Suppression of implanted MDA-MB 231 human breast cancer growth in nude mice by dietary walnut

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WEH was the PI on the grant, designed the experiment, interpreted the results and prepared this report.

GI directed the day to day animal care feeding and measurements, directed the biochemical and image analyses assays and provided input on preparation of this report.

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Abstract

Walnuts contain components which may slow cancer growth including: omega 3 fatty acids, phytosterols, polyphenols, carotenoids and melatonin. A pilot study was performed to determine whether consumption of walnuts could affect growth of MDA-MB 231 human breast cancers implanted into nude mice. Tumor cells were injected into nude mice that were consuming an AIN-76A diet slightly modified to contain 10% corn oil. After the tumors reached 3-5 mm diameter, the diet of one group of mice was changed to include ground walnuts, equivalent to 56 g (2 oz) per day in humans. The tumor growth rate from day 10, when tumor sizes began to diverge, until the end of the study of the group that consumed walnuts (2.9 ± 1.1 mm^3/day; mean ± SEM) was significantly less ($p<0.05$, T-test of the growth rates) than that of the group that did not consume walnuts (14.6 ± 1.3 mm^3/day). The eicosapentaenoic and docosahexaenoic acid fractions of the livers of the group that consumed walnuts were significantly higher than that of the group that did not consume walnuts. Tumor cell proliferation was decreased but apoptosis was not altered due to walnut consumption. Further work is merited to investigate applications to cancer in humans.

Key words: omega 3 fatty acids, melatonin, antioxidant, animal models

Introduction:

Consumption of long chain [20C eicosapentaenoic acid (EPA) and 22C docosahexanoic acid (DHA)] omega 3 fatty acids has consistently slowed the growth of cancer cells or tumors(1-3). Slowed cancer growth was associated with a) incorporation of EPA and DHA in cell membranes(4), b) reduced inflammation or decreased expression of inflammatory cytokines(5,6), c) slower proliferation of cancer cells(7,8), and d) increased death of cancer cells(9,10).

Walnuts contain 2.6 g of alpha linolenic acid (ALA) an 18 carbon, omega 3 fatty acid per 1 ounce serving (11). Preclinical studies show that consumption of flaxseed oil (about 50% ALA), canola oil (about 10% ALA) (12), or mistol seed oil (about 25% ALA)(13) slowed the growth of cancers in rodent models thus providing background for a hypothesis that increased walnut consumption might slow the growth of cancers.

It is difficult to determine *in vivo* whether the ALA itself slows the growth of cancers or if the ALA must be converted to a longer chain fatty acid for effect. Both rodents and humans have the metabolizing enzymes required to elongate and desaturate 18C fatty acids to 20 or 22 C fatty acids. It is known that rodents readily convert ALA to EPA and DHA, however, in the past, there has been question as to how active these enzymes are in human metabolism. The results of recent studies using ^{13}C labeled ALA have demonstrated that both men and women can convert ALA to EPA with variable conversion to DHA (14,15). In clinical diet supplementation trials, 1 to 2 servings of walnuts per day plus flaxseed oil were associated with increased serum levels of EPA and docosapentaenoic acid (DPA, 22:5n3) and decreased serum levels of total n-6 fatty acids(16,17), further supporting the notion that human can effectively convert ALA to EPA. Since many of the hypothesized mechanisms for slowing the growth of cancers require EPA as substrate, these studies indicating that humans do effectively convert ALA to the longer chain omega 3 fatty acids, including EPA, suggest that an ALA containing food such as walnuts may contribute to slowing the growth of cancers. If a serving or two per day of walnuts provides enough ALA to increase serum levels of EPA and DPA, then consumption of walnuts may favorably alter parameters associated with slowing cancer growth.

To our knowledge, the influence of walnut consumption on cancer growth has not been investigated, however the influence of some of the components of walnuts on cancer growth has been investigated. Walnut components that individually have been found to slow cancer growth and that might be expected to contribute to an anticancer potential of walnuts compounds include: phytosterols(18), melatonin(19), ellagic acid(20-23), gamma-tocopherol(24,25), carotenoids(26), and polyphenolic compounds(27).

This experiment was designed to determine if consumption by mice of a clinically relevant amount of walnuts, equivalent to about 2 servings per day in humans, could slow tumor growth and alter parameters associated with tumorigenesis. The MDA-MB 231 breast cancer cell line implanted in nude mice was chosen as a model because we have previous experience with this cancer model(2,4) and because breast cancer has been linked with diet, especially dietary fat(28-32). Slowing tumor growth by consumption of one or two servings of walnuts a day as part of a healthy diet could have benefit for prevention of primary cancer or slowing the recurrence of cancer.

