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Short-term calorie and protein restriction provide partial protection from chemotoxicity but do not delay glioma progression

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Abstract

Short-term starvation (STS) protects normal cells while simultaneously sensitizing malignant cells to high-dose chemotherapeutic drugs in mice and possibly patients. The fasting-dependent protection of normal cells and sensitization of malignant cells depends, in part, on reduced levels of insulin-like growth factor-1 (IGF-1) and glucose. Calorie restricted diets with defined macronutrient (carbohydrate, protein, fat) ratios were evaluated for the effects on stress sensitization markers and protection in mice treated with high-dose chemotherapy. We show that short-term CR significantly reduced both glucose and IGF-1 levels, but when specific macronutrient deficiencies were tested, only the complete lack of proteins reduced IGF-1 levels. Short-term 50% CR combined with either severe protein-deficiency or ketogenic diets improved chemotoxicity resistance similarly to the standard 50% CR, but did not result in the high protection caused by STS. Notably, a high protein diet reversed the beneficial effects of short-term CR. In a subcutaneous mouse model of glioma, feeding a low protein (4% calories from protein) diet for more than 20 days did not delay tumor progression once the tumor became palpable. Also, cycles of short-term (3 days) 50% CR did not augment the chemotherapy efficacy of cisplatin in a murine breast cancer model. These results indicate that the protection from chemotoxicity and retardation of the progression of certain tumors achieved with fasting is not obtained with short-term calorie and/or macronutrient restriction.

Keywords

Calorie restriction; Protein restriction; Fasting; Macronutrients; Stress resistance; Tumor progression; Insulin-like growth factor 1; Chemotherapy

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Conflict of interest

VDL and TEM have equity interest in L-Nutra, a company that develops medical food.

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1. Introduction

Calorie restriction (CR), defined as a reduction in calorie intake without malnutrition, is the most potent and reproducible intervention known to simultaneously protect against age-related diseases (Omodei and Fontana, 2011), including cancer (Longo and Fontana, 2010), and increase the median and maximum lifespan in mammals (Fontana et al., 2010). From an evolutionary point of view, the effects of CR may be explained by the ability of organisms to maximize survival during periods of food shortage (Kirkwood et al., 2000). CR decreases serum levels of IGF-1 and insulin, and reduces the activity of the TOR (target of rapamycin) pathway, an intracellular signal-transduction cascade conserved from yeast and plants to mammals (mTOR), where it integrates nutrient availability, as well as hormone-, growth factor-,and stress-signaling. Similarly to CR, the inhibition of TOR by genetic or pharmacological means extends lifespan (Blagosklonny, 2010; Fabrizio et al., 2001; Sharp and Bartke, 2005). CR reduces oxidative stress (Sohal and Weindruch, 1996; Youngman et al., 1992) and cell proliferation while enhancing autophagy (Cuervo et al., 2005; Wohlgemuth et al., 2007) and certain DNA repair processes (Weraarchakul et al., 1989).

Macronutrient defined diets, with altered proportions of fat, carbohydrates and protein, do not generally have major effects on the lifespan and healthspan of rodents unless calorie restriction is applied to these modified diets (Iwasaki et al., 1988; Masoro, 1990; Ross and Bras, 1973). Of note, a severe restriction of dietary protein (or specific amino acids) can extend the lifespan of rodents by up to 20% independently of the caloric intake (Pamplona and Barja, 2006), may reduce tumorigenesis (Youngman, 1993) and protects against renal and hepatic ischemic injury, resulting in reduced inflammation and preserved organ function (Peng et al., 2012). Reduced levels of serum IGF-1 in rats and mice fed with protein-restricted diets might explain the beneficial effects on longevity (Sonntag et al., 1999).

Based on the ability of complete starvation to have more potent and rapid effects on cellular protection, resistance to ischemia reperfusion injury and longevity of certain organisms compared to CR (Longo et al., 1997; Mitchell et al., 2010; Wei et al., 2008), we have described the beneficial role of cycles of several days of a complete lack of calories (short-term starvation, STS), followed by a period of *ad lib* feeding that allows rodents and humans to rapidly regain normal weight, in cancer treatment (Lee and Longo, 2011). STS selectively protects normal cells, mice, and possibly patients from chemotoxicity without interfering with the therapeutic outcome on cancer cells, an effect we termed Differential Stress Resistance (DSR) (Lee et al., 2010; Raffaghello et al., 2008; Safdie et al., 2009). Furthermore, fasting sensitized 15 out of 17 malignant cell lines tested to chemotherapeutic treatment *in vitro* and augmented the efficacy of chemotherapeutic agents in mouse models of tumor progression, including breast cancer, melanoma, neuroblastoma and glioblastoma multiforme (Differential Stress Sensitization or DSS) (Lee et al., 2012; Safdie et al., 2012). The fasting-induced DSR may be attributed to the redistribution of finite energy and resources from reproduction/growth to cellular protection/maintenance in normal, but not cancer cells, when nutrients are scarce or absent (Kirkwood, 2005), driven in part by differential regulation of the nutrient-sensing TOR network (Blagosklonny, 2010). The enhanced stress resistance of normal cells and the sensitization of tumor cells are in part modulated by reduced glucose availability and dampening of IGF-1 levels (Lee and Longo, 2011; Lee et al., 2010, 2012; Raffaghello et al., 2008).

Circulating IGF-1, acting synergistically with other hormones and growth factors, regulates energy metabolism, cell proliferation and differentiation, body size and lifespan in response to calorie and protein availability (Flototto et al., 2001; Giovannucci et al., 2003; Prisco et al., 1999; Yu et al., 2003). In addition, IGF-1 exerts a potent tumorigenic effect on a variety of cancer cells by promoting proliferation and inhibiting apoptosis (Prisco et al., 1999;

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Ramsey et al., 2002). The reduction in IGF-1 plays a key role in protecting against cancer and slowing aging in mammals (Colbert et al., 2009; Hursting et al., 1999; Sonntag et al., 1999). However, in humans, long-term CR causes a modest reduction in fasting glucose and has no significant effect on IGF-1 if not combined with protein restriction (Fontana et al., 2008). Further, CR requires months to years to be effective in humans and is not a practical preventive or treatment strategy for cancer patients since it may exacerbate weight loss in patients prone to it and cause weight loss in patients who may otherwise not lose and even gain weight (Lee and Longo, 2011). In contrast, fasting for an average of 60 h prior to and 24 h post chemotherapy, which has been shown to lower IGF-1 by 40% or more and cause a major reduction in glucose levels, was well tolerated by patients receiving a variety of chemotherapy drugs. These patients reported a reduction in common side effects caused by chemotoxicity (Safdie et al., 2009).

Here we have begun to address the question of whether different types of macronutrient restriction or CR can partially mimic the effects of fasting on serum levels of IGF-1 and glucose, protection of mice and sensitization of cancer cells in response to chemotherapy treatment.

