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Vitamin A and D3 combinations reduce breast cancer tumor load in a postmenopausal MCF-7 xenograft mouse model in a dose- and time- dependent manner

Nishikant A. Raut[1,2], Temitope O. Lawal[3,4], Bolanle A. Adeniyi[3,4], Pinal N. Kanabar[5], Mark Maienschein-Cline[5], Nina S. Los[6], Zarema Arbieva[6], Gail B. Mahady[7#]

[1]Raman Visiting Scholar, Department of Pharmacy Practice, University of Illinois at Chicago, Chicago, IL 60612, USA; [2]Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, India; [3]Schlumberger Fellow, Department of Pharmacy Practice, University of Illinois at Chicago, Chicago, IL USA 60612; [4]Department of Pharmaceutical Microbiology and Biotechnology, University of Ibadan, Ibadan, Nigeria; [3]MacArthur Fellow, Department of Pharmacy Practice, University of Illinois at Chicago, Chicago, IL USA 60612; [4]Department of Pharmaceutical Microbiology and Biotechnology, University of Ibadan, Ibadan, Nigeria; [5]Research Informatics Core, Research Resources Center, University of Illinois at Chicago, Chicago, IL, USA 60612; [6]Core Genomics Facility, Research Resource Center, University of Illinois at Chicago, Chicago, IL, USA, 60612; [7]Department of Pharmacy Practice, UIC Retsky College of Pharmacy, World Health Organization Collaborating Centre for Traditional Medicine, University of Illinois at Chicago, Chicago, IL, 60612 USA.

***Corresponding author:** Gail B. Mahady, Ph.D. Department of Pharmacy Practice, UIC Retsky College of Pharmacy, World Health Organization Collaborating Centre for Traditional Medicine, University of Illinois at Chicago, Chicago, IL, 60612 USA

Submission Date: November 19th, 2024; **Acceptance Date:** December 12th, 2024; **Publication Date:** December 17th, 2024

Please cite this article as: Raut N. A., Lawal T. O., Adeniyi B. A., Kanabar P. N., Maienschein-Cline M., Los N. S., Arbieva Z., Mahady G. B. Vitamin A and D3 combinations reduce breast cancer tumor load in a postmenopausal MCF-7 xenograft mouse model in a dose- and time- dependent manner. *Functional Foods in Health and Disease* 2024; 14(12): 984-1003. DOI: https://doi.org/10.31989/ffhd.v14i12.1523

ABSTRACT

Introduction: Earlier, we documented that a combination of vitamins A and D3 synergistically inhibited the growth of MCF-7, T48:A18 and SKBR3 breast cancer cells with the best activity seen in the ER+ cell line MCF-7. Transcriptomic analysis of treated MCF-7 cells also showed that the combination significantly upregulated the apoptosis and unfolded protein response canonical pathways, and reduced estrogen signaling.

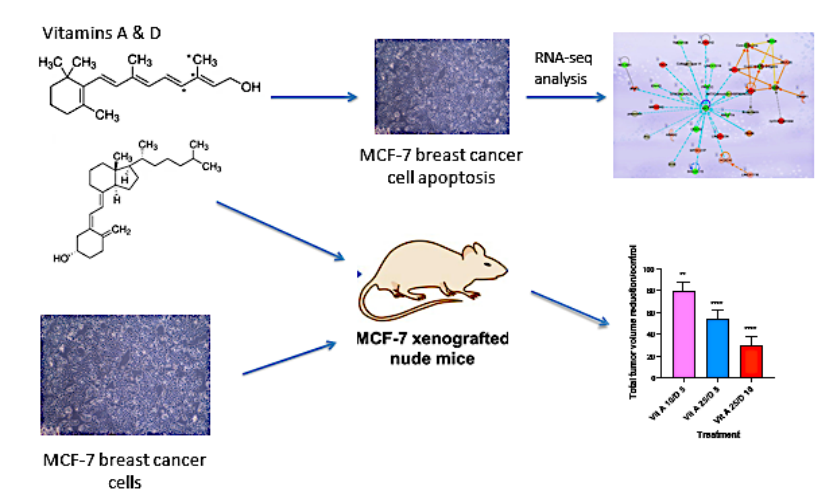
Objective: This study aimed to explore the impact of increasing vitamin A and D3 dose combinations over time in a postmenopausal model of breast cancer using ovariectomized athymic female mice bearing MCF-7 xenografts and further analyze mechanisms of action in MCF-7 cells using RNA-seq analysis.