Materials and Methods:

Mice: Forty, female athymic nude (nu/nu) mice, 6 weeks old were obtained from Charles River Laboratories (Wilmington, MA). Mice were quarantined for 2 weeks, then moved to a study room and allowed to acclimatize for one week before implantation of tumor cells. Mice

were individually numbered for unique identification, and weighed three times weekly during the entire experiment and terminally. Mice were housed, 4 per cage, in an isolation room (temperature controlled at 24°C, 12 h light/dark cycles) in the Marshall University Animal Research Facility. Fresh sterile cages, bedding and water were provided twice weekly. All animal use and handling was approved by the Marshall University Institutional Animal Care and Use Committee.

Tumors: MDA-MB 231 human breast cancer cells were cultured using standard cell culture techniques. One million cells were injected s.c. between the scapulae of each mouse (20 mice/final diet to allow for the expected fraction of tumor take of about 60% based on our past experience with this cell line). MDA-MB 231 are ER negative and do not require added estrogen for growth. The mice were fed an AIN-76 semipurified diet for two weeks, until the tumor was 3 to 5 mm in diameter, to allow the tumor to become established prior to diet change. Palpable tumors were measured using digital calipers three times weekly during the entire experiment to develop tumor growth curves. Twenty two mice had growing tumors, at least 3 mm in diameter, at the time of division into diet groups. Mice were divided into the diet groups such that the mean tumor size and the numbers of larger or smaller tumors were equal in each group.

Diets: Diets were prepared in the Marshall University animal diet prep room. Diet composition is shown in Table 1 and was formulated to be isocaloric, isonutrient and relevant to human consumption. Two servings (2 ounces) of walnuts per day in humans would provide 370 calories. This is 18.5% of a 2000 calorie/day diet. Thus, the walnut diet for the mice was formulated to provide 18% of calories from walnuts. The AIN76 diet is adequate for the nutritional support of the mice(33). The dry ingredients of the diet were obtained in bulk from MP Biomedicals (Solon, Ohio), the corn oil was purchased locally (100% corn oil, no additives or preservatives). Walnuts kernels (a gift from California Walnut Commission) were received in a single batch and were stored in at -20º freezer until incorporated into the diet. Whole walnut kernels, including the brown husk but not the shell, were finely ground in a food processor and immediately mixed with the remainder of the dry ingredients of the diet to prepare the walnut containing diet. Batches of diet were prepared as needed, about each two weeks. The diet mixture was pressed into trays and cut into small squares. Individual cage sized portions (25-30 grams) were stored in sealed containers at -20^{o}C to prevent oxidation of the fat and bacterial growth in the food. Mice had free access to food and water and were fed fresh food 6 days per week. Food removed from the cages was discarded. Mice were fed the experimental diets from day 14 after tumor cell injection until the end of the study on day 49, thus mice consumed the experimental diet for 35 days.

Sacrifice and tissue handling: The experiment was ended when the tumors of some mice in the corn oil fed group reached the maximum allowable size of 1500mm^3. Mice were deeply anesthetized using isoflurane, cervically dislocated, then were exsanguinated by cardiac puncture. Blood was collected into an EDTA containing vacutainer, separated and the plasma was frozen for further analyses.

The tumor, inguinal fat and liver were removed. If the tumor was large enough, it was bisected, a cross-section from the center was removed for fixation in 10% neutral buffered formalin followed by paraffin embedding. The embedded tumor was cut 4μm thick, cut sections were placed on microscope slides for histological and immunohistochemical analyses. Many of the tumors of the walnut fed group were so small they could not be divided, limiting further analyses of that tumor.

Portions of the tumor (if available), fat and liver were flash frozen in liquid nitrogen, placed in individual, labeled microtubes and stored at -80^{o}C until further processing could be performed.

Frozen tissues were thawed and homogenized individually in ice cold 0.9% saline with 0.1% BHT to prevent lipid peroxidation (10% homogenate). The homogenate was allocated for use in TBARS, gas chromatography and total protein assays.

Body weight and tumor growth: Body weight (an indicator of overall animal health) and tumor size was measured three times weekly. An electronic scale was used to weigh mice, electronic digital calipers were used to measure the tumor size. Tumor volume was calculated using the formula: volume = (length X width X depth)/2. Body weight curves and tumor growth curves were calculated for each diet group. Curves were analyzed using Prism© (Graphpad, Inc) software.

