumor growth in KD vs. SD. KD, when administered alone, also trended toward decreased microvascular density vs. SD (data not shown).

Strict insulin inhibition can result in two principle effects, both of which have the potential to induce cancer cell programmed cell death and to reduce proliferation of cancer cells. First, reduced blood insulin concentrations at the cancer cell membrane results in less binding to the insulin receptor with resulting downstream inhibition of the PI3K-Akt-mTOR (PAM) signaling cascade [22], as well as the RAS-RAF-MEK-ERK pathway [23]. We therefore propose that reduced insulin concentration due to a ketogenic diet provides the potential to enhance programmed cell death by inhibiting the PAM cascade, and to reduce proliferation via both pathways [24] [25]. A general caveat, of course, is that well-known, common mutations causing constitutive activation of PAM

protein signals (e.g. PI3KCa) will resist programmed cell death and allow proliferation to continue [26]. Nonetheless those malignancies without these mutations can be therapeutically susceptible to the insulin inhibiting effects.

Second, hepatic ketogenesis due to insulin inhibition increases blood levels of the ketone bodies beta-hydroxybutyrate and acetoacetate, both of which have demonstrated histone deacetylase inhibitor (HDACi) effects at the cellular level. HDAC inhibitors are known to be capable of reducing cancer cell proliferation as well as enhancing programmed cell death [27] [28].

Reduced proliferation was indeed observed as seen in the reduced extent of lung metastatic mass to total body mass ratios with KD in all groups. However, while necrosis in the KD groups was also detected in tumor specimens, meaningful differences with SD could not be identified definitively with our small sample size. The potential of ketogenic diets to inhibit cancers has been suggested, mainly in animal models, for at least four decades [29] [30]. Human data has been sparse, limited mostly to case reports or small clinical trials [5] [31] [32] [33] [34]. Meanwhile, in the past decade, interest has grown in insulin inhibition as a potential cancer therapeutic adjunct. Metformin, for example, has been applied toward this end [35] [36]. Dietary carbohydrate restriction and metformin both reduce insulin secretion and glucose concentration, but the overall effect of carbohydrate restriction may have additional effects beyond those of metformin. This is observed in its ability to induce formation of ketone bodies, known histone deacetylase inhibitors.

The greatest value of ketogenic diets in cancer therapy likely lies in coupling their use with existing agents, permitting additive or synergistic effects with toxic drugs.

These effects can result in reduction of drug doses while improving overall efficacy, thus extending patient survival while improving the quality of life during a period of greater longevity.

A ketogenic diet combined with existing drugs provides a promising approach to increase the therapeutic efficacy of existing cancer therapies at a lower level of overall toxicity

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References

- 326 1. Ketogenic diets as an adjuvant cancer therapy: History and potential mechanism: Hearing
327 before the Redox Biol(2014).
- 328 2. Allen BG, Bhatia SK, Buatti JM, Brandt KE, Lindholm KE, Button AM, et al. Ketogenic
329 diets enhance oxidative stress and radio-chemo-therapy responses in lung cancer xenografts. Clin
330 Cancer Res. 2013;19(14):3905-13. doi: 10.1158/1078-0432.CCR-12-0287. PubMed PMID:
331 23743570; PubMed Central PMCID: PMC3954599.
- 332 3. Klement RJ. Beneficial effects of ketogenic diets for cancer patients: a realist review with
333 focus on evidence and confirmation. Med Oncol. 2017;34(8):132. Epub 2017/06/28. doi:
334 10.1007/s12032-017-0991-5. PubMed PMID: 28653283.
- 335 4. Zahra A, Fath MA, Opat E, Mapuskar KA, Bhatia SK, Ma DC, et al. Consuming a
336 Ketogenic Diet while Receiving Radiation and Chemotherapy for Locally Advanced Lung
337 Cancer and Pancreatic Cancer: The University of Iowa Experience of Two Phase 1 Clinical
338 Trials. Radiat Res. 2017;187(6):743-54. Epub 2017/04/25. doi: 10.1667/RR14668.1. PubMed
339 PMID: 28437190; PubMed Central PMCID: PMCPMC5510645.
- 340 5. Fine EJ, Segal-Isaacson CJ, Feinman RD, Herszkopf S, Romano MC, Tomuta N, et al.
341 Targeting insulin inhibition as a metabolic therapy in advanced cancer: a pilot safety and
342 feasibility dietary trial in 10 patients. Nutrition. 2012;28(10):1028-35. doi:
343 10.1016/j.nut.2012.05.001. PubMed PMID: 22840388.
- 344 6. Hsieh MH, Choe JH, Gadhvi J, Kim YJ, Arguez MA, Palmer M, et al. p63 and SOX2
345 Dictate Glucose Reliance and Metabolic Vulnerabilities in Squamous Cell Carcinomas. Cell
346 Rep. 2019;28(7):1860-78 e9. doi: 10.1016/j.celrep.2019.07.027. PubMed PMID: 31412252.
- 347 7. Hopkins BD, Pauli C, Du X, Wang DG, Li X, Wu D, et al. Suppression of insulin
348 feedback enhances the efficacy of PI3K inhibitors. Nature. 2018;560(7719):499-503. Epub