Methods: MCF-7 breast cancer cells were grown in culture for the xenograft experiments. Athymic female mice were injected with MCF-7 cells (1 x 10^6 in 100 µl of 50% Matrigel mixed with sterile PBS) via subcutaneous injection. Once the tumors reached an average volume of 100 mm^3, the mice were randomly divided into four groups and treated with different vitamin A and D3 combinations. Tumor sizes and mouse body weights were monitored on a biweekly basis. After the treatment period, the mice were euthanized, and the tumors were surgically removed and measured. RNA-seq data from the treated MCF-7 cells were then further evaluated using IPA.

Results: As compared with controls, treatment with vitamin A (25,000 IU) and vitamin D (10,000 IU) led to a significant reduction in tumor volume >70%, ($p < 0.05$-0.01) in OVX athymic mice with MCF-7 xenografts as determined by a two-tailed Student T test. Over the treatment period, the tumor volume in mice treated with vitamin A (10,000 IU) and vitamin D (5,000 IU) or vitamin A (25,000 IU) and vitamin D (5,000 IU) also trended downward and was statistically significant using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test ($p<0.05$ and $p<0.0001$, respectively) but was not significant using a two-tailed Student T test. In cultured MCF-7 cells, Ingenuity Pathway Analysis of mRNA-seq data showed that the vitamin A and D combination significantly altered the expression of 101 genes out of 864 in the molecular mechanisms in cancer canonical pathway, downregulating gene expression in the integrin/P13K/Akt/mTOR pathway.

Conclusions: The findings showed that the combination of vitamins A and D3 effectively reduced tumor burden in a postmenopausal MCF-7 xenograft mouse model, with effects that were both dose-dependent and time-dependent. The combination also significantly altered the expression of genes in the molecular mechanisms of cancer canonical pathway in cultured MCF-7 cells. These preclinical data support the use of vitamins A and D3 in the management of estrogen-dependent breast cancers, with the caveat that higher doses and longer treatment periods may be needed to observe anti-tumor effects.

Keywords: Apoptosis, autophagy, breast cancer, cell cycle, integrin, postmenopausal, P13K, tumor load, xenograft



Graphical Abstract: Vitamin A and D3 combinations reduce breast cancer tumor load in a postmenopausal MCF-7 xenograft mouse model

INTRODUCTION

In 2022, approximately 20 million new cases of cancer were diagnosed globally, and nearly 10 million deaths occurred due to cancer-related causes [1,2]. Lung and breast cancers are the most common in women, and in the United States, breast cancer is the second most frequently diagnosed cancer and the second leading cause of cancer-related deaths. However, in non-Hispanic Black and Hispanic women, breast cancer remains the most common cause of cancer-related deaths [3]. Approximately 5-10% of breast cancers are associated with genetic mutations and familial history, while 20% to 30% of cases may be attributed to modifiable risk factors, including poor nutrition, lack of exercise, alcohol use and other environmental causes [4-5]. In low to middle income countries, the death rate for women with breast cancer is much higher than in Western nations, due to the lack of access to screening and modern healthcare [6]. In fact, approximately 80% of deaths from breast and cervical cancers occur in women from low to middle income countries [6]. Although the death rate for breast cancer has declined in Western countries over the past few years due to increased screening and more personalized treatment strategies, the incidence of breast cancer continues to increase annually [4-5]. Thus, it is critical to investigate new prevention and treatment strategies for breast cancer to develop new ways to lower the both the incidence and mortality rate in all countries.