2. Material and methods

2.1. Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California. 12–15 week old female CD-1, BalB/C or C57BL/6N mice (Charles River) were maintained in a pathogen-free environment throughout the experiments.

2.2. Macronutrient defined diets

AIN93G standard chow (Harlan) was used as the reference diet and supplied to all mice if not indicated otherwise. Diets modified in the macronutrient composition (fat, protein and carbohydrates) were all based on AIN93G (Fig. 1 and Table S1). Diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) had calories from protein sources reduced to 20% compared to the AIN93G formulation; the 0% P diet contained no protein; all these diets were isocaloric to the AIN93G standard chow. The low-carbohydrate LCHP diet had calories from carbohydrates reduced to 20% compared to the AIN93G formulation (13% *vs.* 63.9%) but contained more protein (45.2%) and fat (41.8%). The ketogenic high fat diet 60% HF was designed to supply 60% of the consumed calories from fat sources, the calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet was a ketogenic diet which contains 90% of fat while supplying only minimal carbohydrates (less than 1%) and half of the protein content (9%).Detailed diet composition and calorie content are summarized in Table S2. Mice were fed with the AIN93G control diet before commencing the experiments and based on their initial bodyweight grouped into the experimental groups (N = 5/group). Mice were acclimated to the test diets one week prior to the experiments (adjustment schedule is shown in Table S3). All diets were supplied *ad lib* unless indicated otherwise.

2.3. Calorie restriction (CR) and short-term starvation (STS)

For calorie restriction using the AIN93G diet, the standard chow was grounded into a powder and mixed in hydrogel (clear H_2O) in the necessary amounts to achieve 60%, 50%, 40%, 20%, and 10% calorie density of AIN93G (Table S4). The calorie restricted macronutrient modified diets were prepared similarly (Table S5). To avoid malnutrition, all diets were supplemented with vitamins, minerals, fiber and essential fatty acids matching those in AIN93G. Baseline food intake (3.7 g or 14 kcal/day) was determined with AIN93G

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feeding prior to the experiment (data not shown). For the short-term starvation (STS) regimen, mice had no access to food for up to 60 h.

For all CR and STS experiments, mice were single caged in standard shoebox-cages which were refreshed daily to avoid coprophragy or feeding on residual chow. Animals had access to water at all times and were supplied with hydrogel to ensure sufficient hydration. Bodyweight of each individual animal was measured routinely during the CR or STS regimens.

2.4. Blood collection for glucose and IGF-1 measurements

Mice were anesthetized with 2% inhalant isoflurane and blood was collected by left ventricular cardiac puncture. Blood was collected in tubes coated with K^2-EDTA for serum preparation (BD). Blood glucose was measured with the Precision Xtra blood glucose monitoring system (Abbott Laboratories). IGF-1 was measured using a mouse specific ELISA kit (R&D Systems).

2.5. Resistance to high-dose chemotherapy

12–15 week-old female CD-1 mice weighing 25–32 g were starved for up to 60 h (STS) or fed with the macronutrient modified 50% CR diets for 3 days, followed by an intravenous injection of 24 mg/kg Doxorubicin (DXR, Bedford Laboratories). In all experiments, mice were offered AIN93G standard chow after chemodrug injection and monitored daily. Animals showing signs of severe stress and/or deteriorating health status were designated as moribund and were euthanized.

2.6. Subcutaneous tumor model

Murine 4T1 breast cancer and GL26 glioma cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2. Cells in log phase growth were washed and suspended in PBS at 2×10^6 cells/mL and injected subcutaneously (s.c., 2×10^5 cells/mouse in 100 μL PBS) in the lower back region of the mouse. Tumor size was measured using a caliper. To mimic multi-cycle treatments in humans, mice were treated intravenously (i.v., lateral tail vein) with Cisplatin (Teva Parenteral Medicines Inc.) three times on days 15, 33 and 44 after tumor inoculation at 12, 8 and 8 mg/kg body weight, respectively. Mice were monitored daily and animals showing signs of severe stress, deteriorating health status or excess tumor load (2000 mm^3) were designated as moribund and euthanized.

2.7. Statistical analysis

Comparisons between groups in the glucose and IGF-1 measurements were done with ANOVA, followed by Tukey's multiple comparison using GraphPad Prism v.5. All statistical analyses were two-sided and P values < 0.05 were considered significant.

3. Results

3.1. Effects of calorie restriction on glucose and IGF-1 levels

Short-term starvation (STS) reduces serum levels of glucose and IGF-1, increases cellular protection against high-dose chemotherapy (Lee et al., 2010; Raffaghello et al., 2008), and sensitizes malignant cells to chemotherapeutic drugs (Lee et al., 2012). STS effects on glucose and IGF-1 are usually achieved once animals lost approximately 20% bodyweight. Thus, we used the 20% weight-loss as a criterion to compare glucose and IGF-1 levels of calorie restricted diets to those obtained from a 60 h STS regimen.

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The 20% weight-loss threshold was reached at 4 days for 90% CR, 6 days for 80% CR, 9 days for 60% CR, or 13 days for 40% CR (Fig. 2A and Fig. S1A). As expected, the time to achieve 20% weight-loss strongly depends on the severity of the calorie restriction (linear fit with $r^2 = 0.9976$; Fig.2B). At 48 h, the reduction in blood glucose levels correlates with the severity of the calorie restriction (linear fit with $r^2 = 0.7931$; Supplementary Fig. S1B). The 60 h fasting regimen (STS) reduces blood glucose levels by 70% compared to that in *ad lib* fed mice (Fig. 2C, $p < 0.001$). The 4 day 90% CR regime reduced blood glucose by approximately 40%, significantly less than STS ($p < 0.05$). In addition, we observed a trend for the effect of CR in lowering blood glucose depending on the length of the CR-feeding: the glucose level in the 13-day 40% CR feeding was significantly ($p < 0.05$) lower than in the 4-day long 90% CR group. However, no calorie restricted group resulted in blood glucose levels that were lower than in the 60 h fasting group; and 9 or more days of CR were required to obtain glucose lowering effects in the range of those in the fasted group (Fig. 2C). Mice of all experimental CR groups, independently of the severity of the restriction, reached similar serum IGF-1 levels at the 20% weight-loss margin and had significantly ($p < 0.001$) lower IGF-1 levels than mice in the ad lib control group (Fig. 2D).

3.2. Effect of macronutrient defined diets on glucose and IGF-1 levels

We designed a set of macronutrient-defined diets (Fig. 1 and Table S1) based on the AIN93G rodent chow to determine whether the restriction of specific dietary constituents could mimic the effects of STS or short-term CR on blood glucose and/or serum IGF-1. The low protein diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) have calories from protein sources reduced to 20% compared to the original AIN93G formulation while carbohydrates and fat are increased to maintain the diets isocaloric to AIN93G. The 0% P diet contains no protein; carbohydrates as well as fat are increased proportionally to keep the diet isocaloric to the standard chow. The LCHP diet has the calories from carbohydrate sources reduced to 20% compared to the original AIN93G formulation (13% *vs.* 63.9%) but supplies more protein and fat. The high fat ketogenic diet 60% HF was designed to supply 60% of the consumed calories from fat sources, the calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet is a ketogenic diet that contains 90% of the calories from fat while supplying only minimal (less than 1%) carbohydrates and has 9% of the calories from protein. Due to the higher fat proportions, the LCHP, 60% HF and 90% HF diets have a high caloric-density compared to the AIN93G standard chow. Detailed diet composition and calorie content are summarized in Table S2.