Determination of fatty acids in tissues. Dietary fatty acids are incorporated into the tissues as consumed in the diet or may be elongated and desaturated prior to incorporation. The fatty acid composition of tumor, liver and fat from mice of each dietary group was assayed using gas chromatography to determine changes in the lipid composition of these tissues due to the diet. Tissue was homogenized in 0.1% BHT in distilled water to prevent any fatty acid oxidation. Lipids were extracted with chloroform/methanol, the fatty acids were methylated followed by separation and identification using gas chromatography, as previously described(4,12,34). Gas chromatography was done using a Perkin-Elmer Clarus 500 Gas Chromatograph (Shelton, CT) with a PerkinElmer Elite-5 (5% Diphenyl) Dimethylpolysiloxane Series Capillary Column (Length: 30m, Inner Diameter: 0.25mm), under the following conditions: initial temperation of 150 ^{o}C, ramp 1 at 175^{o} C for 15min, ramp 2 at 225^{o} C for 50min, ramp 3 at 250^{o} C for 10min, helium carrier gas flow rate of 1.60mL/min. Fatty acid methyl ester standards (Nu-Chek-Prep, Elysian, MN) was used for peak identification. To better identify peaks we use two standards: GLC #464 which contains 52 fatty acids and a custom preparation, GLC 704, which contains 10 fatty acids, methyl esters of stearate, oleate, linoleate, alpha linolenate, gamma linolenate, homogamma linolenate, arachidonate, eicosapentaenoate, docosapentaenoate, and docosahexaenoate. The fatty acid methyl esters were reported as the percent of the total methylated fatty acids (area under the curve). A T-test was used to determine statistical differences.

Antioxidant capacity of plasma: The total antioxidant capacity in the plasma of six mice from each dietary group was assayed using the Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, Michigan). This assay compares the antioxidant capacity of the plasma to the capacity of a synthetic antioxidant, Trolox. Plasma was separated from anticoagulated (EDTA treated) whole blood by centrifugation and stored at -80^{o}C. Trolox standards of different concentrations were prepared from reconstituted Trolox and assay buffer to generate a standard curve. The assay was set up and performed according to manufacture's direction followed by absorbance reading at 750nm. The antioxidant capacity of each plasma sample was calculated from the Trolox standard curve. A Kruskal-Wallis test was used to determine statistical differences.

Determination of tumor oxidative damage: Oxidative damage in five tumors from each group was determined by assay of thiobarbituric acid reactive substances (TBARS). Hydroperoxides are formed following oxidation of polyunsaturated lipids. Malondialdehyde (MDA) is the major degradation product of lipid hydroperoxides and can be quantitated spectrophotometrically at 535 nm after reaction with thiobarbituric acid(2,35). The accuracy of the spectrophotometric assay for quantification of MDA has been confirmed by HPLC(35,36). A malondialdehyde

standard curve was prepared to quantify MDA in the samples. The protein content of the homogenate was determined using a BioRad protein assay for normalization of TBARS results. TBARS are reported as nmoles TBARS/mg protein. A T-test was used to determine statistical differences.

Tumor cell proliferation and apoptosis: Proliferation of cancer cells was determined by proliferating cell nuclear antigen (PCNA) immunohistochemistry. PCNA antibody detects a protein component of DNA polymerase delta and stains cells that are actively synthesizing DNA in preparation for mitosis. Standard immunohistochemical technique was used with anti-PCNA (Biogenex, San Ramon, CA) as the primary antibody. Image analysis was used to determine the fraction of viable area that was positive for PCNA in each tumor. Digital images were taken using a Leica microscope equipped with a 40X lens and a Leica DFC 480 digital camera. Using Photoshop, the number of pixels in each of five 40X fields were determined for each of 5 tumors per diet group. Areas in each field that were not viable tumor or that were not cellular were subtracted. The number of pixels in PCNA positive nuclei was determined. The PCNA positive fraction was defined as the number of PCNA positive pixels divided by the total of pixels in the field. A T-test was used to determine statistical differences between groups. WEH was blinded to the group of origin at the time of image analyses of PCNA slides.

Death (apoptosis) of cancer cells was assayed by morphological identification of apoptotic cells. The numbers of cells undergoing apoptosis according to morphological indicators: nuclear and cellular condensation (dark condensed nuclei with a clear space around the cell), fragmentation of nuclei (nuclear fragments, sometimes engulfed by surrounding cells) and/or membrane blebbing (blebs surrounding cytoplasm fragments) and fragmentation of the cell into apoptotic bodies in five 40X fields in viable areas of 5 tumors per diet group were counted to estimate an apoptotic index. Observing viable area of tumor assures that there are approximately the same number of cells per field thus the apoptotic index represents the number of apoptotic cells per X number of cells. Morphological identification of apoptosis has been previously used and validated to quantify apoptosis(37,38). Three individuals were blinded to the group of origin at the time of independent counting of apoptotic figures.

A T-test was used to determine statistical differences in the number of apoptotic cells.

