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Impact of dietary walnuts, a nutraceutical option, on circulating markers of metabolic dysregulation in a rodent cachectic tumor model

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Abstract

Background: Nutraceutical foods, like walnuts which are rich in immunonutrients, can have medicinal benefits. Dietary walnuts have been shown to slow or prevent tumor growth in mice genetically programmed to grow breast or prostate tumors. This study investigated whether walnuts could exert the same preventable effect in a transplantable carcinoma rat model.

Methods: Eighteen rats were randomly fed a diet containing walnuts (10% of food by weight), and 36 were fed a diet without walnuts (control) for 21 days. On day 22, 18 control diet rats were switched to the walnut diet. All other animals remained on their same diet. Within each diet group, 6 rats were implanted with the Ward colon carcinoma (TB), and 12 were sham-operated. Five days

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Conflict of interest statement

The authors declare no conflict of interest.

Ethics approval and consent to participate

This study was approved by the Institutional Care and Use Committee at the Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, LA.

CRediT authorship contribution statement

Lauri O. Byerley: Conceptualization, Funding acquisition, Investigation, Formal analysis, Data curation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Hsiao-Man Chang:** Visualization, Writing – review & editing. **Brittany Lorenzen:** Methodology, Data curation. **Jessie Guidry:** Methodology, Data curation, Formal analysis. **W. Elaine Hardman:** Conceptualization, Investigation, Writing – review & editing, Funding acquisition.

Consent for publication

All authors have carefully read and revised the full text and agreed to publication.

Author statement

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later, 6 sham-operated animals were weight-matched to a TB and then pair-fed for the remainder of the study. The remaining 6 sham-operated, or non-tumor-bearing rats, were ad-lib fed.

Results: The tissue of the walnut-eating rats showed higher omega-3 fatty acid (immunonutrient) content which did not slow or prevent tumor growth or the loss of lean and fat mass typical of this TB model. In addition, blood glucose, insulin, IGF-1, and adiponectin levels were significantly lower in the TB, demonstrating metabolic dysregulation. Again, these changes were unaltered by consuming walnuts. Plasma proteomics identified six proteins elevated in the TB, but none could be connected with the observed metabolic dysregulation.

Conclusion: Although walnuts' rich immunonutrient content prevented tumor growth in genetically programmed mice models, there was no effect in this model.

Keywords

Walnuts; Cancer; Cachexia; Weight loss; Rodent tumor model; Metabolic dysregulation

1. Introduction

Cachexia is the unexplained loss of body mass (fat-free and fat) that occurs in more than 50% of cancer patients [1]. This weight loss can be devastating, resulting in a decreased quality and length of life [2], poor response to chemotherapy, and decreased survival [3]. The cachectic syndrome is associated more with certain cancers such as pancreatic and gastric cancer than others like breast and prostate [4]. There are three states of cancer cachexia: precachexia, cachexia, and refractory cachexia [5].

Since there is no known cure for cancer-associated cachexia, prevention would be an optimal investment for patient prognosis. Despite decades of research, the etiology of cancer-associated cachexia remains a mystery. The hallmark of the cachectic syndrome is unexplained loss of body mass, particularly skeletal muscle, but fat mass can be lost as well [6]. An imbalance between protein synthesis and protein breakdown that favors catabolism seems to drive the loss of skeletal muscle mass. Recent research efforts have focused on inappropriate skeletal muscle apoptosis as the cause [7]. Using a transplantable rodent tumor model, our studies suggest the loss of fat mass loss occurs before the loss of lean body mass [8]. Fouladiun et al. demonstrated that body fat loss was more rapid than lean tissue in progressive cancer cachexia [9]. Regardless of the body mass stores lost, cancer-associated cachexia has been established as an energy balance disorder in which other tissues, organs, and biosynthetic substrates compete with the tumor for fuel [10].

Although several therapies have been proposed and tested, no treatment options that eliminate or reverse cachexia exist. Many treatments investigated have involved dietary constituents such as omega-3 fatty acids [11], branch-chain amino acids [12], glutamine [13], and antioxidants [14]. Usually given as purified supplements, some of these components alone or in combination have helped to improve cachexia temporarily [15]. Very few studies have investigated the effect of whole foods. This study investigated the effect of adding a modest amount of walnuts to the diet.

Walnuts were selected due to their high content of alpha-linolenic acid, an omega-3 fatty acid. In addition, walnuts are an excellent source of antioxidants. Both omega-3 fatty acids and antioxidants are known to have anti-cachectic [16,17] and anti-inflammatory properties [18]. Because cancer-associated cachexia is characterized by systemic inflammation [3], food rich in antioxidants and anti-inflammatory compounds is expected to be beneficial.

Several studies have also shown that walnuts can slow or prevent breast [19] and prostate tumor growth in genetically programmed mice [20] and xenografts [21]. These studies added walnuts to the animal's diet without compromising nutritional quality or host growth. These effects, along with walnut's anti-inflammatory properties, made it an excellent option to test as a whole food nutraceutical.

This report summarizes the effect of adding walnuts to the diets of a rodent tumor model on tumor growth, cachexia development, and adipose and glucose dysregulation, all of which are commonly observed in cancer-associated cachexia. A pair-fed group was included to tease out anorectic effects, common during the development of cancer-associated cachexia, from tumor-driven changes. Pair-fed animals received the amount their matched tumor-bearing pair ate 24 h earlier. Animals consuming walnuts had more omega-3 fatty acids incorporated into their tissues as well as had higher blood antioxidant capacity. Although omega-3 fatty acids and antioxidants are known to have health benefits, neither prevented the loss of body lean and fat mass, slowed or prevented tumor growth, or abated the observed metabolic dysregulation. In addition, serum proteomics could not identify proteins involved in glucose and adipose tissue metabolic dysregulation.

2. Methods

2.1. Study design

The Institutional Care and Use Committee at the Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, LA, approved this study. Fifty-four male Fischer 344 rats (Harlan Laboratories) were group-housed for one week and maintained on rat chow upon arrival at the LSUHSC vivarium. Then, the animals were weighed, and their weight was used to randomly assign them to one of two diet groups: 1) walnut (18 animals) and 2) control (36 animals) (Fig. 1). The diets are described under "Diets." Each rat was singly housed and fed daily according to its assigned diet and treatment group for the remainder of the study. The animals were allowed to adjust to their single housing and diet for 21 days.

On day 22, the animals within each diet group were randomly assigned to one of three treatment groups: 1) a tumor-bearing (TB), where the animals were implanted with a tumor and fed ad libitum; 2) a non-tumor-bearing (NTB) that was sham-operated and fed ad libitum; and, 3) a pair-fed (PF) that was sham-operated but given the amount of food the TB animal ate in the previous 24 h. After random assignment to one of the treatment groups, a PF animal was assigned by weight to a TB animal; a PF animal's weight was no more than 2 g less than its matched TB animal and did not exceed its match. The NTB animals were also weight-matched to PF and TB animals (Fig. 1).

After assignment to their treatment group, each animal was anesthetized using isoflurane. Next, a 2 × 2×2 mm piece of the Ward colon carcinoma was implanted subcutaneously on the left hind flank of the animals assigned to a TB group. Dr. Vickie Baracos at the University of Alberta, Canada, graciously supplied cells for this tumor line. These cells were grown in a donor animal before being transplanted into the study animals. PF and NTB animals received the same operation but had no tumor cells implanted.

Following the operation, 18 animals on the walnut diet remained on the walnut diet (W/W), 18 animals on the control diet remained on the control diet (C/C), and 18 animals on the control diet were switched to the walnut diet (C/W). Each animal consumed their respective diet for the remainder of the study. All animals continued to be weighed and fed daily. Pair feeding started ten days after surgery to allow the animals first to recover. Half the food was given in the morning around 8 am and the second half in the afternoon around 4 pm to avoid binging. Our previous studies have shown that the TB animal's food intake does not decrease until fifteen days after tumor implantation [8].

At the end of 49 days, the animals were sacrificed. Twelve hours prior to sacrifice, food was removed from the animal's cage, so the animals were in a similar metabolic state. At sacrifice, the animal was anesthetized using isoflurane, and blood was collected by cardiac puncture. The abdominal aorta was cut to assure death. The liver, epididymal fat pad, posterior subcutaneous adipose tissue, gastrocnemius muscle, and tumor were removed, weighed, and immediately frozen in liquid nitrogen for later analysis. The same investigator collected all tissues.