Female CD-1 mice were fed *ad lib* with the experimental diets for nine consecutive days to establish bodyweight profiles (Fig. 3A, B) and to monitor the caloric intake (Fig. 3C, D). We did not observe any significant food aversion but noticed that mice fed with the diet lacking proteins completely (0% P) reduced food consumption after 6 days (Fig. 3C). The reduced calorie intake caused weight-loss for animals in this experimental group (Fig. 3A). Mice in the ketogenic high-fat groups (60% HF and 90% HF) consumed more calories during the 9 days of feeding than mice fed with the AIN93G standard chow (Fig. 3D) and mice fed *ad lib* with the ketogenic 90% HF diet rapidly gained weight after 4–5 days (Fig. 3B). CD-1 mice in the experimental groups fed with diets 20% P and LCHP showed no difference in calorie intake or bodyweight compared to the mice fed with the AIN93G control diet (Fig. 3A, C).

Blood glucose levels at day 2, day 5 and at day 9 from mice on the macronutrient modified diets were not different from those on the standard chow diet (Fig. S2 and data not shown). By contrast, serum IGF-1 levels were significantly elevated ($p < 0.05$) in mice on the ketogenic 60%HF diet for 9 days but not for mice fed with the ketogenic 90% HF diet (Fig. 3E). Interestingly, not only the macronutrient composition (e.g. the protein content) but also

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the fatty acid source differentially modulated circulating IGF-1 levels: the low protein diet 20% P-1 (containing soybean oil as the only fat source) did not reduce IGF-1 levels but the low protein diet 20% P-2 (coconut oil as the only fat source) significantly ($p < 0.05$) reduced IGF-1 levels and there are no differences in these diets other than the fat source. The most noticeable effect on serum IGF-1 was in mice fed with the protein deficient diet 0% P for 9 days. Circulating IGF-1 was reduced to approximately 30% of that in mice on the standard chow (Fig. 3E). The protein deficient diet 0% P was the only diet that reduced serum IGF-1 levels similarly to the 60 h short-term starvation.

3.3. Short-term calorie restriction and fasting improve stress resistance

In mice, reduced serum IGF-1 and blood glucose levels promote the capability to cope with toxicity induced by high-dosed chemotherapeutic agents (Lee et al., 2010; Raffaghello et al., 2008). Since short-term calorie restriction, but not the macronutrient defined diets (except for complete protein removal), reduced IGF-1 and glucose levels, we used a combinatorial approach to test whether diets with defined macronutrient deficiency fed at 50% of the regular daily calorie intake could result in enhanced chemotoxicity protection. 20% P diets were not included in the stress resistance experiments due to the fact that diet 0% P showed much more pronounced effects on serum IGF-1.

Stress resistance was tested in CD-1 mice fed either *ad lib* with AIN93G standard chow or with macronutrient defined diets reduced to 50% of the normal calorie intake for three days prior to doxorubicin (DXR, 24 mg/kg, i.v.) treatment (Fig. 4A). In the 50% calorie restricted groups mice lost 12–15% of their initial bodyweight after 3 days, whereas in the STS group mice lost 20% of their weight after 60 h. Following DXR treatment, AIN93G chow was provided *ad lib* for all animals and the mice regained weight until chemotoxicity-induced weight-loss set in (Fig. S3A, B). The weight-loss continued in all experimental groups until day 8 post injection, after which many animals slowly recovered. Mice fed with the calorie restricted 0% P and LCHP diets never fully recovered their initial weight (Fig. S3A). Animals started to succumb to chemotoxicity 9–18 days post injection (Fig. 4A), in agreement with the reported onset and nadir days of myelo-suppression after DXR treatment (http://dailymed.nlm.nih.gov). Mice were considered survivors if they were alive 25 days post DXR injection. Mice fed *ad lib* with the AIN93G diet 3 days prior to DXR injection showed the worst outcome with only 16% surviving by day 25 (Fig. 4A). In contrast to the *ad lib* fed mice, the great majority (89%)of fasted (60 h)mice survived the high-dose chemotherapy. Control mice treated with DXR showed signs of toxicity including reduced mobility, ruffled hair and hunched back posture whereas mice in the STS group showed no visible signs of stress or pain after the treatment (data not shown). Three days feeding of the combination of 50% CR with macronutrient modification prior to DXR injection improved the stress resistance in mice and resulted in 45–55% survival (Fig. 4A). There was no indication that fat or carbohydrate content affected the results because all diets achieved a similar rate of protection. The data indicates that short-term CR, not the fat or carbohydrate composition of the diet, confers partial chemo-protection which is not as potent as to those caused by fasting. Mice fed with the 50% CR LCHP diet performed worse than all other CR fed groups, presumably because of the effect of the high protein content of this diet on IGF-1 (Lee et al., 2010).

Blood glucose measurements revealed that three-days feeding of the calorie restricted modified diets was not sufficient to significantly reduce glucose levels, with the exception of the 50% CR ketogenic 90% HF diet (Fig. 4B). The reduction in glucose levels in the ketogenic group however was not associated with enhanced stress resistance. Mice in the STS group had significantly lower blood glucose levels than all other experimental groups (Fig. 4B).

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3.4. A low protein diet does not delay GL26 glioma progression

Diets low in protein have been shown to lower cancer risks while high-calorie and high-protein diets are linked to obesity and promote hormonal, metabolic, and inflammatory alterations that modulate carcinogenesis (Calle and Kaaks, 2004; Kaaks and Lukanova, 2002). To test the effects of a low protein diet in a glioma model, we switched mice from the standard chow (18.8% of calories are from protein, Table S1) to a diet low in protein (20% P-1, 3.9% of calories are from protein) 10 days after the implantation of GL26 cells, when the tumor was palpable (Fig. 5A). Low protein diet fed mice displayed tumor progression that was not distinguishable from that in mice fed *ad lib* with the AIN93G diet (Fig. 5A). These results indicate that the tumor progression could not be retarded by protein-restriction once the tumor was established.