Results:

Body weight: Figure 1 shows the mean body weight of each group of mice during the experiment. Consumption of the walnut diet did not significantly change the body weight of the mice compared to the control group.

Determination of tissue lipid composition: The results of analyses of lipid composition of the liver, inguinal fat and tumor by gas chromatography is shown in Figure 2. The diet of the mice did not contain either EPA or DHA. These results indicate that there was significantly less linoleic acid (18C, n-6) and significantly more eicosapentaenoic acid (20C, n-3) and docosahexaenoic acid (22C, n-3) in the lipids of the livers of mice that consumed the walnut containing diet. Overall, the omega 3 content of the liver was significantly increased in mice that consumed walnuts in the diet.

The lipids of the inguinal fat of the walnut fed mice contained significantly more linoleic acid and significantly less arachidonic acid than did the inguinal fat of the corn oil fed mice. The total omega 3 content of the inguinal fat was slightly but not significantly more in the mice fed the walnut diet.

Our custom standard demonstrated that oleic acid (18:1) and α-linolenic acid (ALA) were not adequately separated to accurately quantify ALA. Since the ALA was clearly being converted to EPA and DHA it would be difficult to make meaningful conclusions from ALA content of these tissues had we been able to quantify ALA.

Plasma antioxidant capacity: The Trolox assay compares the antioxidant capacity of an aliquot of plasma to the antioxidant capacity of a standard preparation of Trolox. Figure 3 shows that there was slightly but not significantly higher antioxidant capacity in the plasma of the mice that consumed walnut than in the plasma of mice that did not consume walnut. The lowest value for blood total antioxidant capacity of the walnut fed group was higher than the median blood antioxidant capacity of the corm oil fed mice.

Tumor growth rate: Figure 4 illustrates the growth rate of the implanted MDA-MB 231 tumors in control or walnut fed mice. The data indicate that the tumor sizes began to diverge about 10 days after the diet change. Non-linear regression analyses, using an exponential growth fit, of mean tumor sizes of each group with time, showed that the mean doubling time tumors of the corn oil fed, control group was 11.1 days (95% confidence interval 9.5 to 13.2 days, mean volume 438 mm^3 at 34 days after diet change) whereas the mean doubling time of the tumors of the walnut fed group was significantly longer at 23.3 days (95% confidence interval 16.4 to 40.6 days, mean volume 185 mm^3 at 34 days after diet change).

Figures 5 and 6 show the results of assays to obtain information about potential mechanisms for slowing of tumor growth by consumption of walnut. In past studies, omega 3 fatty acids have been shown to slow proliferation, to increase apoptosis and to increase lipid peroxidation in tumors(1,39). The PCNA positive fraction (Figure 5) of the tumor was significantly less in the mice that consumed walnuts (T-test, $p<0.05$). There were no significant differences in number of apoptotic figures/field in the tumors (Figure 5) due to the diet. Lipid peroxidation has been proposed as a cause of increased tumor cell death by omega 3 fatty acids in past studies(39). In keeping with the lack of apoptosis induction, there were no significant differences in the amounts of thiobarbituric acid reactive substance, a measure of lipid peroxidation, (TBARS, Figure 6) in the tumors due to the diet of the mice. The specimen sizes were inadequate for complete analyses on every animal in the study, yet since there is overlap in the results of specimens that were available, it is unlikely that an increase in the n from 5 or 6 to 10 or 11 would have resulted in significant differences in antioxidant capacity, apoptosis or TBARS.

Discussion:

The notion that foods may have components that could influence the risk for cancer was noted as early as the Song dynasty (960-1279 AD) by Yong-He Yan[(40) p.13]. This idea has been repeated by physicians through the centuries, often based on clinical observations(40). More recently, epidemiology studies have provided evidence that diet patterns or specific diet components can alter cancer risk. Using *in vivo* and *in vitro* models we are beginning to link dietary components and cancer risk and to uncover some of the mechanisms of action of these components to increase or decrease cancer risk.

Walnuts (California walnuts, English walnuts, *Juglans regia*) have a unique composition (Table 2) of components that could be beneficial for prevention or slowing the growth of cancer. Walnuts have a much higher amount of omega 3 fatty acid per serving than other common nuts: (g of 18:3 per 1 ounce: almonds – 0.00; brazil nuts – 0.017; cashew – 0.046; European chestnuts – 0.015; filberts 0 0.025; macadamia – 0.058; peanuts – 0.001; pecans 0.28; pistachio – 0.072;

English walnuts - 2.57(11)) and contain significant amounts of antioxidants, phytosterols, polyphenols, selenium and vitamins. Many of these components have been shown, individually to slow cancer growth.