An adiposity index was calculated by adding the weight of the epididymal and posterior subcutaneous fat depots together [22], multiplying by two, and dividing by the animal's body weight. For the TB animals, body weight minus the weight of the tumor (TB-tumor) was calculated.

2.2. Diets

The diets were identical to those previously reported by Hardman et al. [13]. This diet is based on the AIN-76 diet and was previously reported by our group [23]. The protein, fat, carbohydrate, and fiber content in the control diet was adjusted using corn oil, corn starch, casein, and cellulose, respectively, so the walnut and control diet had a similar macronutrient profile. All ingredients except the sugar, corn oil, and walnuts were purchased from Dyets (Bethlehem, PA). The sugar and corn oil were purchased from a local grocery store in bulk (Albertsons, Mandeville, LA). The California Walnut Commission graciously provided shelled, whole walnuts. Immediately upon receipt, the walnuts were vacuumed-sealed in one kg freezer-proof bags and stored in a 4 °C walk-in cooler. Then, small batches of the following diet were made. First, the walnuts were finely ground using an electric grinder, then mixed with the other diet ingredients in an industrial mixer (Hobart, Troy, OH). Once the mix was the consistency of cookie dough, a portion was rolled out into 1-inch-thick sheet, then vacuum-sealed, and frozen at − 20 °C until used to feed the animals. At feeding time, a diet batch was thawed, cut into 1-inch cubes, weighed, and given to the animal.

The diets were analyzed for protein, fat, and ash content by CoVance (Madison, WI). The carbohydrate and calorie content of the diet was calculated from the analytically derived values. The walnut diet provided per 100 g: 15.6 g protein; 4.3 g fat; 3.67 g fiber; 16.2% moisture; 2.2 g ash; and 61.7 g carbohydrate (calculated). The control diet (358 kcal/100 g diet) was slightly denser calorically than the walnut diet (348 kcal/100 g diet). In both diets, the predominant fatty acid was linoleic acid, 1011.6 ug/g, and 844.9 ug/g, respectively. In addition, the control and walnut diet had an omega-3 fatty acids/g diet content of 43.5 ug/g and 187.9 ug/g, respectively, and an omega-6/omega-3 ratio of 23.3 and 4.5, respectively.

2.3. Fatty acid analysis

Liver fatty acid content was quantified by gas chromatography following lipid extraction using the Folch method with slight modifications as previously described [24]. Briefly, the frozen tissue was homogenized in a chloroform/methanol/KCl mixture, and the lipid was isolated further with changing volumes of chloroform/methanol/KCl. The extracted lipid was saponified using ethanol and KOH, and the fatty acids were extracted using PET ether. A known amount of heptadecanoic acid was added to quantify the fatty acids. Fatty acids were analyzed by gas chromatography as previously described [21].

2.4. Blood metabolites

Glucose was measured using a kit from Cayman (Ann Arbor, MI). Vascular endothelial growth factor (VEGF) (RnDSystems, Minneapolis, MN), insulin (Alpco, Salem, NH), total adiponectin (Alpco, Salem, NH), leptin (EMD Millipore, St Charles, MO), IGF-1 (RnDSystems, Minneapolis, MN), TNF (Invitrogen, Camarillo, CA) and IL-6 (Invitrogen, Camarillo, CA) were quantitated using Elisa kits. HOMA-IR [25] and Quicki [26] were calculated from the glucose and insulin values. Trolox equivalent antioxidant capacity (TEAC) was measured using a kit from Cayman (Ann Arbor, MI). A ferric reducing antioxidant power (FRAP) assay was developed from an assay obtained from The Robert Gordon University (Aberdeen, Scotland) [27]. Adiponectin-leptin ratio, a functional biomarker of adipose tissue inflammation, was calculated by dividing the serum adiponectin value by serum leptin [28].

2.5. Depletion of greater abundance proteins for serum proteomic analysis

In order to detect proteins that were present in lower abundance, the more abundant proteins, like albumin, were removed from the serum using a procedure described by Liu et al. [29]. This protocol removes the bulk of the over-abundant proteins. Briefly, serum from each animal in each treatment group (NTB, TB, and PF) was pooled because a pilot study determined that diet did not alter the expression of serum proteins. To each pooled serum, 1% trichloroacetic acid in isopropanol was added [30]. The mixture was vortexed and centrifuged, and the resultant pellet was suspended in methanol to wash the excess TCA. The pellet was dried, resuspended in 1% sodium dodecyl sulfate (SDS) in triethylammonium bicarbonate (TEAB), and incubated. Protein concentration was quantitated using Bicinchoninic Acid (BCA) Protein Assay Kit (ThermoFisher Scientific, Waltham, MA).

2.6. Cysteine blocking, desalting, and trypsin digestion

100 μg of each protein sample adjusted to a final volume of 100 μL using 100 mM TEAB was reduced using 10 mM Tris (2-Carboxyethyl) phosphine hydrochloride (TCEP) at 55 °C for 1 h. Subsequently, the reduced samples were alkylated using 5 μL of 375 mM iodoacetamide at room temperature for 30 min. Next, chloroform-methanol extraction was used to precipitate the protein [31], which was air-dried, then digested with 2 μg of trypsin (Trypsin Protease, MS Grade, Thermo Scientific Pierce, Waltham, MA) at 37 °C overnight.

2.7. Tandem mass tag (TMT) labeling and basic pH reverse phase fractionation

TMT 6plex reagent set (Thermo Scientific Pierce, Waltham, MA), according to the manufacturer's protocol, was used to label the tryptic peptides. An equal amount of each TMT-labeled sample was pooled together in a single tube and SepPak purified (Waters, Ireland) using acidic reverse phase conditions; acetonitrile [32] and trifluoroacetic acid (TFA). An offline fractionation step was employed after drying to completion to reduce the complexity of the sample. Briefly, 260ul of 10 mM ammonium hydroxide, pH 10, was added to the sample to resuspend it. Then the mixture was subjected to basic pH reverse phase chromatography (Dionex U3000, Thermo Scientific Pierce Waltham, MA). Briefly, UV was monitored at 215 nm for an injection of 100ul at 0.1 ml/min with a gradient developed from 10 mM Ammonium Hydroxide, pH= 10–100% acetonitrile in 10 mM ammonium hydroxide, pH 10 over 90 min. As a result, 12 "super fractions" were created from 48 fractions (200ul each) that were collected in a 96-well microplate using a checkerboard fashion. For example, original fractions 1, 13, 25, and 37 became new super fraction #1; while original fractions 2, 14, 26, and 38 became new super fraction # 2, etc. [33].

2.8. Acidic pH reverse phase chromatography and tandem mass spectrometry

A Dionex U3000 nanoflow system coupled to a Thermo Fusion mass spectrometer (Waltham, MA) was used to analyze the 12 "super fractions." Each fraction was subjected to a 120-minute chromatographic method employing a gradient from 2% to 25% acetonitrile in 0.1% formic acid (ACN/FA) over the course of 100 min, a gradient to 50% ACN/FA for an additional 10 min, a step to 90% ACN/FA for 5 min and a re-equilibration into 2% ACN/FA. The separation was based on a "trap- and-load" configuration. The trap column was an Acclaim C18 PepMap100, 5 μm, 100 A, and the separation column was an Acclaim PepMap RSLC 75 μm × 15 cm (Thermo Fisher Dionex, Sunnyvale, CA). The entire run was 0.3ul/min flow rate, and the sample was ionized through a Thermo Nanospray Flex Ion Source.

2.9. Protein identification and TMT quantitative analysis

An Orbitrap with a resolution of 120,000 was used to collect survey scans with TMT data acquisition using an MS3 approach. Data-dependent MS2 scans were performed in the linear ion trap using collision-induced dissociation (CID) of 25%. Reporter ions were fragmented using a high energy collision dissociation (HCD) of 65% and detected using a resolution of 30,000. Three technical replicates were collected, and Proteome Discoverer 2.2 was performed using TMT data analysis. A SEQUEST search was utilized after the three runs of 12 "super fractions" were merged. We used the Protein FASTA database,

Rattus norvegicus (SwissProt Tax ID=10116) version 2017–07–05. TMT reagents were used to make static modifications on lysine and N-terminus (+229.163), carbamidomethyl on cysteines (=57.021), and dynamic modification of oxidation of methionine (=15.9949). The parent ion tolerance was set at 10 ppm, fragment mass tolerance at 0.6 Da, and the maximum number of missed cleavages at 2. The false discovery rate (FDR) was set at 1%, and only high-scoring peptides were considered. T-test p-values were determined for the three experimental replicate runs.