3.5. Short-term intermittent calorie restriction does not enhance efficacy of chemotherapy against breast cancer

The efficacy of STS in augmenting the treatment of various cancers is twofold: it protects against chemotherapy-induced toxicity to normal cells/tissues and sensitizes malignant cells to chemotherapeutic agents (Lee et al., 2010, 2012). Nonetheless, even short interval fasting (e.g. 4 days) can be a challenge for the majority of people and thus the “milder” calorie restricted approach could be a more feasible solution. To test whether a short-term intermittent 50% CR (ICR) diet could result in similar beneficial effects as the established fasting protocols, we implanted murine 4T1 breast cancer cells subcutaneously into female BalB/C mice and monitored the tumor progression. Twelve days after tumor implantation, the tumor volume was measured and mice were assigned to either the untreated control group (AIN93G), a group treated with cisplatin (CDDP) or a group intermittingly fed with 50% CR (ICR) for three days prior to cisplatin treatment. The tumor in the untreated control group progressed rapidly and reached the experimental endpoint volume of 2000 mm^3 54 days after tumor implantation (Fig. 5B, black circles). Three cycles of cisplatin treatment delayed the tumor progression; the tumor volume of these mice was approximately half the size of that in untreated mice (Fig. 5B, blue squares). In contrast to STS (Lee et al., 2012), an intermittent 50% calorie restricted AIN93G feeding regimen fed to mice for three days prior to the cisplatin injections did not result in the sensitization of the tumor and did not augment the chemotherapy (Fig. 5B, orange triangle). Tumor volumes in this experimental group did not significantly differ from tumor volumes in mice that were treated with cisplatin alone.

4. Discussion

We have previously shown that a major reduction in blood glucose and IGF-1 levels is partly responsible for the beneficial effects of 2–3 days of fasting in animal cancer models. In mice, short-term fasting reduces bodyweight by 20% or more, serum IGF-1 by up to 75%, and glucose by up to 70%. Under these conditions, animals become highly stress resistant (Lee et al., 2010; Raffaghello et al., 2008), in agreement with the results in yeast (Longo, 1999). Also, fasting sensitizes a variety of tumors to chemo-and radio-therapy (Lee et al., 2012; Safdie et al., 2012). When we applied 20% weight-loss as an endpoint, as expected, various degrees of CR regimens resulted in progressively quicker weight losses but also reductionsinIGF-1 and glucose. However, we observed that the much shorter STS regimen had more pronounced effects on glucose than most of the CR diets, even when the CR diets were maintained for 9–13 days and caused an equivalent 20% weight-loss. The less pronounced effects of calorie restricted diets, when compared to short-term starvation, might be explained by a distinct physiological response that is unique to conditions under which nutrients are completely absent (Lee and Longo, 2011). For example, the decrease in blood

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glucose caused by short-term fasting in this study was 70% and occurred within 60 h vs. the 40% glucose reduction caused by a 90% CR diet after 96 h.

When deprived of food, mammals generally undergo three metabolic stages (Wang et al., 2006): 1) a post-absorptive phase, lasting for 10 or more hours following food ingestion, which involves the use of glycogen as the main stored energy source, 2) an amino acid-dependent glucose generation by gluconeogenesis once the liver glycogen storage has been depleted, and 3) a phase in which the remaining glucose is mostly consumed by the brain while glycerol and fatty acids are released from adipose tissue and become the major energy source. The fat-derived ketonebodies also become a major carbon source in a matter of days of fasting. Within the body, these changes trigger a cellular response including the down-regulation of pathways involved in proliferation, cell growth and the reduced production of reactive oxygen species while simultaneously increasing genomic stability and cellular stress resistance (Holzenberger et al., 2003; Hursting et al., 1999; Lee and Longo, 2011; Longo and Fontana, 2010; Sohal and Weindruch, 1996). Glucose is the major energy source for proliferating cells such as malignant cells and elevated blood glucose has been associated with increased cancer risk (Rapp et al., 2006; Stocks et al., 2009). Many cancer cells have elevated glucose uptake rates and rely on glycolysis followed by lactic acid fermentation even in the presence of oxygen, instead of glycolysis followed by oxidation of pyruvate, a phenomenon known as the Warburg effect (Oudard et al., 1997; Warburg, 1956). In normal cells, the reduction of blood glucose as well as IGF-1 likely contributes to a differential regulation of the activation of stress resistance transcription factors that are negatively affected by nutrient sensing pathways (Fontana et al., 2010; Longo et al., 2008) and cell cycle progression (Blagosklonny and Pardee, 2001). In cancer cells, the low glucose instead presents a specific and major challenge; particularly when chemotherapy drugs are also present.

In agreement with the partial effects on blood glucose and IGF-1, the results of this study indicate that 72 h of 50% CR, but also of diets restricted in carbohydrates or proteins, have only partial effects on stress resistance. The combination of a short-term intermittent 50% CR regimen and cisplatin treatment did not result in the augmentation of chemotherapy efficacy in contrast to the combination of STS and chemotherapy. The results described here suggest that three days of a 50% ICR did not significantly reduce blood glucose levels and thus might not cause a sufficient reduction in the carbon sources metabolized by murine breast cancer cells within this interval. None of the 50% dietary restricted and macronutrient defined diets fed for 3 days, except for the ketogenic 90% HF diet, lowered blood glucose levels, which we have shown to promote host-protection and tumor sensitization. Interestingly, a 50% reduction in the calories consumed on a ketogenic diet leads to a 30% reduction in blood glucose levels after three days of feeding, an effect presumably due to the very low carbohydrate content (less than 1%) of this diet. However, our stress resistance experiment indicates that this reduction did not improve survival. It is noteworthy, that no mice from any of the CR diets achieved protection equivalent to that caused by 60 h fasting (STS). Thus it appears that the limited changes in blood glucose and IGF-1, caused by specific carbohydrates or protein-restricted diets, will most likely not improve stress-resistance and/or reduce tumor progression or have limited effects on them. However, additional studies with extended feeding regimes and larger experimental group size will be necessary to understand whether specific diets may be sufficient to achieve DSR and DSS effects that are close to those caused by fasting cycles. Future studies could also evaluate the effects of various macronutrient-defined and CR diets on ROS production, tumor progression and stress resistance.

Dietary protein and resulting amino acid-content seems to affect longevity and healthy aging (Pamplona and Barja, 2006; Ross and Bras, 1973). Restricting protein intake shares some of