In this study, consumption of 18% of the dietary calories from walnuts significantly decreased the growth rate of the implanted MDA-MB 231 human breast cancer tumors in mice. A likely mechanism for the lower growth is the suppression of proliferation in the tumor by omega 3 fatty acids. Many of the mechanisms proposed for suppression of cancer growth by omega 3 fatty acids require the presence of long chain (20 or 22C) omega 3 fatty acids (5,41-43). In this study, there was essentially no EPA and DHA in the diet, however, there were significantly higher levels of EPA and DHA in the liver of the mice that consumed walnuts than in the controls. Since the only source of the omega 3 bond was the α-linolenic acid of the walnuts, the increased EPA and DHA indicates that the α-linolenic acid of the walnut was being effectively elongated and desaturated to EPA and DHA in the liver.

Inflammation is increasingly recognized as promotional to cancer(44-47). Zhao, et al. reported that dietary ALA decreased systemic inflammatory cytokines in hypercholesterolemic subjects (48) raising the possibility that inflammatory cytokines could have been decreased in the walnut fed mice. This is an area for further investigation. Even though we could not statistically analyze EPA and AA in the tumors of walnut fed mice due to the small amount of remaining specimen, it is likely that AA was significantly decreased, reducing the amount of substrate available for synthesis of inflammatory prostaglandin E_2 (PGE_2) in the tumor. PGE_2 has been found to be promotional to tumor cell proliferation(49,50).

Walnuts contain a number of substances that may contribute to their overall antioxidant capacity, including melatonin, gamma-tocopherol, carotenoids, phytosterols, and polyphenolic compounds. Reiter, et al (51) reported that melatonin from walnuts was absorbed into the blood and the antioxidant capacity of blood was significantly higher in rats that were fed walnuts after a 24 hour fast compared to the rats that were fed chow. Mice in the current study were fed much less walnut (18% of calories vs 100% of calories) than in the study of Reiter, et al. Even though the antioxidant capacity of the blood was not statistically significantly increased, it is notable that the antioxidant capacity of all of the mice of the walnut fed group was above the median of the control group. As such, walnuts would be a beneficial addition to a diet already incorporating good levels of antioxidant containing fruits and vegetables and would be expected to add to the antioxidant capacity of the blood.

Phytosterols are another food component that have potential as anticancer compounds(18). Walnuts contain about 20 mg/serving of phytosterols, predominately β-sitosterol (18.14 mg β-sitosterol/ 28 g serving). Phytosterols, at a level of 16 μM in cell culture, have been shown to induce apoptosis in MDA-MB-231 cells(52) and phytosterols may slow cancer cell proliferation(53). Humans that consumed about 200 mg phytosterol/day from dietary supplements showed serum phytosterol levels of about 0.1 to 0.4 μM(54). Thus consumption of 1 to 2 servings/day of walnuts, containing 20 to 40 mg of phytosterols, might result in a serum level of about 0.01 to 0.04μM of phytosterol. That level of phytosterol is far below the amount that was required to induce cancer cell apoptosis in cell culture, making it unlikely that the phytosterol content of 1 to 2 servings per day of walnuts could induce cancer cell apoptosis.

Ellagic acid is a polyphenol released by the hydrolizable tannins found in walnuts(21). It has been found to be a potent antioxidant with apoptosis inducing properties(20) and to induce cell cycle arrest(23). Even though the amount of individual components is small, this does not rule out the possibility that β-sitosterol, ellagic acid and omega 3 fatty acid could act

synergistically for induction of cancer cell apoptosis or slowing proliferation in the MDA-MB 231 tumors.

There might be concern that the caloric content of the walnuts would increase body weight, in itself a significant risk for developing cancer(55). In one study, the diet of diabetic subjects was supplemented with one ounce of walnuts per day for 6 months, there was no significant difference in energy intake between groups that did or did not consume walnuts(56). In a review, Sabaté noted that nut consumption was not associated with weight gain in either epidemiology studies or clinical trials(57). There is also evidence from human studies that consumption of omega 3 fatty acids, as reflected by higher serum EPA and DHA, may help protect against obesity(58,59). Thus the addition of walnuts to the diet did not cause weight gain in the mice and would not be expected to result in weight gain in humans.

Conclusion:

The results of this pilot study demonstrate that the addition of walnuts slowed the growth of cancers in a mouse model possibly by slowing proliferation of tumor cells. Suppression of proliferation of cells that might transform to cancer cells or suppression of the growth of metastatic sites could reduce cancer incidence and mortality. Additional work should be done to determine whether the addition of walnuts to a healthy diet could be beneficial to preventing or slowing the growth of cancer.