2.10. Bioinformatics of differentially expressed proteins

To help elucidate the functional relevance of proteins at least 2-fold differentially expressed between groups, we employed a Panther Gene Ontology (GO)-Slim analysis (Version 13.1, released 2018–02–03) [34]. In addition, a protein interaction prediction was performed employing a STRING analysis (Version 10.5 2017–05–14) [35].

2.11. Statistical analysis

Data was statistically analyzed using GraphPad Prism 6 (LaJolla, CA) and SPSS (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp). Variables were tested for homogeneity before analysis. Insulin, HOMA-IR, and FRAP were log-transformed. The actual values are shown in the graphs. Linear regression parameters among adiposity index, leptin, adiponectin, insulin, and the adiponectin/leptin ratio were determined using GraphPad Prism. Differences among the three diet groups (C/C, C/W, and W/W) and three treatment groups (NTB, TB, and PF) for tissue weights, blood metabolites, and tissue fatty acids were determined using a mixed effect model with a factorial arrangement of diet and tumor status as fixed effects and the effects of the initial matching of animals as a random effect. Statistical significance for comparisons between treatment groups or diet groups was derived from tests of differences between marginal means. In all cases, significance was set at a 5% Type I error rate but adjusted for multiple comparisons. Data are presented as means. Error bars are the standard error of the mean, and statistical significance was defined as $p < 0.05$.

3. Results

3.1. Diet

The animals on the walnut diet ate slightly more food than the animals on the control diet, but the walnut diet was slightly lower in calories than the control diet. Therefore, caloric intake for the NTB animals was remarkably similar throughout the study among the three diet groups (data not shown). The average total intake of the NTB animals from day 22 to day 70 was 741 + 18 g. There were no differences in total food intake among the three diet groups, so the value is reported as an average for all diets. Total food intake for the TB and PF animals was significantly less compared to the NTB (NTB (741 + 16 g) vs PF (667 + 16 g), $p = 0.002$; NTB (741 + 16 g) vs TB (660 + 16 g), $p < 0.001$).

3.2. Animal weight

Fig. 2 shows the host body weight for each treatment group on each diet. The average body weight of each group did not differ significantly at the start of the study. Before tumor

implantation, the animals grew at a similar rate (1.7 + 0.4 g/day), gained, on average, 32 + 8 g, and had indistinguishable body weights (all groups). At the time of tumor implantation, there was no difference in body weight among the groups regardless of diet; adding walnuts to the diet did not significantly increase body weight.

Around day 15, the tumor became palpable. Within 23 days after tumor implantation, all the TB animals, regardless of diet, were losing weight. This loss continued until sacrifice. The rate of weight loss for the TB animals during this period was the same, 0.9 + 0.6 g/day, regardless of diet.

3.3. Tumor growth and weight

At sacrifice, tumor weight was not significantly different among the three diet groups (Fig. 3A). For the animals eating the control diet (C/C), the greatest number of palpable tumors occurred sixteen days after tumor implant. There was a one-day lag for the animals eating the walnut diet (W/W), and the greatest number of palpable tumors occurred on day 17. By day 20, all TB animals in the C/C and C/W groups had palpable tumors. It took one day longer for all tumors to be palpable in the W/W group. VEGF was positively correlated with tumor size (Fig. 3B), and low or undetectable levels were observed in the NTB and PF animals for both diets (data not shown).

3.4. Tissue weights

The weights of organs and tissues at the time of sacrifice are shown in Fig. 3C–E. No differences in tissue weights were observed among the diet groups (C/C, C/W, and W/W) within each treatment group (NTB, PF, and TB); thus, the diet groups were consolidated into each treatment group. Liver weight was significantly lower for the PF animals compared to the NTB animals (Fig. 3C) but was not significantly different from the TB animals.

Both the PF and TB animals lost a similar amount of gastrocnemius muscle weight compared to the NTB animals. There was no significant difference between the PF and TB. Two fat pads, epididymal (Fig. 3D) and posterior subcutaneous inguinal (Fig. 3E), were measured; both fat pads were significantly reduced in the TB animals compared to the NTB and PF.

3.5. Fatty acids

Neither diet nor treatment significantly affected the total amount of fatty acids found in the liver of the NTB, PF, and TB animals (Fig. 4A). Both diets contain omega-6 and omega-3 fatty acids, but the walnut diet contains four times more omega-3 fatty acids than the control diet. The predominant omega-3 fatty acid in walnuts is alpha-linolenic acid, which was also the most predominant liver fatty acid composition (data not shown). There was no difference in liver linoleic acid content or the total n6 fatty acid content among the groups (Fig. 4B and C). However, consumption of walnuts significantly increased the omega-3 content of the livers. The C/W and W/W diets had significantly more liver omega-3 fatty acids than the C/C diet (Fig. 4D). Walnuts contained the omega-3 fatty acid, alpha-linolenic acid (ALA), while the control diet contained very low levels of this particular fatty acid. Neither diet contained eicosapentaenoic acid (EPA) nor docosahexaenoic acid (DHA), so these two fatty

acids were generated enzymatically in vivo from ALA. As expected, the liver's omega-6 to omega-3 fatty acids ratio was significantly lower when walnuts were added to the diet (Fig. 4E), indicating a higher omega-3 content.

3.6. Blood antioxidants and metabolites

Antioxidant status was measured by two assays, FRAP and TEAC. No significant differences in antioxidant capacity were observed for the TEAC assay. For FRAP, there was no significant difference based on treatment (NTB, PF, and TB) (Fig. 4E). Instead, the animals that consumed walnuts during the entire study (W/W) had significantly lower values than those that consumed the control diet (C/C), even just for 21 days before tumor implantation (C/W).

Blood glucose, insulin, and several measures of insulin responsiveness (HOMA-IR, Quicki, and FG-IR) were measured (Fig. 5). NTB had significantly higher blood glucose (Fig. 5A) and insulin (Fig. 5B) than TB, regardless of diet. Still, animals consuming the C/C diet had higher values than the W/W, primarily due to one animal's value. This animal also had higher plasma insulin as well. These differences also carried through to the HOMA-IR (Fig. 5C), Quicki (Fig. 5D), and FG-IR (Fig. 5E) values. The NTB animals had a significantly higher HOMA-IR score and significantly lower Quicki and FG-IR compared to the TB. Serum IGF-1 levels were significantly higher in the NTB compared to the TB and PF. There was no significant difference between the PF and TB (Fig. 5E).

For adiponectin (Fig. 6A) and leptin (Fig. 6B), a reduced food intake increased circulating levels of these adipokines (PF groups) while bearing a tumor reduced blood levels (TB groups). Leptin and adiponectin were positively correlated ($y = 271.6 *x + 7178$, $R^2 = 0.2988$, $p < 0.001$).

An adiposity index was calculated from the subcutaneous and epididymal fat pad weights. The TB animals had significantly less adiposity than the NTB and PF (Fig. 6C). Those animals with a higher adiposity index had higher leptin levels (Fig. 6D) and unexpectedly higher adiponectin (Fig. 6E). The ratio of adiponectin to leptin has been suggested as a marker of adipose tissue dysfunction. As the adiposity index declined, the adiponectin-leptin ratio significantly increased (Fig. 6F). Also, leptin was not significantly correlated with insulin ($R^2 = 0.06$, $p = 0.09$).

No significant differences in TNF and Il-6 were observed among the TB, PF, and NTB animals on either diet (data not shown).

3.7. Serum proteins

A total of 171 proteins were identified utilizing a discovery-based proteomics workflow with a false discovery rate (FDR) of 1% or less. One hundred sixty-three of these proteins were quantitated across all samples. A pair-wise analysis was employed to determine any protein that was differentially expressed. A 2-fold threshold was employed and yielded a total of 47 proteins that were flagged as differentially expressed between at least two groups. Six proteins were identified to have significantly higher expression in the TB and PF animals

compared to the NTB animals (Fig. 7). These six proteins had more than two-fold higher expression in the TB animals than the PF.

The 47 proteins were explored further utilizing a Gene Ontology analysis through PANTHER GO-Slim. PANTHER classified the proteins according to three categories: molecular function, biological processes, and cellular components (Fig. 8). For molecular function, 32% were binding, 9% were structural molecules, and 59% were catalytic activity. For biological processes, 5% were cellular component organization or biogenesis, 26% were cellular process, 10% were localization, 14% were biological regulation, 7% were a response to stimulus, 2% were developmental process, 5% were multicellular organismal process, 5% were biological adhesion, 19% were metabolic process, and 7% were immune system process. Finally, for cellular components, 9% were membrane, 17% were macromolecular complex, 22% were cell parts, 13% were organelle, and 39% were extracellular regions. Pie charts representing the classification distribution are presented in Fig. 8, along with GO ID definitions.