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the physiological effects of CR, including a decreased metabolic rate, reduced oxidative damage, enhanced hepatic resistance to toxins and oncogenic insults, improved surgical stress response, decreased preneoplastic lesions and tumors (Ayala et al., 2007; Maeda et al., 1985; Peng et al, 2012; Rodrigues et al., 1991; Youngman et al., 1992). Furthermore, both CR and protein restriction reduce serum IGF-1 levels (Spindler, 2010), which might be one of the contributors to longevity extension as the IGF-1-like signaling pathways regulate lifespan in various model organisms such as *Caenorhabditis elegans, Drosophila melanogaster* and mice (Bartke, 2008; Fontana et al., 2010; Guarente and Kenyon, 2000; Kenyon, 2005; Russell and Kahn, 2007). The IGF-1 pathway has been shown to affect both animal life span and sensitivity to oxidative stress, consistently with the greater resistance to oxidative stress in IGF-1 receptor deficient mice (Holzenberger et al., 2003). The forkhead box protein O1 (FOXO1), a down-stream target of IGF-1/AKT signaling, can enter the nucleus in the absence/reduction of IGF-1/AKT signaling where it can modulate a wide array of genes involved in oxidative stress resistance, longevity, and metabolism (Guevara-Aguirre et al., 2011; Kim et al., 2008; Partridge and Bruning, 2008), and thus it is a key mechanism involved in protection against age associated stress and disease development. Previous work from our lab suggested that a reduction in IGF-1 results in improved stress resistance to high dose chemotherapy as well as tumor sensitization (Lee et al., 2010, 2012; Longo and Fontana, 2010; Raffaghello et al., 2008). IGF-I exerts a potent tumorigenic effect on a variety of cancer cells by increasing their proliferative rate and inhibiting apoptosis (Prisco et al., 1999; Ramsey et al., 2002). Studies in mice with deficiencies in the downstream effectors of IGF-R signaling, including mTOR inhibition by rapamycin (Harrison et al., 2009) and S6K1 (Selman et al., 2009), demonstrate the central role of intracellular mitogenic pathways downstream of IGF-I in regulating lifespan and stress resistance while simultaneously reducing tumor growth (Garcia et al., 2008; Ikeno et al., 2009; Pinkston et al., 2006); in agreement with our yeast studies (Fabrizio et al., 2001). In addition, humans with growth hormone receptor deficiency have significantly lower circulating IGF-1 levels, and also exhibit drastically reduced incidences of cancer and diabetes, which are common among age matched relatives with intact growth hormone receptor (Guevara-Aguirre et al., 2011).

Despite the well-established effects of protein deficient diets in reducing IGF-1 and inducing cellular stress resistance, we did not detect any benefits in short-term protein restricted mice (0% P and 20% P, 72h to allow a comparison to STS) with regard to stress resistance and tumor progression. In a subcutaneous glioma model, *ad lib* feeding of a diet low in protein did not significantly reduce tumor progression once the tumor was palpable; possibly because this diet did not reduce glucose and/or IGF-1 levels during the feeding regimen. However, glioma is a particularly aggressive tumor, so many additional cancer models should be tested in combination with protein restricted, or both CR and protein restricted, diets to establish whether these interventions can be effective in delaying cancer progression. Mice in the group fed with a calorie restricted high protein diet (LCHP) had the worst survival of all CR groups, comparable to that of mice in the control group. The fact that mice in this group consumed similar, or higher, amounts of fat-derived calories (20.9% in 50% CR LCHP vs. 17.2% *ad lib* AIN93G) and more importantly protein-derived calories (22.6% in 50% CR LCHP vs. 18.8% *ad lib* AIN93G) during three days of feeding, might explain this lack of protection. Of note is that the results presented on the induction of stress resistance are based on relatively short (72 h) feeding periods, thus we cannot exclude that longer CR regimens with either altered calorie and/or macronutrient restrictions could result in an improved stress resistance.

Ketogenic diets are used extensively in the treatment of refractory epilepsy in children, but have also been studied in cancer treatment (Seyfried et al., 2003). To determine how this approach would compare with our stress resistance and potentially tumor sensitization

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experiments, we designed two ketogenic diets: our 90% HF diet (% calorie ratio of fat: carbohydrates: protein of 90%: 1%: 9%; Fig. 1) is nearly identical (±0.5% variation) to the classic ketogenic diet with a ratio of fat: carbohydrates: protein of 90%: 1.4%: 8.6% respectively (Fig. S4). The high-fat diet 60% HF (% calorie ratio of fat: carbohydrates: protein of 60%: 31%: 9%) contains fat ratios similar to the fat ratio used in the modified Atkins diet (% calorie ratio of fat: carbohydrates: protein of 60%: 5%: 35%; Fig. S4) (Kossoff and Dorward, 2008), but we reduced the protein content because previous work has established that protein, and not carbohydrates, regulate IGF-1 levels in human (Fontana et al., 2008; Thissen et al., 1994). The results we described here demonstrate that neither glucose nor IGF-1 levels were significantly reduced after feeding both ketogenic diets *ad lib* for 9 consecutive days.

To evaluate the effects of saturated *vs.* unsaturated fatty acids, as well as medium- *vs.* long-chain fatty acids in cancer treatment, we designed two diets that were isocaloric to the control diet with soybean oil or coconut oil as a fat source but had low protein content. Long-chain unsaturated fatty acids are found in most commonly used dietary fats and vegetable oils such as soybean oil, while short-and medium-chain saturated fatty acids (*e.g.* lauric acid and myristic acid) are found in relatively high abundance in palm kernel oil and coconut oil. The medium-chain triglycerides (MCT) can easily be hydrolyzed in the gastro-intestinal tract and can be transported through the portal venous system towards the hepatocytes, while most of the long-chain fatty acids are transported as chylomicrons in the lymphatic system and packaged into triglycerides in the liver. MCTs can easily be fed into the mitochondrial β-oxidation (Aoyama et al., 2007), while LCTs rely on transporters, such as carnitine, to enter the mitochondrial matrix in hepatocytes (Calabrese et al., 1999). Data from human studies have indicated that consumption of MCTs, or diets with higher unsaturated to saturated fatty acid ratio, are associated with decreasing blood glucose, improving lipid profile, and reducing obesity (Ghosh, 2007; Xue et al., 2009). In a study of biochemical and anthropometric profiles in women with abdominal obesity, dietary supplementation with coconut oil promoted a reduction in abdominal obesity (Assuncao et al., 2009).

The beneficial effects of prolonged CR are known for over a century now (McCay and Maynard, 1935; Moreschi, 1909). The problems associated with translating CR into any clinical application is that long-term CR delays but does not stop the progression of many malignant diseases (Bonorden et al., 2009; Mukherjee et al., 2004) and is associated with a chronic reduced weight state that might be detrimental for cachectic cancer patients, or patients at risk of becoming cachectic, but also might chronically reduce fat and other reserves that may increase frailty particularly in elderly patients (Seyfried et al., 2003). In fact, prolonged CR can delay wound healing and immune function, which might present an additional hurdle for the great majority of patients receiving chemotherapy or undergoing surgery (Fontana et al., 2010; Kim and Demetri, 1996; Kristan, 2008; Reed et al., 1996). Furthermore, the 75% reduction in serum IGF-1 caused by a 2 to 5-day fast in mice and humans cannot be achieved by a more moderate CR which does not reduce IGF-1 levels in humans unless the protein intake is also restricted (Clemmons and Underwood, 1991; Fontana et al., 2008; Lee et al., 2010). Even when combined with protein restriction, chronic CR only causes a 30% reduction of IGF-1 in humans (Fontana et al., 2008). Because of the consistent effects on glucose and IGF-1, and consequent effects on protection of normal and sensitization of cancer cells without the chronic under-weight, periodic fasting cycles appear to have the highest potential to protect patients treated with a variety of chemotherapy drugs while augmenting their efficacy in the treatment of many tumors.