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Table legends

Table 1
Composition of the diets: The AIN-76-A was modified to 10% w/dry w corn oil or 2.63% corn oil and 18% of calories from walnut. The control (corn oil) and walnut diets were balanced for nutrients, protein, fat, carbohydrate and calories.

Table 2
Walnut nutrient composition: The nutrient composition of walnuts as given in the USDA Nutrient Database for Standard Reference, release 19, 2006. (http://riley.nal.usda.gov/NDL/cgi-bin/list_nut_edit.pl)

Figure Legends

Figure 1
Body weights of the groups of tumor bearing mice (mean ± SEM),
Mice were weighed three times per week, mean + SEM of body weights of each group, n=11 mice/group, at each time point are shown. The initial mean body weight is the day of injection of tumor cells. The diets of the mice were changed to the experimental diets 14 days after injection of MDA-MB 231 cells, study was ended 49 days after injection of the tumor cells. There were no significant differences between the body weights of the mice due to the diet.

Figure 2
Lipid composition of liver, inguinal fat pad and tumor.
The fraction of the total lipids of major omega 6 and omega 3 fatty acids of the liver (n=5/group), inguinal fat pad (n=5/group) and tumor (n=2/group) are shown. Total lipid was analyzed by gas chromatography. The results illustrate significant differences in the lipid content of liver or inguinal fat due to the diet. No T-test was performed for tumor lipids since the n was only 2. A split scale is used to better illustrate the changes in the omega 3 fatty acid fractions.

Figure 3
Antioxidative capacity of the plasma.
The antioxidative capacity of the blood, expressed as Trolox equivalents, of mice that consumed either corn oil or walnut containing diets is shown, n=6/group. The median antioxidant capacity of walnut consuming mice is slightly but not significantly higher than the antioxidant capacity of mice that did not consume walnuts.

Figure 4
Mean tumor size of groups of mice.
Tumors were measured three times weekly, volumes were calculated as volume = (length X width X depth)/2, the mean tumor volumes of mice each group (n=11/group) of mice at each time point is shown. The mean tumor sizes of the groups of mice begins to diverge about 10 to 14 days after initiation of the experimental diet. The dashed line indicates the non-linear regression using an exponential growth fit.

Figure 5
Proliferating cell nuclear antigen and apoptotic fractions of the tumor.
Analyses of the proliferating cell nuclear antigen (PCNA) fraction of the tumors (n = 5 tumors/group, 5 fields/tumor) demonstrated that proliferation was significantly lower in the tumors of mice that consumed the walnut diet than in mice that consumed the corn oil diet. There were no differences in the number of apoptotic figures per field due to the diet, n=5 tumors/group, 5 fields/tumor.

Figure 6
Lipid peroxidation in the tumor.
Analyses of lipid peroxidation (n=5 tumors per group) as indicated by generation of thiobarbituric acid reactive substances (TBARS) showed that consumption of the walnut containing diet did not alter lipid peroxidation in the tumor.

Table 1

	Control diet (Corn oil diet)		Walnut diet (18% of calories from walnut)		
Ingredient	% of wt	Amount /100g	Amount /100g	Additional nutrient contained in 11.3 g walnut/100 g diet	Calories/ 100g
Casein (protein)	20%	20 g	18.3g	Protein -1.72g	80
Sucrose	45%	45 g	45g		180
Corn starch (carbohydrate)	15%	15 g	13.5g	Carbohydrate - 1.55g	60
Alphacel (fiber)	5%	5 g	4.8g	Fiber - 0.2g	0
Choline bitartrate	0.2%	0.2 g	0.2g		0
DL-methionine	0.3%	0.3 g	0.3g		0
Mineral mix	3.5%	3.5 g	3.5g		0
Vitamin mix	1.0%	1.0 g	1.0g		0
Ground walnut		0	11.1g		0
Corn oil (fat)	10%	10 g	2.63g	Fat - 7.37g	90
Total	100%	100 g	100.3	(0.46g water in walnut)	410
n3/n6	0% n3, 50% fat is n6		(1.02g n3)/(1.3g+4.3g n6) = 0.18		
Total fat	10g		10.0g		90
Total protein	20g		20.0g		80
Total carbohydrate	60g		60.0g		240