Fig. 9 shows the differentially expressed proteins known to have direct connections. An asterisk designates proteins (A2m, Apob, C4bpa, ENSRNOG, Kng1, and Kng2) with a statistically significant fold difference between PF and TB compared to the matched NTB animal.

4. Discussion

During the last several decades, numerous studies have demonstrated the power of dietary constituents and/or whole foods, including walnuts, to influence the early steps of tumor formation. Studies with mouse models showed walnuts could slow or prevent tumor growth. Hardman et al. [19] used the C[3]1 Tag mouse model, a well-characterized breast cancer model in which the female develops mammary gland cancer at a predictable rate [36,37]. At weaning, the mice were fed a walnut-containing diet or a control diet (the same control diet used for this study). Consumption of walnuts significantly reduced tumor incidence, multiplicity, and size. Davis et al. [20] found similar results using the TRAMP mouse model, a transgenic adenocarcinoma of mouse prostate. Prostate tumor weight and growth rate were reduced in the animals eating walnuts. In our study, the transplantable tumor represents later stages of tumor development, when initiation, promotion, malignant conversion, and progression have occurred [38]. The cells used are tumor cells well advanced in their development. Despite prior evidence that walnuts can impact genetically programmed tumor growth, they did not change the growth rate or tumor weight of the well-defined Ward colon carcinoma in our study.

The popular belief is that eating walnuts can cause weight gain [39]. However, several published human studies have shown that walnuts do not promote weight gain in healthy humans [40,41]. In this study, adding walnuts to the animal’s diet did not cause weight gain, supporting the observations in human studies. We did not select walnuts based on their weight gain potential.

Shifts in body composition have been observed in numerous studies of cancer cachexia. This study also observed loss of skeletal muscle and two adipose depots in tumor-bearing animals. Including a pair-fed group allowed us to determine if this was tumor-driven or due to anorexia. The tumor-bearing animal's reduction in gastrocnemius muscle mass was due to a reduction in food intake instead of its tumor-bearing status since this skeletal mass did not differ significantly between the TB and PF animals. In contrast, both adipose depots were reduced in the TB animals compared to the NTB and PF. Since there was no difference in fat pad weight between the NTB and PF animals, the reduction in fat mass can be attributed to bearing a tumor and not to a reduction in food intake. We have observed this in the MCA sarcoma model [8].

Cancer-associated cachexia is associated with adipose tissue secretion of adipokines [42]. Adiponectin and leptin were significantly lower in the blood of the tumor-bearing animals regardless of the diet they ate. This decrease was driven by the presence of the tumor as pair feeding, regardless of diet composition, increased blood levels of adiponectin and leptin. Batista et al. investigated plasma adiponectin and epididymal white adipose tissue adiponectin gene expression in rats bearing the Walker 256 carcinoma with intermediate and terminal cachexia [43]. They found lower adiponectin levels in terminal cachectic rats, as we report here. We have previously reported lower adiponectin and leptin levels in cachectic rats bearing the MCA-sarcoma [8]. In that study, we measured blood levels before body weight and food intake decreased; the results showed significantly lower adiponectin levels in the TB animals, which was not the case for leptin. These observations suggest adiponectin is unrelated to body fat depot size, like leptin in tumor-bearing rats. Several research groups have investigated the loss of adipose tissue-derived adiponectin using mice with congenital loss of adiponectin [44] or genetically induced acute depletion of adiponectin [45]. In these situations, the loss of adiponectin is detrimental to lipid homeostasis, including elevated serum triglyceride. In cachectic Fischer 344 rats bearing the MCA sarcoma, we have reported elevated serum triglycerides before developing the cachectic syndrome [8]. We did not measure serum triglyceride levels in this study.

We then calculated an adiposity index. In non-tumor-bearing animals, this index should be positively correlated with leptin and negatively correlated with adiponectin. As expected, our non-tumor-bearing group had the highest adiposity index, and the tumor-bearing animals had the lowest number. Since adipose is the main site of adiponectin production and secretion [46], we investigated its relationship to the adiposity index. Several published studies have shown adiponectin levels increase with decreasing adipose stores [47]; however, the opposite was observed in our study. The tumor-bearing animals had a lower adiposity index and lower total adiponectin blood levels. Since these levels were significantly lower than the pair-fed animals, it suggests that tumor-derived factors may impact adiponectin secretion. We measured total adiponectin, but adiponectin is released into the blood as a trimer (67 kDa), hexamer (140 kDa), and high molecular weight (300 kDa) [46]. We did not measure its isoforms, which may have been increased.

Interestingly, dietary intake of fish oil or omega-3 supplementation has also been shown to increase adiponectin levels by 14–60% [47]. However, in our study, although walnuts increased cellular omega-3 content, there was no impact on adiponectin levels for the TB

animals. This observation suggests that walnuts cannot change adipose tissue dysfunction when a tumor is present.

Leptin is primarily produced by adipose tissue and circulating levels of leptin correlate closely with the amount of body fat [28]. Serum leptin levels were significantly lower in the TB animals compared to the NTB. We found a positive correlation between serum leptin levels and the adiposity index. We also found the same relationship in MCA sarcoma-bearing rats [8]. This change in serum leptin can be attributed to the presence of the tumor since blood levels of adiponectin were not significantly different between the pair-fed and non-tumor-bearing animals.

A ratio of serum adiponectin to serum leptin has been suggested as a marker of adipose tissue dysfunction [48]. BMI is considered a measure of adiposity. As BMI decreases, blood adiponectin increases, leptin decreases, and the ratio of adiponectin to leptin increases [28]. In this study, adiponectin levels unexpectedly decreased with decreasing body fat in TB animals. Because adiponectin levels increased in pair-fed animals, this inappropriate adiponectin response can be attributed to the tumor. The PF animals also experienced body fat loss, just not as great as the TB animals. Therefore, we found the adiponectin to leptin ratio was not a marker of adipose tissue dysfunction.

We calculated three measures of insulin resistance and β-cell function: HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), Quicki (Quantitative Insulin Sensitivity Check Index), and FG-IR (fasting glucose to insulin ratio). Several studies have demonstrated insulin resistance in cachectic cancer patients [49]. In our study, those animals bearing a tumor had significantly higher Quicki and FG-IR scores, suggesting that something uniquely associated with the presence of the tumor rather than a decrease in food intake drives the insulin insensitivity. These changes could not be altered by including a whole functional food in the animal's diet.

IGF-1 is a circulating hormone that works through autocrine and paracrine mechanisms to promote tissue growth. Costelli et al. reported lower plasma IFG-1 levels within two days after implantation in rats bearing the Yoshida AH-130 ascites hepatoma [50] and suggest this hormone is involved in the pathogenesis of cancer cachexia. Davis et al. [20] observed lower plasma IGF-1 levels in the TRAMP mouse prostate cancer model after consuming a diet rich in walnuts for 24 weeks. The downregulation we saw was unique to the tumor-bearing rats, not associated with the characteristic anorexia or inclusion of walnuts in the diet. The difference may be due to the 1) tumor model as one was transgenic (TRAMP) and the other transplanted (Ward colon carcinoma), and/or 2) the point at which the tumor is exposed to walnuts - during its early initiation or later progression when it is well formed.

Six proteins were highly upregulated in the blood of the tumor-bearing animals; each has been associated with cancer, most by proteomic analysis. However, a particular role in cancer pathogenesis has not been identified. Alpha-2-macroglobulin is a serum protease inhibitor that is synthesized in the liver as well as macrophages and fibroblasts. Although its physiological importance is unknown, several studies have identified elevated levels of

alpha-2-macroglobulin in tumor-bearing mice [51] and humans [52] and suggest it may play an important role in immune-cell function [53].

Two kininogens were elevated, form 2, and the low molecular weight form 1. Kininogens are actively being investigated as a biomarker of hepatocellular carcinoma [54], but no specific biological function has been associated with them. We previously identified kininogen as one of seven proteins that could distinguish three different age cohorts (20–34 y, 60–74 y, and >90 y) [55].