349 2018/07/28. doi: 10.1038/s41586-018-0343-4. PubMed PMID: 30051890; PubMed Central
350 PMCID: PMCPMC6197057.

351 8. Basho RK, Yam C, Gilcrease M, Murthy RK, Helgason T, Karp DD, et al. Comparative
352 Effectiveness of an mTOR-Based Systemic Therapy Regimen in Advanced, Metaplastic and
353 Nonmetaplastic Triple-Negative Breast Cancer. *Oncologist*. 2018;23(11):1300-9. Epub
354 2018/08/25. doi: 10.1634/theoncologist.2017-0498. PubMed PMID: 30139837; PubMed Central
355 PMCID: PMCPMC6291334.

356 9. Jain V, Patel RK, Kapadia Z, Galiveeti S, Banerji M, Hope L. Drugs and hyperglycemia:
357 A practical guide. *Maturitas*. 2017;104:80-3. Epub 2017/09/20. doi:
358 10.1016/j.maturitas.2017.08.006. PubMed PMID: 28923179.

359 10. Schindler CE, Partap U, Patchen BK, Swoap SJ. Chronic rapamycin treatment causes
360 diabetes in male mice. *Am J Physiol Regul Integr Comp Physiol*. 2014;307(4):R434-43. doi:
361 10.1152/ajpregu.00123.2014. PubMed PMID: 24965794; PubMed Central PMCID:
362 PMC4137151.

363 11. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab*. 2014;19(3):373-
364 9. Epub 2014/02/11. doi: 10.1016/j.cmet.2014.01.001. PubMed PMID: 24508508; PubMed
365 Central PMCID: PMCPMC3972801.

366 12. Iyikesici MS. Feasibility study of metabolically supported chemotherapy with weekly
367 carboplatin/paclitaxel combined with ketogenic diet, hyperthermia and hyperbaric oxygen
368 therapy in metastatic non-small cell lung cancer. *Int J Hyperthermia*. 2019;36(1):446-55. doi:
369 10.1080/02656736.2019.1589584. PubMed PMID: 30931666.

370 13. Gluschnaider U, Hertz R, Ohayon S, Smeir E, Smets M, Pikarsky E, et al. Long-chain
371 fatty acid analogues suppress breast tumorigenesis and progression. *Cancer Res*.