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Supplementary Material

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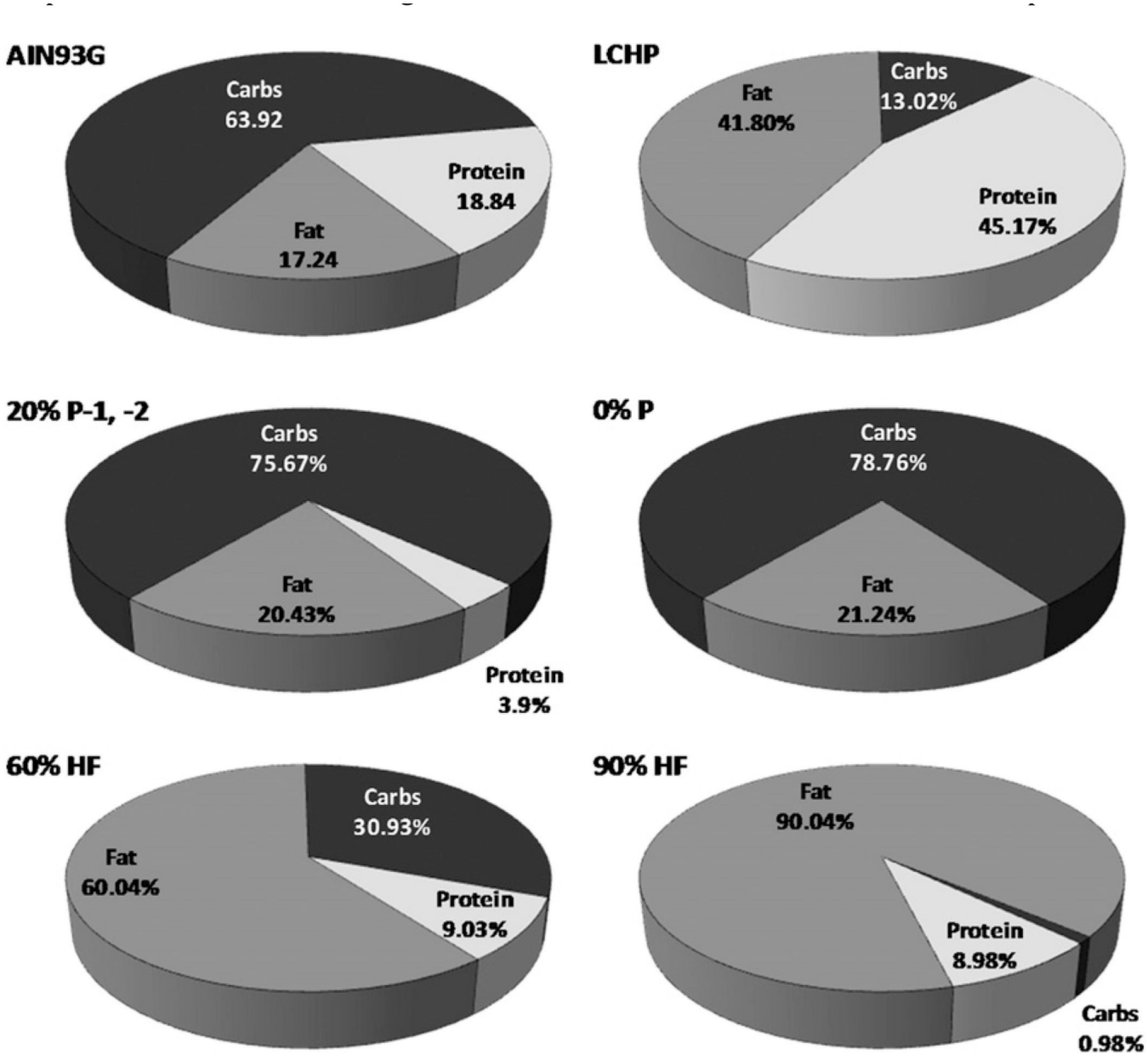

Fig. 1.

Calories supplied by macronutrients of the experimental diets in %. AIN93G standard chow was the reference diet supplied to mice. The experimental diets modified in the macronutrient composition (fat, protein and carbohydrates) were all based on this diet. The low-carbohydrate LCHP diet had calories from carbohydrates reduced to 20% compared to the AIN93G formulation (13% vs. 63.9%) but contained more protein (45.2%) and fat (41.8%). Diets 20% P-1 (soybean oil as fat source) and 20% P-2 (coconut oil as fat source) had calories from protein sources reduced to 20% compared to the AIN93G formulation; the 0% P diet contained no protein; all these diets were isocaloric to the AIN93G standard chow. The ketogenic high fat diet 60% HF was designed to supply 60% of the consumed calories from fat sources, the calories coming from protein and carbohydrates were reduced proportionally. The 90% HF diet was a ketogenic diet which contains 90% of fat while

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supplying only minimal carbohydrates (less than 1%) and half of the protein content (9%). Detailed diet composition and calorie content are summarized in Table S2.

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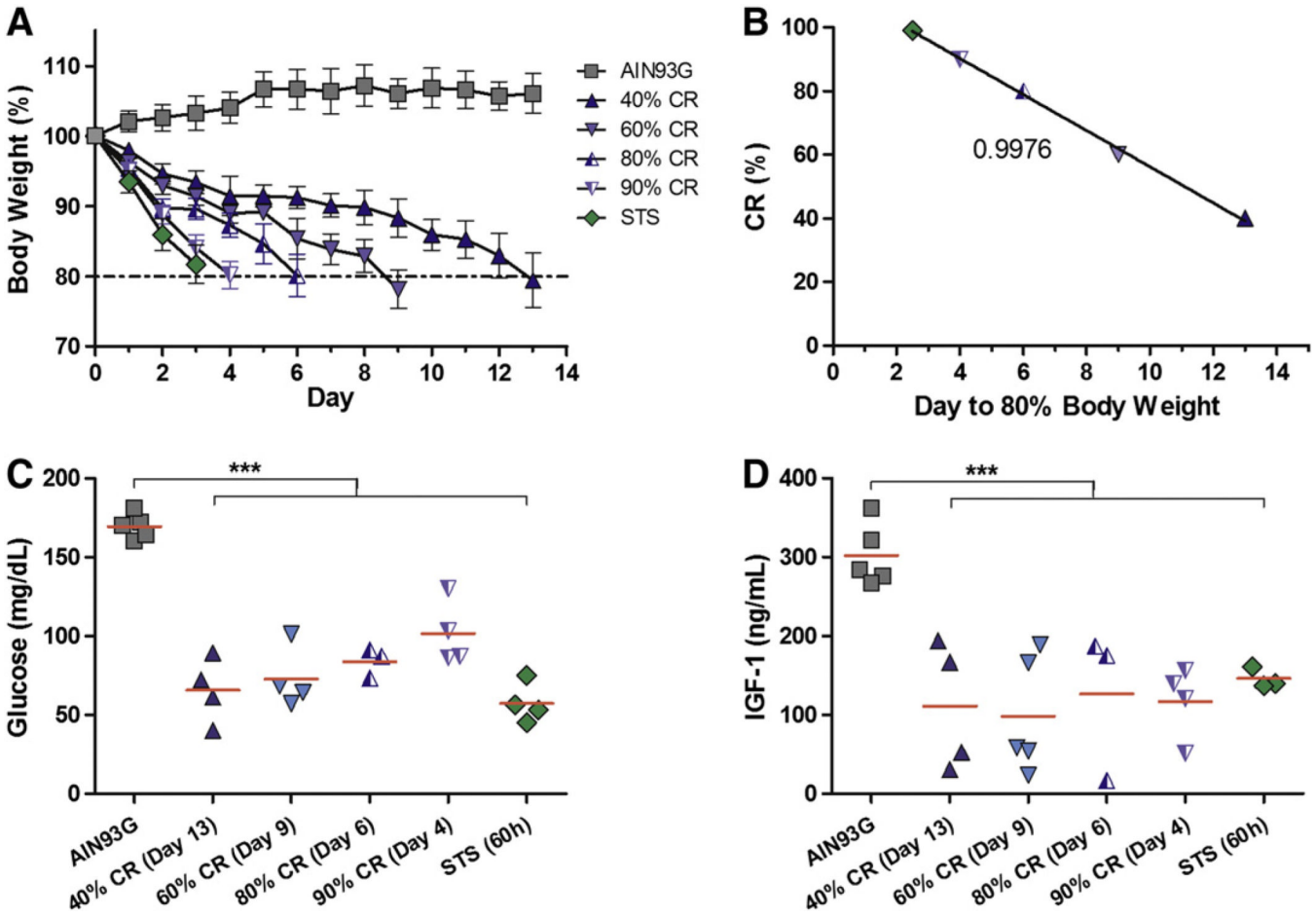