Table 2

Walnut Nutrient Profile

NUTRIENTS IN 1 OUNCE (28.3 g) OF WALNUTS **(14 unsalted & unroasted halves)**

Nutrients	Units	Walnuts	Nutrients	Units	Walnuts
Calories	kcal	185	Selenium	mcg	1.4
Protein	g	4.3	Vitamin C	%DV	0
Total Fat	g	18.5	Thiamin	%DV	6
Saturated Fat	g	1.7	Riboflavin	%DV	2
Monounsaturated Fat	g	2.5	Niacin	%DV	2
			Pantothenic acid	%DV	2
Polyunsaturated Fat	g	13.3	Vitamin B6	%DV	8
Linoleic acid (18:2)	g	10.8	Folate	%DV	6
Linolenic acid (18:3)	g	2.6	Vitamin B12	%DV	0
			Vitamin A	%DV	0
Cholesterol	mg	0	Vitamin E	%DV	4
Carbohydrate	g	3.9	mg ATE****		0.83
Fiber	g	1.9	Tocopherol, alpha	mg	0.20
Calcium	mg	28	Tocopherol, beta	mg	0.04
Iron	mg	0.8	Tocopherol, gamma	mg	5.91
Magnesium	mg	45	Tocopherol, delta	mg	0.54
Phosphorus	mg	98	**Total Phytosterols**	mg	20
Potassium	Mg	125	Stigmasterol	mg	0.0
Sodium	mg	1	Campesterol	mg	2.0
Zinc	mg	0.9	Beta-sitosterol	mg	18.
Copper	mg	0.45			
Manganese	mg	0.97			

g = gram; mg = milligram; mcg= microgram, %DV = percent Daily Recommended Value;

Figure 1

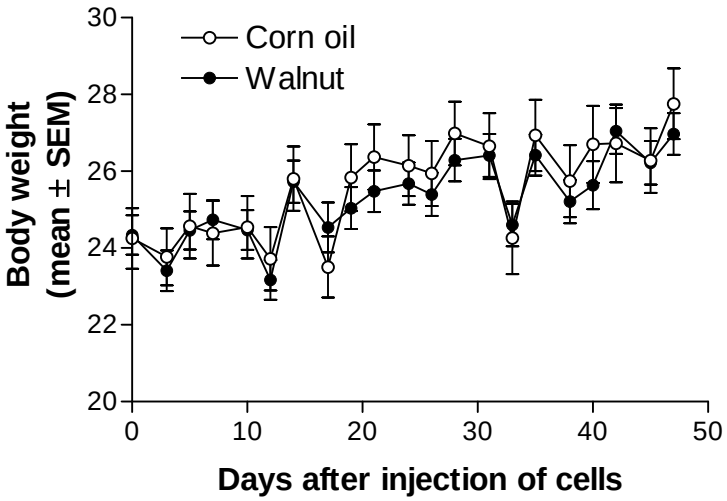
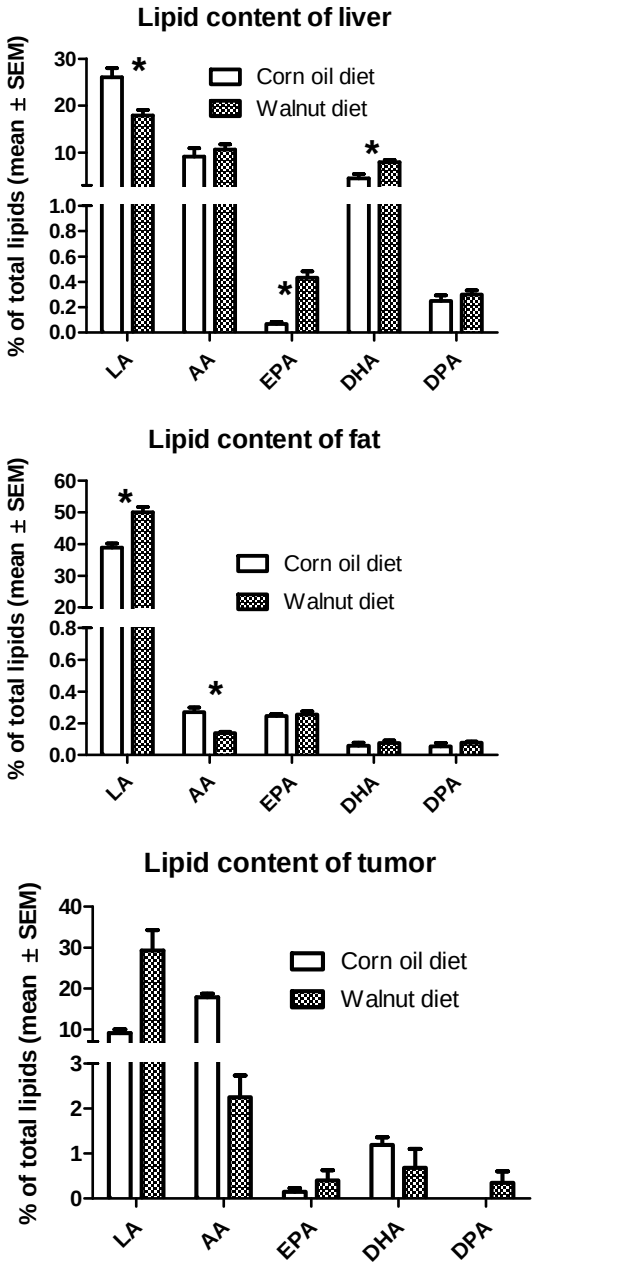