- 2014;74(23):6991-7002. Epub 2014/10/12. doi: 10.1158/0008-5472.CAN-14-0385. PubMed
PMID: 25304261.
14. Schwartz L, Seyfried T, Alfarouk KO, Da Veiga Moreira J, Fais S. Out of Warburg
effect: An effective cancer treatment targeting the tumor specific metabolism and dysregulated
pH. *Semin Cancer Biol.* 2017;43:134-8. Epub 2017/01/26. doi:
10.1016/j.semcancer.2017.01.005. PubMed PMID: 28122260.
15. van der Louw E, Olieman JF, van den Bemt P, Bromberg JEC, Oomen-de Hoop E,
Neuteboom RF, et al. Ketogenic diet treatment as adjuvant to standard treatment of glioblastoma
multiforme: a feasibility and safety study. *Ther Adv Med Oncol.* 2019;11:1758835919853958.
Epub 2019/07/02. doi: 10.1177/1758835919853958. PubMed PMID: 31258628; PubMed
Central PMCID: PMC6589986.
16. Khodabakhshi A, Akbari ME, Mirzaei HR, Mehrad-Majd H, Kalamian M, Davoodi SH.
Feasibility, Safety, and Beneficial Effects of MCT-Based Ketogenic Diet for Breast Cancer
Treatment: A Randomized Controlled Trial Study. *Nutr Cancer.* 2019:1-8. Epub 2019/09/10. doi:
10.1080/01635581.2019.1650942. PubMed PMID: 31496287.
17. Woodhouse C, Ward T, Gaskill-Shiple M, Chaudhary R. Feasibility of a modified
Atkins diet in glioma patients during radiation and its effect on radiation sensitization. *Curr
Oncol.* 2019;26(4):e433-e8. Epub 2019/09/25. doi: 10.3747/co.26.4889. PubMed PMID:
31548811; PubMed Central PMCID: PMC6726259.
18. Nutrient Requirements of Laboratory Animals: Fourth Revised Edition, 1995.
Washington (DC)1995.
19. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of
polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell*

- 395 Biol. 1992;12(3):954-61. doi: 10.1128/mcb.12.3.954. PubMed PMID: 1312220; PubMed Central
396 PMCID: PMC369527.
- 397 20. Shishido SN, Delahaye A, Beck A, Nguyen TA. The anticancer effect of PQ1 in the
398 MMTV-PyVT mouse model. Int J Cancer. 2014;134(6):1474-83. doi: 10.1002/ijc.28461.
399 PubMed PMID: 24038078; PubMed Central PMCID: PMC3948329.
- 400 21. Zou Y, Zong G, Ling YH, Hao MM, Lozano G, Hong WK, et al. Effective treatment of
401 early endobronchial cancer with regional administration of liposome-p53 complexes. J Natl
402 Cancer Inst. 1998;90(15):1130-7. doi: 10.1093/jnci/90.15.1130. PubMed PMID: 9701362.
- 403 22. Senapati P, Kato H, Lee M, Leung A, Thai C, Sanchez A, et al. Hyperinsulinemia
404 promotes aberrant histone acetylation in triple-negative breast cancer. Epigenetics Chromatin.
405 2019;12(1):44. Epub 2019/07/19. doi: 10.1186/s13072-019-0290-9. PubMed PMID: 31315653;
406 PubMed Central PMCID: PMCPMC6636093.
- 407 23. Nagini S, Sophia J, Mishra R. Glycogen synthase kinases: Moonlighting proteins with
408 theranostic potential in cancer. Semin Cancer Biol. 2019;56:25-36. Epub 2018/01/09. doi:
409 10.1016/j.semcancer.2017.12.010. PubMed PMID: 29309927.
- 410 24. Radogna F, Dicato M, Diederich M. Cancer-type-specific crosstalk between autophagy,
411 necroptosis and apoptosis as a pharmacological target. Biochem Pharmacol. 2015;94(1):1-11.
412 Epub 2015/01/07. doi: 10.1016/j.bcp.2014.12.018. PubMed PMID: 25562745.
- 413 25. Thakur B, Kumar Y, Bhatia A. Programmed necrosis and its role in management of
414 breast cancer. Pathol Res Pract. 2019;215(11):152652. Epub 2019/10/02. doi:
415 10.1016/j.prp.2019.152652. PubMed PMID: 31570277.
- 416 26. Han S, Witt RM, Santos TM, Polizzano C, Sabatini BL, Ramesh V. Pam (Protein
417 associated with Myc) functions as an E3 ubiquitin ligase and regulates TSC/mTOR signaling.