Fig. 2.

Calorie restriction reduces bodyweight, glucose and IGF-1. A) Female CD-1 mice, age 12–15 weeks were either fed *ad lib* (gray square) with AIN93G rodent standard chow, exposed to 40%, 60%, 80% and 90% calorie restricted AIN93G diets (triangles) or fasted (STS, green rectangle) until mice lost 20% of their initial bodyweight (dotted line). N = 5 per experimental group. All data presented as mean ± SEM. B) Linear fit for the severity of the CR regimen *vs.* the duration (days) until 80% bodyweight was reached. C) Blood glucose levels for mice once 80% bodyweight was reached. Red line represents mean; * $p < 0.05$, *** $p < 0.001$, ANOVA, Tukey's multiple comparison. D) Serum IGF-1 levels for mice once 80% bodyweight was reached. Red line represents mean; *** $p < 0.001$, ANOVA, Tukey's multiple comparison. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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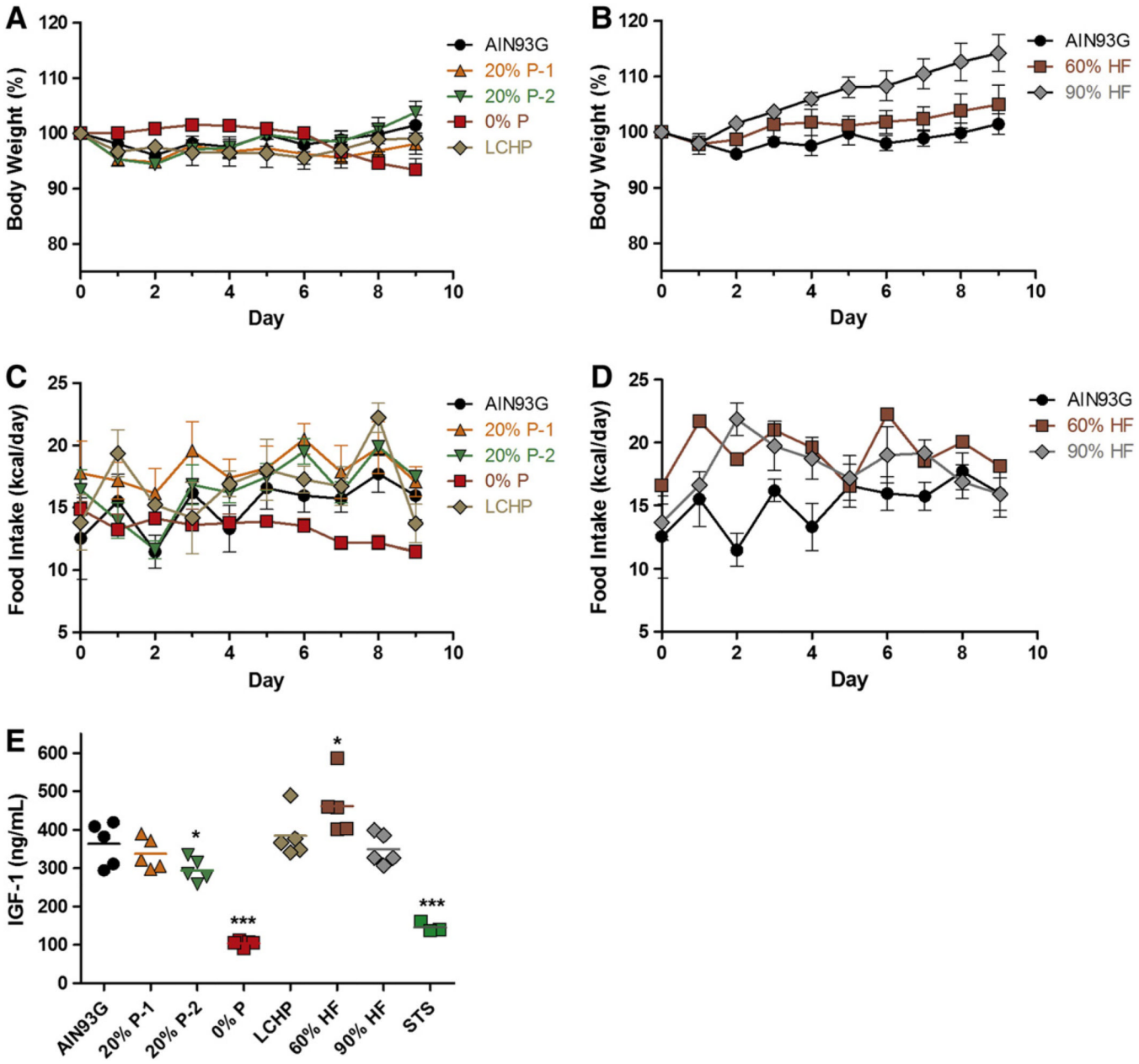

Fig. 3.