Corn oil
Walnut
Body weight
(mean ± SEM)
30
28
26
24
22
20
0
10
20
30
40
50
Days after injection of cells

Figure 2



***significantly different between diets by t-test, p< 0.05, fat and liver n=5/group, tumor n=2/group**

Figure 3

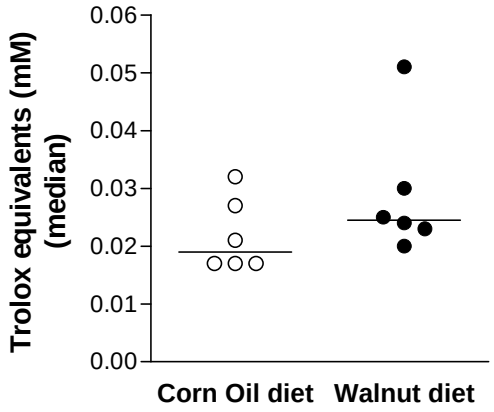

Trolox equivalents (mM)
(median)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Corn Oil diet
Walnut diet

Figure 4

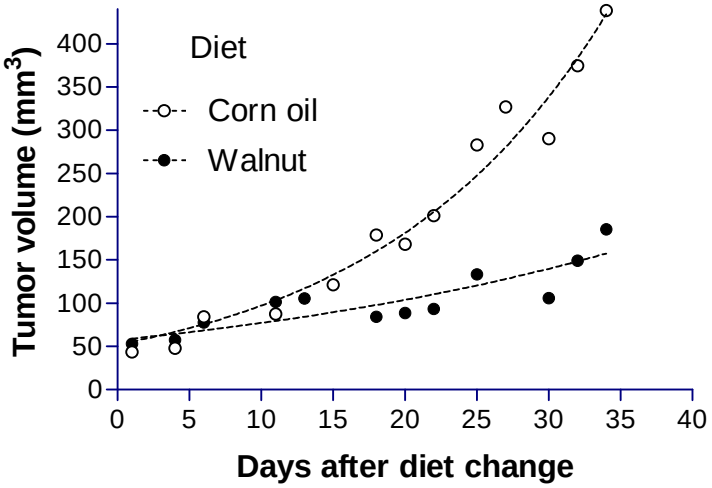
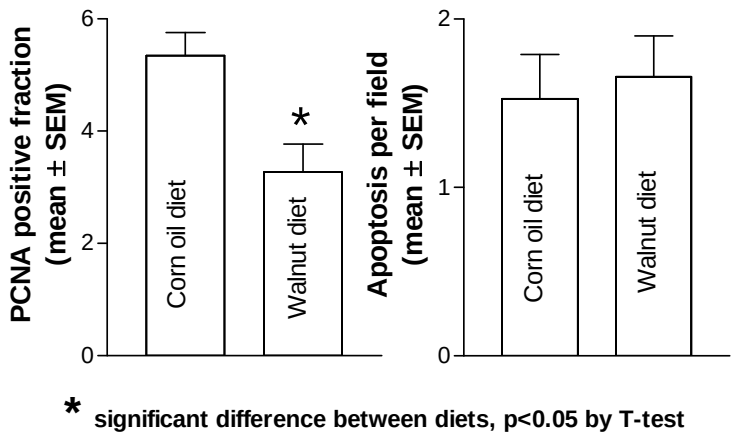
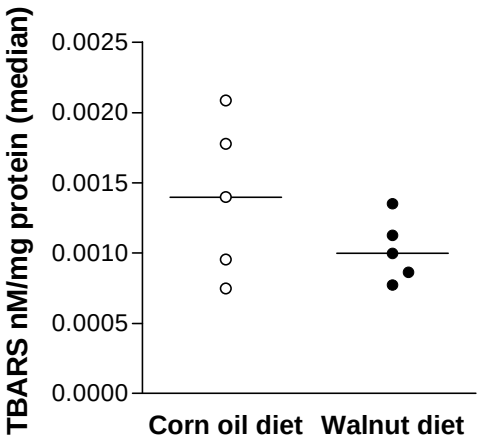

Diet
Corn oil
Walnut
Tumor volume (mm3)
Days after diet change
0
50
100
150
200
250
300
350
400
0
5
10
15
20
25
30
35
40

Figure 5



* significant difference between diets, p<0.05 by T-test

Figure 6


TBARS nM/mg protein (median)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Corn oil diet
Walnut diet