418 Cell Signal. 2008;20(6):1084-91. Epub 2008/03/01. doi: 10.1016/j.cellsig.2008.01.020. PubMed
419 PMID: 18308511; PubMed Central PMCID: PMCPMC2435383.

420 27. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, et al.
421 Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase
422 inhibitor. Science. 2013;339(6116):211-4. Epub 2012/12/12. doi: 10.1126/science.1227166.
423 PubMed PMID: 23223453; PubMed Central PMCID: PMCPMC3735349.

424 28. Wilson AJ, Chueh AC, Togel L, Corner GA, Ahmed N, Goel S, et al. Apoptotic
425 sensitivity of colon cancer cells to histone deacetylase inhibitors is mediated by an Sp1/Sp3-
426 activated transcriptional program involving immediate-early gene induction. Cancer Res.
427 2010;70(2):609-20. Epub 2010/01/14. doi: 10.1158/0008-5472.CAN-09-2327. PubMed PMID:
428 20068171; PubMed Central PMCID: PMCPMC2939837.

429 29. Tisdale MJ, Brennan RA, Fearon KC. Reduction of weight loss and tumour size in a
430 cachexia model by a high fat diet. Br J Cancer. 1987;56(1):39-43. Epub 1987/07/01. doi:
431 10.1038/bjc.1987.149. PubMed PMID: 3620317; PubMed Central PMCID: PMCPMC2001676.

432 30. Beck SA, Tisdale MJ. Nitrogen excretion in cancer cachexia and its modification by a
433 high fat diet in mice. Cancer Res. 1989;49(14):3800-4. Epub 1989/07/15. PubMed PMID:
434 2736521.

435 31. Nebeling LC, Miraldi F, Shurin SB, Lerner E. Effects of a ketogenic diet on tumor
436 metabolism and nutritional status in pediatric oncology patients: two case reports. J Am Coll
437 Nutr. 1995;14(2):202-8. Epub 1995/04/01. doi: 10.1080/07315724.1995.10718495. PubMed
438 PMID: 7790697.

439 32. Champ CE, Palmer JD, Volek JS, Werner-Wasik M, Andrews DW, Evans JJ, et al.
440 Targeting metabolism with a ketogenic diet during the treatment of glioblastoma multiforme. J

441 Neurooncol. 2014;117(1):125-31. Epub 2014/01/21. doi: 10.1007/s11060-014-1362-0. PubMed
 442 PMID: 24442482.

443 33. Klement RJ, Champ CE. Calories, carbohydrates, and cancer therapy with radiation:
 444 exploiting the five R's through dietary manipulation. Cancer Metastasis Rev. 2014;33(1):217-29.
 445 Epub 2014/01/18. doi: 10.1007/s10555-014-9495-3. PubMed PMID: 24436017; PubMed Central
 446 PMCID: PMCPMC3988521.

447 34. Seyfried TN, Flores R, Poff AM, D'Agostino DP, Mukherjee P. Metabolic therapy: a new
 448 paradigm for managing malignant brain cancer. Cancer Lett. 2015;356(2 Pt A):289-300. Epub
 449 2014/07/30. doi: 10.1016/j.canlet.2014.07.015. PubMed PMID: 25069036.

450 35. Goodwin PJ. Obesity, insulin resistance and breast cancer outcomes. Breast. 2015;24
 451 Suppl 2:S56-9. Epub 2015/08/19. doi: 10.1016/j.breast.2015.07.014. PubMed PMID: 26283600.

452 36. Goodwin P, Esplen MJ, Butler K, Winocur J, Pritchard K, Brazel S, et al.
 453 Multidisciplinary weight management in locoregional breast cancer: results of a phase II study.
 454 Breast Cancer Res Treat. 1998;48(1):53-64. Epub 1998/04/16. doi: 10.1023/a:1005942017626.
 455 PubMed PMID: 9541189.

456

Fig 1.

Fig. 1

Fig 2.

Fig. 2

Fig 3A.

Fig. 3A

Fig 3B.

Rapamycin (mg/kg)

0

0.4

4.0

SD

KD

Fig. 3B