Effects of macronutrient defined diets on bodyweight, food intake, glucose and serum IGF-1. Five female CD-1 mice, age 12–15 weeks were either fed *ad lib* with AIN93G rodent standard chow (black circle) or with A) two different low protein diets (20% P-1 and 20% P-2), a diet low in carbohydrates but high in protein (LCHP), a protein deficient diet (0% P) or B) a high fat diet (60% HF) and ketogenic diet (90% HF). A detailed overview over the macronutrients is given in Table S1. C) Daily *ad lib* calorie intake for diets AIN93G, 20% P-1, 20% P-2, LCHP and 0% P. D) Daily *ad lib* calorie intake for diets 60% HF and 90% HF; AIN93G shown as reference. All data presented as mean ± SEM. E) Serum IGF-1 levels after 9 days of *ad lib* feeding. Lines represent mean; $*p < 0.05$,*** $p < 0.001$, ANOVA, Tukey's multiple comparison compared to AIN93G control.

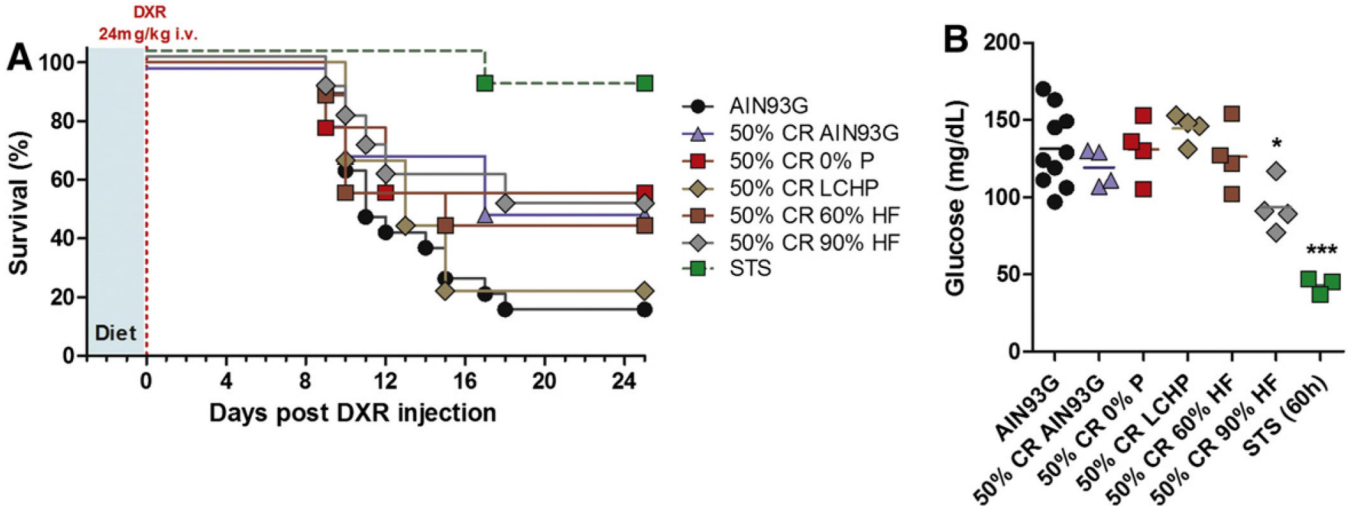

Fig. 4.

Stress resistance test for calorie restricted macronutrient defined diets. Mice were fed *ad lib* (AIN93G), were fasted for 60 h (STS) or fed with 50% calorie restricted diets with defined macronutrient compositions (AIN93G, LCHP, 0% P, 60% HF, 90% HF) for 3 days (green box) prior to an intravenous injection of doxorubicin (24 mg/kg, red dashed line). Survival was followed for 25 days post injection, after which the remaining animals were considered survivors. B) Blood glucose levels after 3 days of feeding ad *lib and* CR diets, as well as after 60 h STS. Lines represent mean. * $p < 0.05$, *** $p < 0.001$, ANOVA, Tukey's multiple comparison. Survival data plotted from pair-matched pooled experiments with the statistical software Prism (GraphPad Software). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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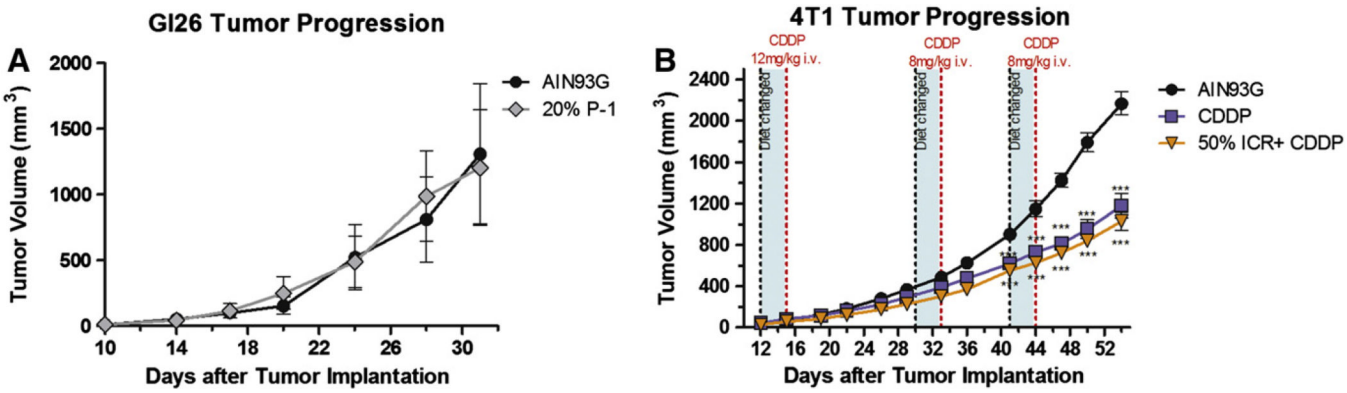

Fig. 5.

Tumor progression of GL26 glioma and 4T1 breast cancer in vivo. A) Subcutaneous tumor progression of murine GL26 glioma is shown by total tumor volume in mm^3. Tumor measurements were started once the tumors became palpable under the skinat day 10. Animals were fed *ad lib* with either AIN93G(N = 5)as a control or with the low protein diet 20% P-1 (N = 6). All data presented as mean ± SEM. B) Subcutaneous tumor progression of murine 4T1 breast cancer is shown by total tumor volume inmm^3. Tumor measurements were started once the tumors became palpableunder skinatday 12. Control animals(N = 10) received no treatment and tumor progressed rapidly, reaching the endpoint volume of $2000mm^3$ by day 54 post tumor implantation. Cisplatin (CDDP) animals (N = 9) were injected at days 15, 33 and 44. The first CDDP dose was delivered at 12 mg/kg by intravenous injection, the two subsequent injections were delivered at 8 mg/kg to avoid chemotoxicity. Mice in the 50% ICR + CDDP group (N = 9) were fed in intermittent regimens with the AIN93G diet reduced to 50% of the daily calorie intake of the control group for three days (ICR, green box) prior to cisplatin injection. Injection schedule was identical as for the CDDP group. All data presented as mean ± SEM; *** $p < 0.001$, ANOVA, Tukey's multiple comparison, compared to control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)