Ig gamma-1(IgG1) chain is the constant region of the immunoglobulin heavy chain and is secreted by B-lymphocytes. Most of the humoral immune response is mediated by IgG immunoglobulins, with IgG1 as the most abundant in the peripheral circulation [56]. Immunoglobulins (Ig) allotypes consist of polymorphic epitopes on the constant domain region. This results in certain IgGs having greater susceptibility to infections, cancers, or autoimmune diseases. This protein is known to be upregulated in Warthin's tumor [57], a benign salivary gland tumor. Our study showed IgG1 is 4-fold higher in tumor-bearing animals.

C4b-binding protein alpha chain (C4BPA) inhibits the complement system, and increased serum levels have been found in pancreatic cancer [58]. Hepatocytes, activated by monocytes, synthesize C4BPA, then activate B cells by mimicking CD40L activity. Therefore, via T-cell antitumor response, C4BPA has been identified as a biomarker for early-stage pancreatic ductal adenocarcinoma (PDAC) [59].

Apolipoprotein B-100 is one of the primary proteins found in chylomicrons, LDL, and VLDL. Elevated blood levels have been observed in breast cancer patients [60]. Our results add to this body of evidence that these proteins are altered by the presence of a tumor.

5. Conclusion

In summary, our tumor model reflects what others have reported. Loss of body fat and skeletal muscle led to unrelenting cachexia in our tumor-bearing animals. Despite similar food intake, the pair-fed animals did not see the same body fat loss pointing to other drivers unique to the tumor-bearing state. Despite losing body fat and skeletal mass, the tumor-bearing animal's tissue reflected their diet. More alpha-linolenic acid was incorporated into the tissue of animals eating walnuts. Our study demonstrated changes in glucose/insulin metabolism and adipocyte function similar to those reported by other investigators for other rodent tumor models. Cachexia is a complex metabolic syndrome that is an integrated physiological response to substrate mobilization. This physiological response is driven by inflammation [61]. Walnuts are rich in anti-inflammatory and antioxidant compounds, making them an excellent whole food nutraceutical choice. However, walnuts did not slow or prevent the growth of the Ward colon carcinoma transplantable tumor, nor did they alter the tumor-driven metabolic changes in adipokines and regulators of glucose metabolism. These tumor-associated changes were too great to be overcome by diet alone.

Acknowledgments

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Data availability

The raw data supporting the conclusion of this article is available upon reasonable request from the corresponding author.

Abbreviations:

NTB	non-tumor-bearing ad lib fed
PF	pair-fed but not tumor bearing
TB	tumor-bearing ad lib fed
C/C	control diet/control diet
C/W	control diet/walnut diet
W/W	walnut diet/walnut diet
FRAP	ferric reducing antioxidant power

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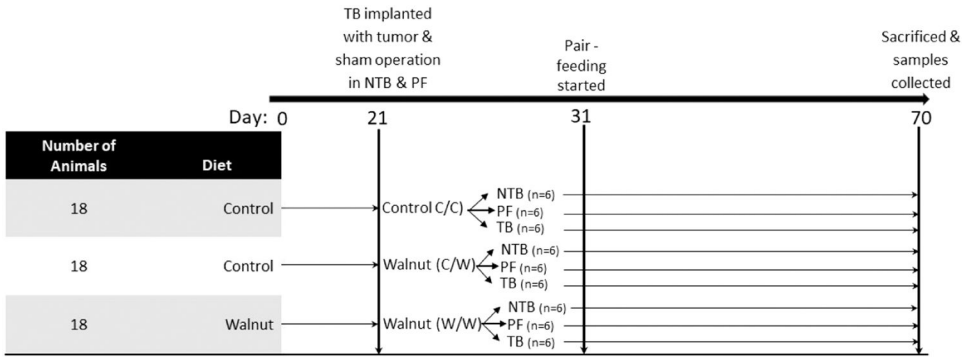

Fig. 1.

Study design and timeline showing the three diet and treatment groups (C/C, C/W, W/W), the diet administered to each, day of tumor implantation or sham operation, start of pair-feeding, and time of sacrifice.

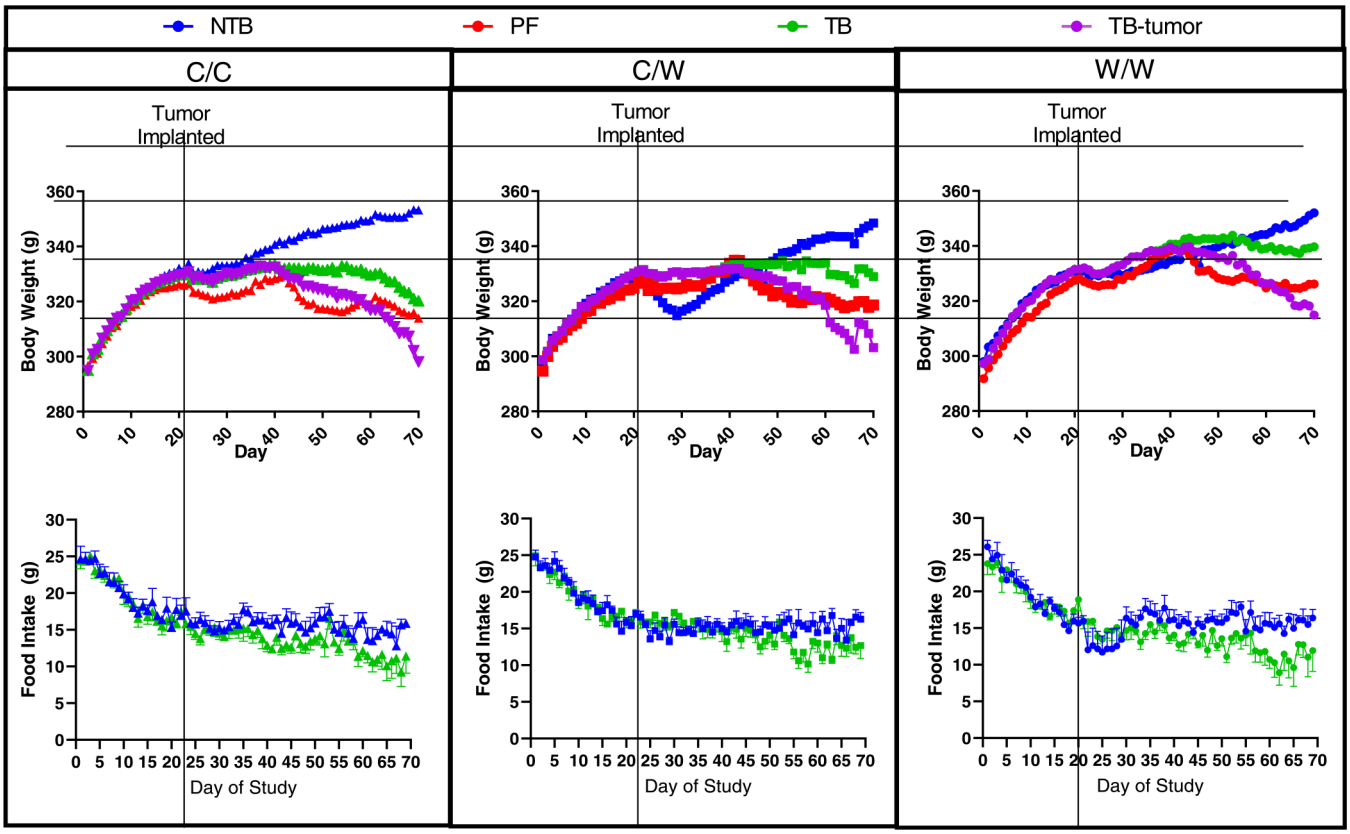

Fig. 2.

Body weight and food intake for each diet and treatment group throughout the study. There were six animals per group. The top three figures show daily body weight 21 days prior to tumor implantation or sham operation and for 49 days after tumor/sham surgery for the non-tumor-bearing (NTB, blue), pair-fed (PF, red), and tumor-bearing (TB, green). Also shown is the host weight of the TB minus tumor weight (TB-tumor, purple). The lower three figures show each diet's food intake for each treatment group. PF are not shown because their food intake was the same as the TB 24-h later. Values are mean (n = 6 per group). Errors bars are the standard error of the mean.