Functional foods play a significant role in health and disease and contain naturally occurring bioactive compounds such as vitamins, minerals and phytochemicals that are well known to reduce disease risk and promote health [8]. Furthermore, food bioactive compounds are known to reduce cancer risk by suppressing specific gene expression, generating reactive oxygen species (ROS), modulating signaling pathways, inducing autophagy, downregulating anti-apoptotic Bcl-2 family proteins, disrupting the microtubule network, promoting apoptosis, and inducing cell cycle arrest, all of which contribute to their anti-cancer effects [9-16]. Thus, functional foods and their bioactive compounds have significant therapeutic potential for development of low costing therapies for cancer prevention and management. Some good examples of this include vitamins A and D. These compounds and their derivatives are present in dairy products such as milk, as well as in fruits, vegetables, and have been reported to reduce the proliferation of various cancers such as prostate, skin, lung, colon, breast, bladder, and head and neck cancers [13-17]. In our previous work, we have reported that combinations of vitamin A and D, were synergistic in MCF-7 cells (ER+) with an IC50 similar to 5-flurouracil (5-FLU) [17]. MCF-7 breast cancer cells treated with this combination exhibited increased caspase 3/7 activities and upregulated the expression of genes in the unfolded protein response and the apoptosis canonical pathways. Notably, treatment with vitamins A and D in MCF-7 cells led to the downregulation of gene expression involved in estrogen-driven S phase entry and estrogen signaling pathways, suggesting the compounds may exert antiestrogenic effects. Therefore, transcriptomic analysis of the treated MCF-7 cells revealed changes in several cancer-related signaling pathways, reinforcing the idea that combined treatment with vitamins A and D could be more effective than treating with either vitamin individually. While vitamins A and D have been tested separately in breast cancer xenograft models, the combined effects of vitamins A and D in vivo have not yet been reported.

In this work, we tested the effects of escalating doses of vitamin A and D combinations in an estrogen-dependent MCF-7 xenograft in ovariectomized female outbred athymic nude mice to determine the in vivo effects, as well as further investigated the mechanisms of action using transcriptomic analysis of treated MCF-7 cells.

METHODS

Cell Culture and Maintenance: The MCF-7 human breast

cancer cell line was sourced from ATCC and maintained using the protocols previously outlined by our team [17]. The cells underwent short tandem repeat (STR) analysis for identification and were tested to ensure they were free from mycoplasma contamination. In brief, the MCF-7 cells were cultured in Eagle's Minimal Essential Medium (EMEM, ATCC, 30-2003) supplemented with 10% fetal bovine serum (FBS, Gibco, 16140-071). After thawing, the cells were transferred to fresh media, centrifuged, and the resulting pellet was resuspended in warm complete culture medium (EMEM + 10% FBS). The cells were then plated into a T175 flask for continued culture. The cells were maintained by media replacement every 3-4 days and passaged when cells reached approximately 75% confluence in 5% CO2 at 37°C [17].

Cell viability measurement*:* The viability of cultured MCF-7 cells was determined using Cell TiterGlo® (Promega) as we have previous described [17]. The IC_{50} concentrations were determined by conducting a log (inhibitor) versus normalized response analysis, using GraphPad Prism 10.3 software (GraphPad Software, Inc., La Jolla, CA, USA).

Total RNA extraction: MCF-7 cells were sub-cultured into 6-well well plates at a density of 1.0 x 10^6 cell/1 ml media/well. The cells were allowed to incubate overnight, after which they were treated with either the vehicle solvent (0.01% DMSO) or a combination of vitamins A and D at the IC50 concentration. RNA was extracted using Trizol and its quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Tech., Palo Alto, CA), with RNA samples having a RIN greater than 9 selected for RNA-seq analysis [17].

Transcriptomic analysis: RNA-seq was carried out following the methodology outlined in our previous studies [17-19]. The RNA-seq libraries were quickly prepared using the Universal Plus mRNA-Seq kit (Tecan). Preparation of the libraries was performed using 250 ng RNA/sample with fifteen PCR cycles [17-19]. Electrophoresis was carried out to purify the amplified libraries and assess their fragment size distribution (ranging from 264 to 294 nt) using an Agilent 2200 TapeStation System with D1000 ScreenTape. The final library concentrations were measured by PCR using the KAPA Library Quantification Kit (Roche, KAPA Biosystems). Library proportions were assessed with a MiniSeq system (Illumina), followed by normalization and pooling of the libraries to a final concentration of 10 nM. Sequencing was performed on a NovaSeq 6000 Sequencing System (Illumina) with an SP flowcell (2x50 nt reads) [17].