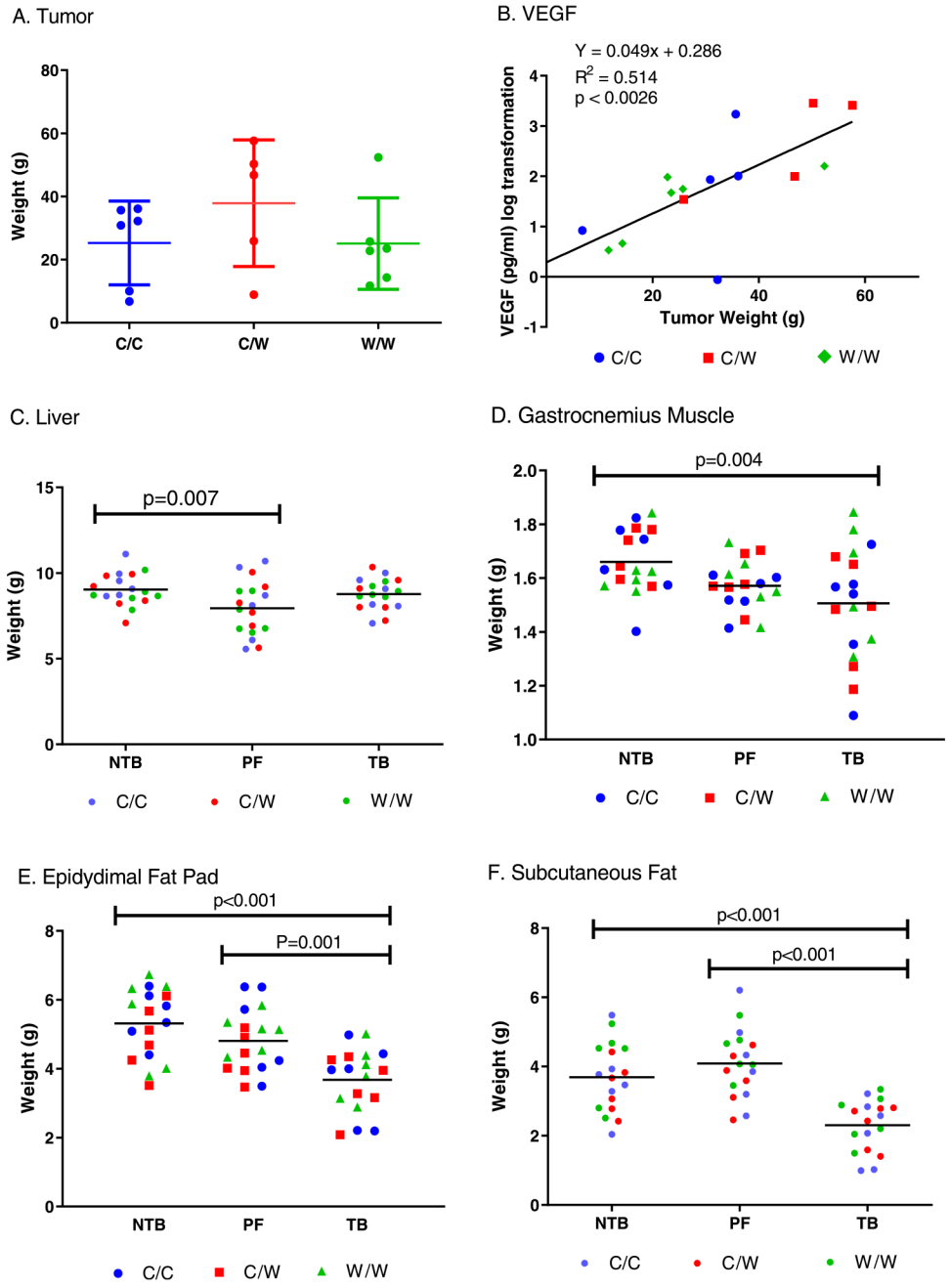

Fig. 3.

Changes in body composition. Final tumor weight is shown in A. Organ weights (C through F) demonstrate TB's muscle and adipose tissue loss. Blood levels of vascular endothelial growth factor (VEGF), a signaling protein that promotes the growth of new blood vessels, correlated positively with tumor size. Blue color is C/C. Red color is C/W and green is W/W.

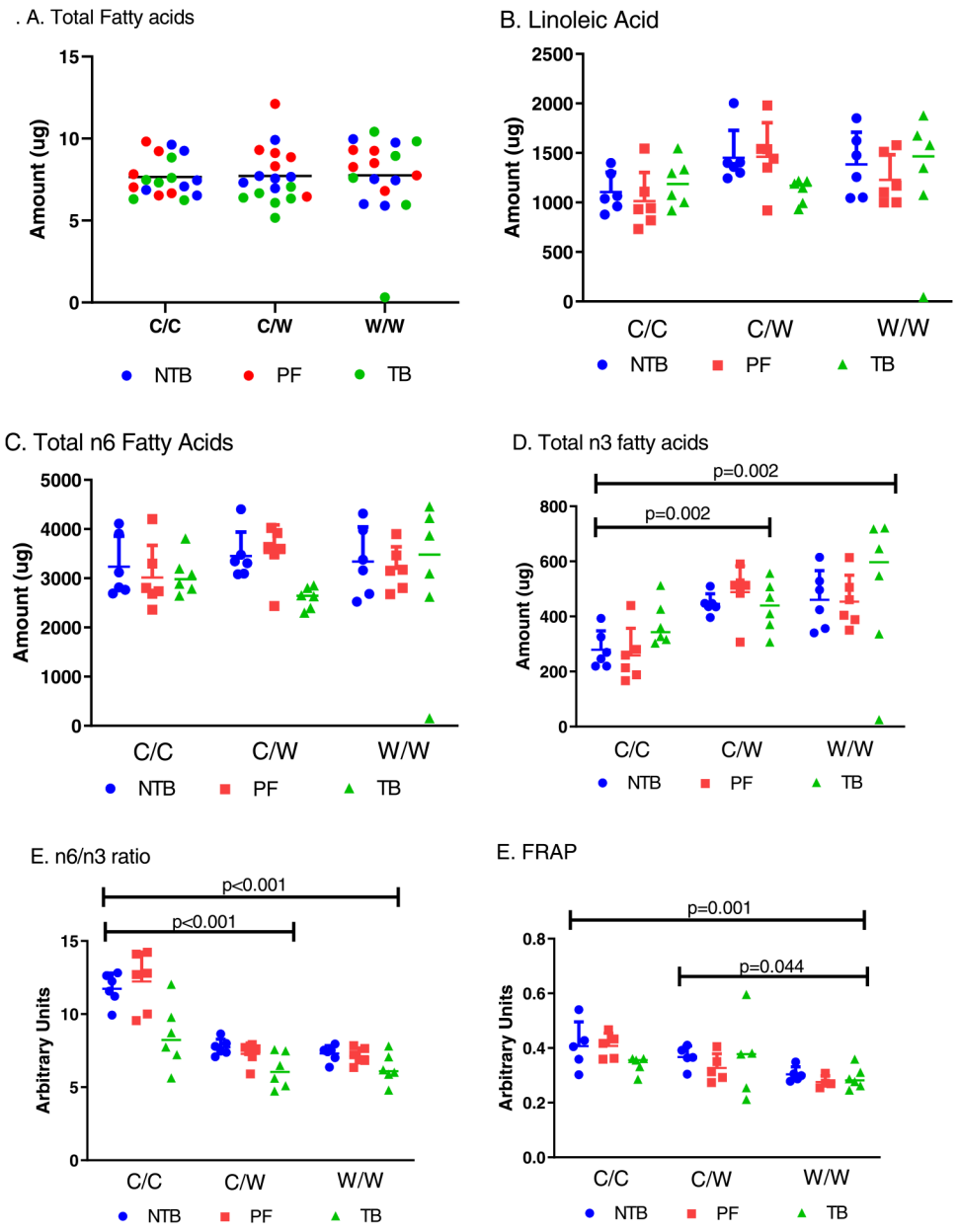

Fig. 4.

Evidence that substances within walnuts were incorporated into body tissues: total fatty acids (A), linoleic acid (B), total n6 fatty acids (C), total n3 fatty acids (D), and n6/n3 ratio (E). Because significant differences were found for diet but not treatment, the x-axis shows diet while the treatment groups are shown in color (NTB blue; PF red; TB green). (E) shows differences in serum FRAP, a measure of blood antioxidant capacity. For all figures, the line above the bars shows significant differences.

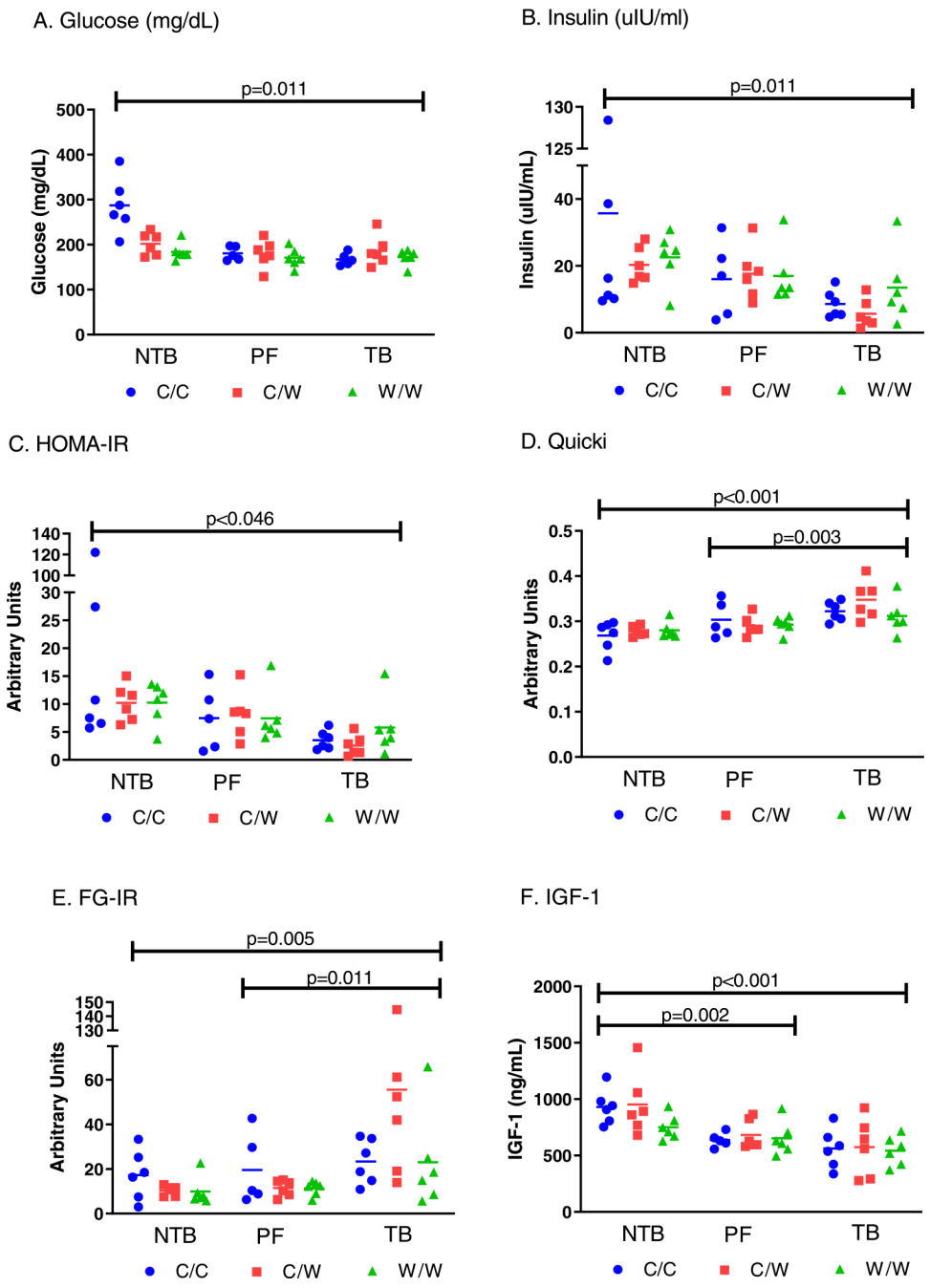

Fig. 5.

Blood measurements associated with glucose metabolism for each diet and treatment group. Diet did not significantly affect any measure, but treatment did, so the data are shown with treatment on the x-axis. For each treatment group, the diet is shown in color (C/C Blue; C/W red; W/W green). For all figures, the bars above the plots show significant differences with the p-value stated above the bar.

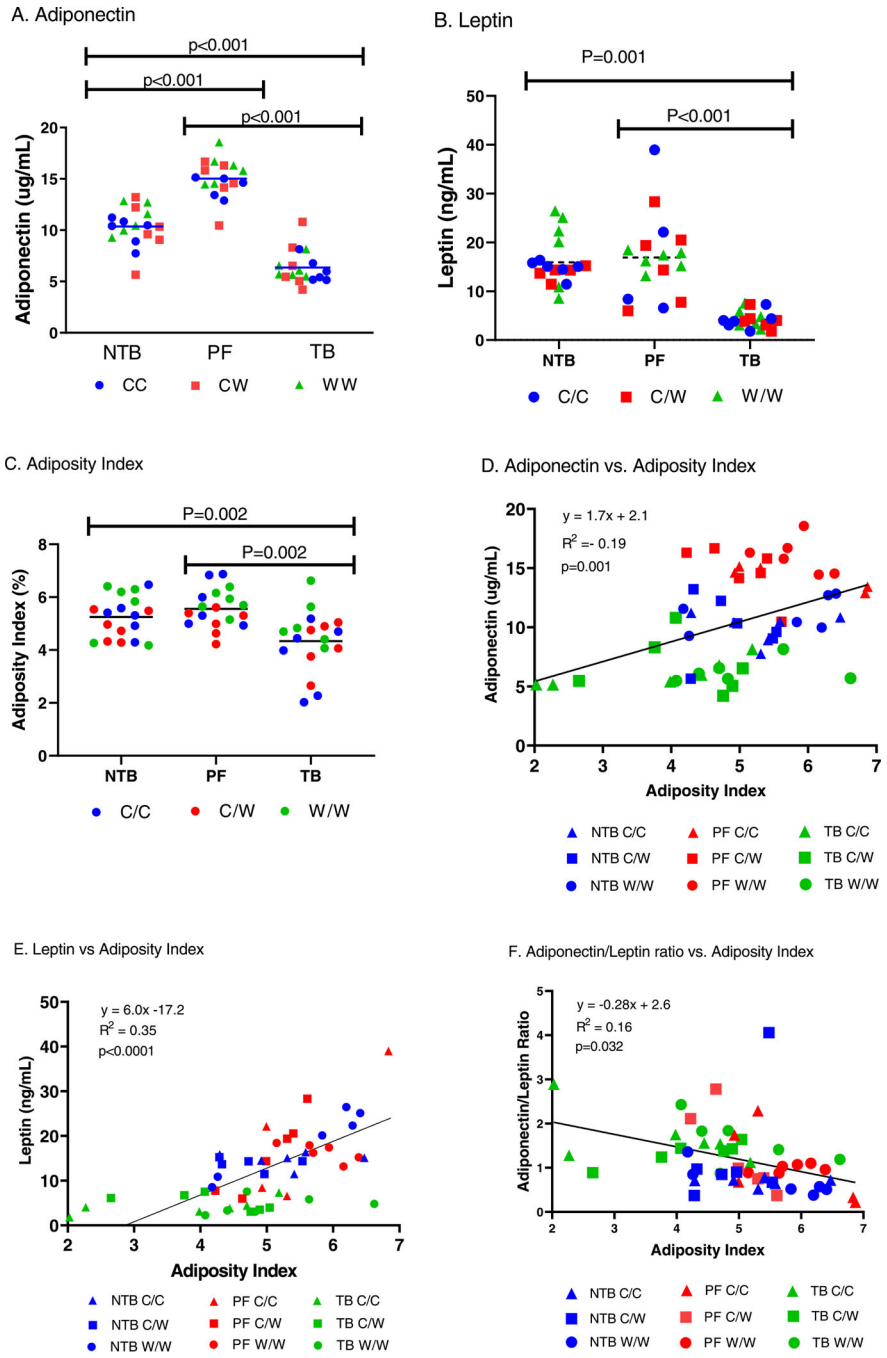

Fig. 6.

Changes in adipokines derived from adipose tissue. Graphs A–C show treatment on the x-axis with the diet color-coded (C/C blue, C/W red, and W/W green). D–F show the correlation between the adiposity index (x-axis) and serum adiponectin, serum leptin, and adiponectin/leptin ratio, respectively (y-axis).

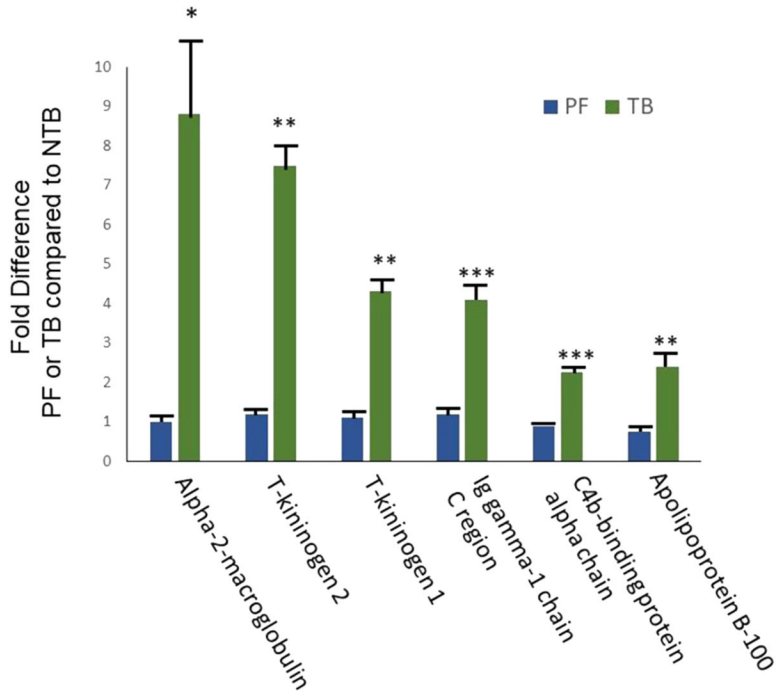

Fig. 7.

Serum proteins for the PF and TB with a significant fold difference compared to their matched NTB animal. Bars show those proteins with a significantly ($p < 0.01$) greater expression in the TB and PF animal's serum compared to their matched NTB. Error bars represent standard error among experimental replicates. T-test comparisons between PF and TB fold-differences were performed; (*, $p < 0.05$), (**, $p < 0.005$), and (***, $p < 0.0005$).

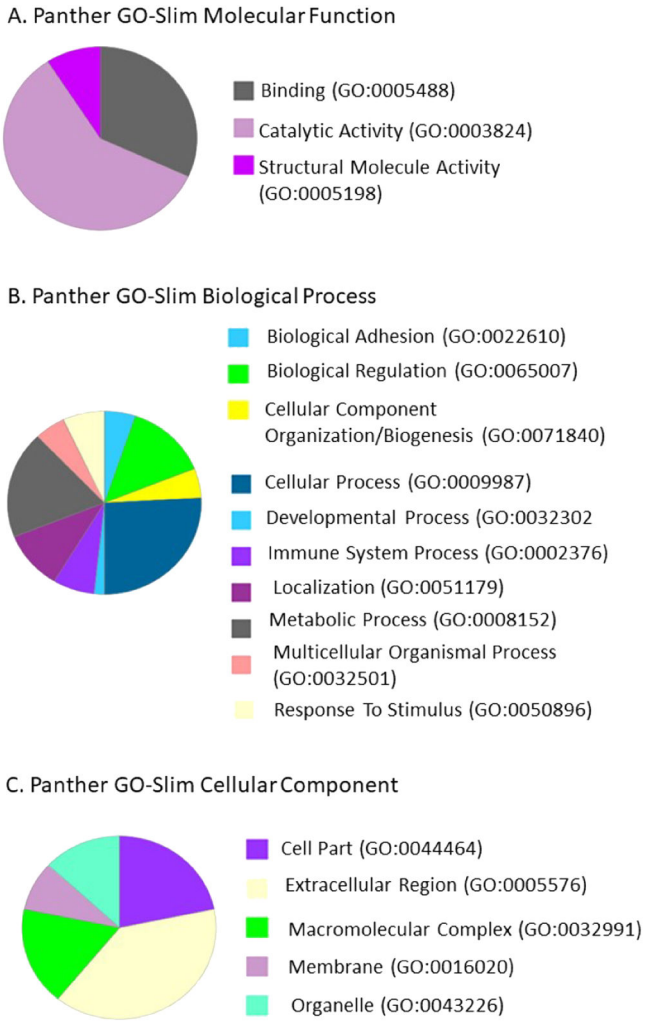

Fig. 8.

Bioinformatic analysis of differentially expressed proteins identified by proteomic analysis. Designations represent PANTHER GO-Slim database entry for each protein [version 13.1, released 2018–02–03] for (a) Molecular function, (b) Biological process, and (c) Cellular component.

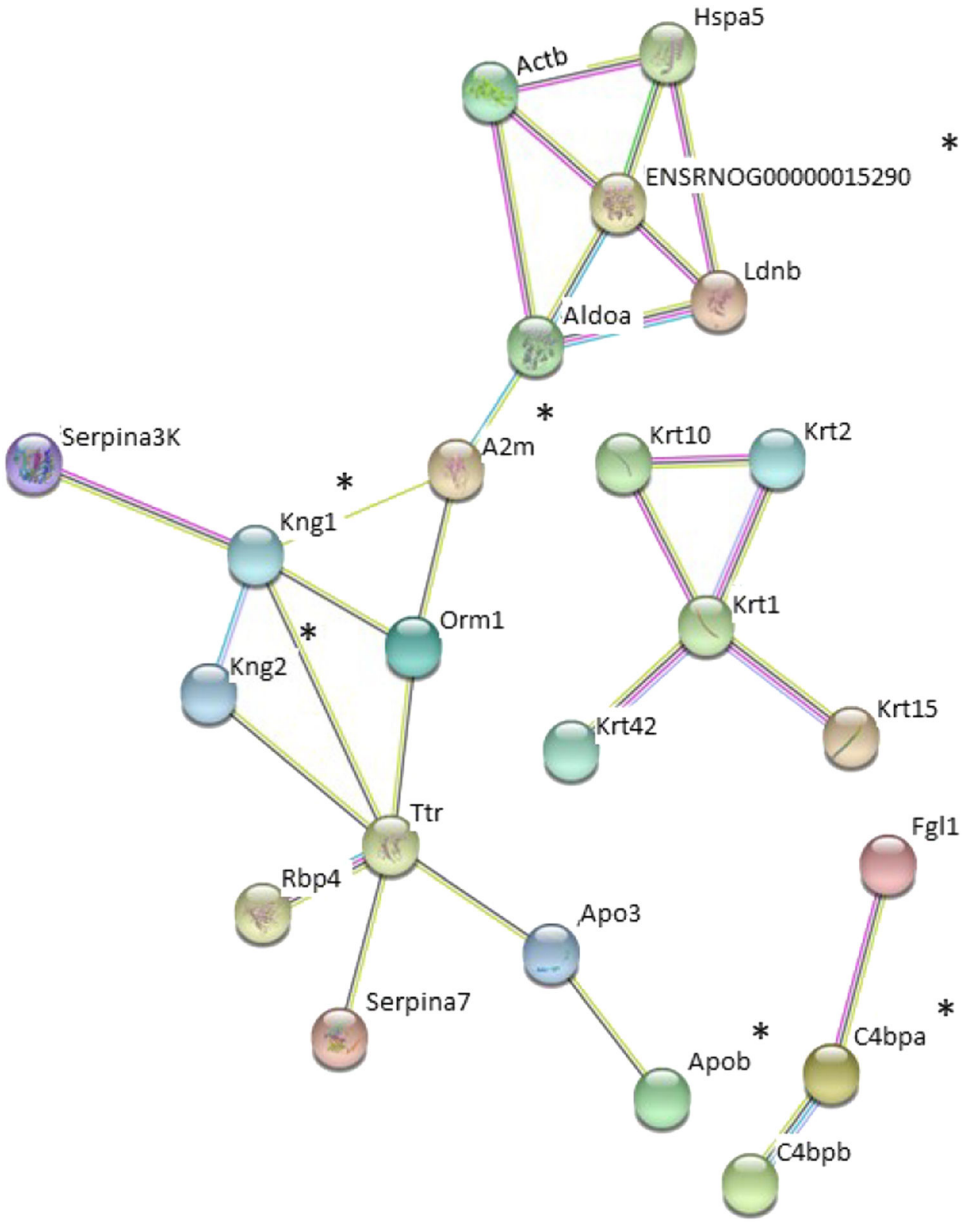

Fig. 9.

STRING analysis (version 10.5 2017–05–14) of differentially expressed proteins identified by proteomic analysis. An asterisk designates proteins with a statistical significance fold difference between PF and TB compared to the matched NTB animal.