Bioinformatics: Raw data processing was performed at the UIC Core for Research Informatics (UICCRI) using FastQC for general quality-control metrics as we have described [17- 19]. In brief, raw reads were aligned to the human reference genome hg38 using STAR and BWA MEM, while FeatureCounts was employed to quantify ENSEMBL genes [20-22]. EdgeR and the exactTest function were used to calculate differential expression statistics for the raw expression counts. Statistical P-values were calculated and then the Benjamini and Hochberg correction was used to adjust for multiple testing using the false discovery rate (FDR; q value) [23].

Ingenuity® Pathway Analysis (IPA): To analyze differentially expressed genes (DEGs) and related biological networks and pathways, we used the predicted protein functions from the Ensembl Database [17]. Statistically processed transcription data were exported as an Excel file and uploaded into Ingenuity® Pathway Analysis software (Qiagen, USA). Genes showing upregulation or downregulation were filtered based on a fold change (FC) greater than 1 or less than -1, a Z-score of 2, and a false discovery rate (FDR) of less than 0.01. This data was then used to map and identify relevant canonical pathways within the Ingenuity Pathway Analysis database [17, 24].

Data sharing and availability: The RNA-seq data supporting the findings of this study have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) database and can be accessed publicly under the GEO accession number GSE221019 at https://www.ncbi.nlm.nih.gov/geo.

In vivo mouse model: All animal protocols related to the MCF-7 xenograft mouse model experiments were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee (UIC IACUC #23-056) prior to initiation of the study. After obtaining UIC IACUC approvals, xenografts were established using forty, six-week-old female ovariectomized (OVX) outbred athymic nude mice (Jackson Laboratories, Sacramento, CA). To promote tumor growth of estrogen-dependent MCF-7 breast cancer cells, 17β-estradiol time-release pellets (1.7 mg/pellet, 60-day release; Innovative Research of America, Sarasota, FL, USA) were implanted subcutaneously at the time of ovariectomy (OVX) to facilitate the development of MCF-7 tumors in the mice. The mice were allowed to recover from OVX surgery for six weeks. The mice were given ad lib access to water and food, (Control diet AIN-76A rodent diet containing low concentrations of vitamin A (retinyl acetate, 5,000 IU/Kg diet) and vitamin D (cholecalciferol, 1000 IU/kg/diet; Research Diets, New Brunswick, NJ).

At the end of week six, and prior to the start of treatment, OVX mice (postmenopausal model) with estradiol (E2) implants were prepared for xenograft injection using standard approved anesthesia (isoflurane, ~2-3%). A solution of MCF-7 cancer cells suspended 1:1 in sterile media:Matrigel™ (Corning Life Sciences) was implanted subcutaneously in the right rear flank of each mouse. Four weeks later, when the average tumor volume reached 100 mm^3, the E2 implants were removed, and the implants were substituted by daily subcutaneous injection of E2 (estradiol) at 100 pg/mouse (this is equivalent to the physiological concentration of estrogen in postmenopausal women) for the remaining treatment period. On treatment day (SD)-1, in a single enrollment, all mice were randomized and distributed into groups to provide an approximately equal tumor volume of 100- 200mm^3 in each group with approximately equal tumor volume variance between groups. The mice were marked for identification with ear notches and kept in individually ventilated polysulfonate cages with HEPA-filtered air, housing up to five mice per cage. The cages were cleaned biweekly. The animals were kept under a controlled 12-hour light/dark cycle (6 am to 6 pm light), with room temperature maintained between 20-26°C and humidity levels between 30-70%. Additionally, the room had 15 air exchanges per hour to ensure proper ventilation. Body weight, clinical assessments, and measurements using digital calipers were documented two times a week. During the study period, all mice received daily cage-side observation for signs of morbidity and mortality. Twice weekly, the mice were removed from the cage for body weight measurements, detailed clinical observations, and digital caliper tumor measurements recorded twice weekly post dose initiation. The animals were euthanized before the scheduled study terminus at the discretion of the veterinary staff, including if they met any of the following criteria:

i) a body condition score of ≤ 2, ii) a body weight loss of > 20%, or iii) the development of ulcerated tumors/tumors with a volume > 2000 mm^3. Mice were euthanized by CO2 asphyxiation and tumor tissues were photographed.

Diet and treatments: The control and treatment diets were prepared by Research Diets (New Brunswick, NJ), AIN-76A rodent diet with different levels of vitamin A and D3. The diet contained 20% protein, 66% carbohydrate and 5% fat per gram. Vitamin A was added as retinyl acetate and vitamin D3 as cholecalciferol.

Table 1. Groups and Treatments:

Group	N	Treatment	Unit/Kg diet	Vitamin ROA	Vitamins Frequency	Estradiol pg/mice
1	10	Vitamin A	5,000IU	Diet	Total study	Daily and 100
		and Vitamin D3	+1,000IU		period	pg/mice (S.C.)
2	10	Vitamin A	10,000IU	Diet	8 weeks	Daily and 100
		and Vitamin D3	+ 5,000IU			pg/mice (S.C.)
3	10	Vitamin A	25,000IU	Diet	8 weeks	Daily and 100
		and Vitamin D3	+ 5,000IU			pg/mice (S.C.)
4	10	Vitamin A	25,000IU	Diet	8 weeks	Daily and 100
		and Vitamin D3	+10,000IU			pg/mice (S.C.)

Statistics: Statistical evaluations were carried out using GraphPad Prism Version 10.3. To determine the significance between treatment groups and the control, a one-way analysis of variance (ANOVA) was performed, followed by Dunnett's post hoc test. Sample size was determined using a power analysis, designed to detect potential differences between treated and untreated groups. A total of 10 mice per group was deemed adequate to identify a 50% reduction in tumor size/volume with 80% statistical power, using a t-test, with a significance threshold set at $P < 0.05$. To compare the control group with each treatment group at the study's endpoint, a two-tailed Student's t-test was utilized. For RNA sequencing analysis, differential gene expression was examined using edgeR, applying the exactTest function to raw expression counts. Multiple testing corrections were made using the false discovery rate (FDR), as outlined by Benjamini and Hochberg [23].

RESULTS

MCF-7 breast cancer cell viability is reduced by vitamin A and D combinations: Combinations of vitamins A and D3 (1:1) reduced the proliferation of cultured MCF-7 cells with an IC50 of 1.5 µg/ml and an IC90 of 5 µg/ml. To further understand possible mechanisms and signaling pathways associated with the antitumor effects of vitamin A and D combination, RNA- seq data from vitamin A and D treated MCF-7 cells was re-analyzed using Ingenuity Pathway Analysis (Qiagen). Analysis of data from vitamin A and D treated MCF-7 breast cancer cells (treated with the IC50 concentration) showed that the molecular mechanisms of canonical cancer pathway, including autophagy and apoptotic signaling was significantly upregulated ($q < 0.01$), and cancer metastatic pathways were significantly downregulated ($q<0.01$) (Figure 1). The molecular mechanisms in cancer canonical pathway showed significant differential expression of 101 of 864 genes in this pathway ($q<0.01$, Figure 2). Treatment of cultured MCF-7 cells with a combination of vitamin A and D downregulated Integrin mRNA expression, inhibited cell cycle progression by reducing gene expression of cyclin-CDK complexes, cAMP, and PI3K/Akt; induced apoptosis by upregulating the expression of caspase 3/7, caspase 8-9, and downregulating expression of MAPK/ERK and PI3K/Akt mRNAs (Figure 2). It also upregulated autophagy signaling through increased ceramide, ER stress, and downregulating mTORC1/2, supporting our previous results [17].

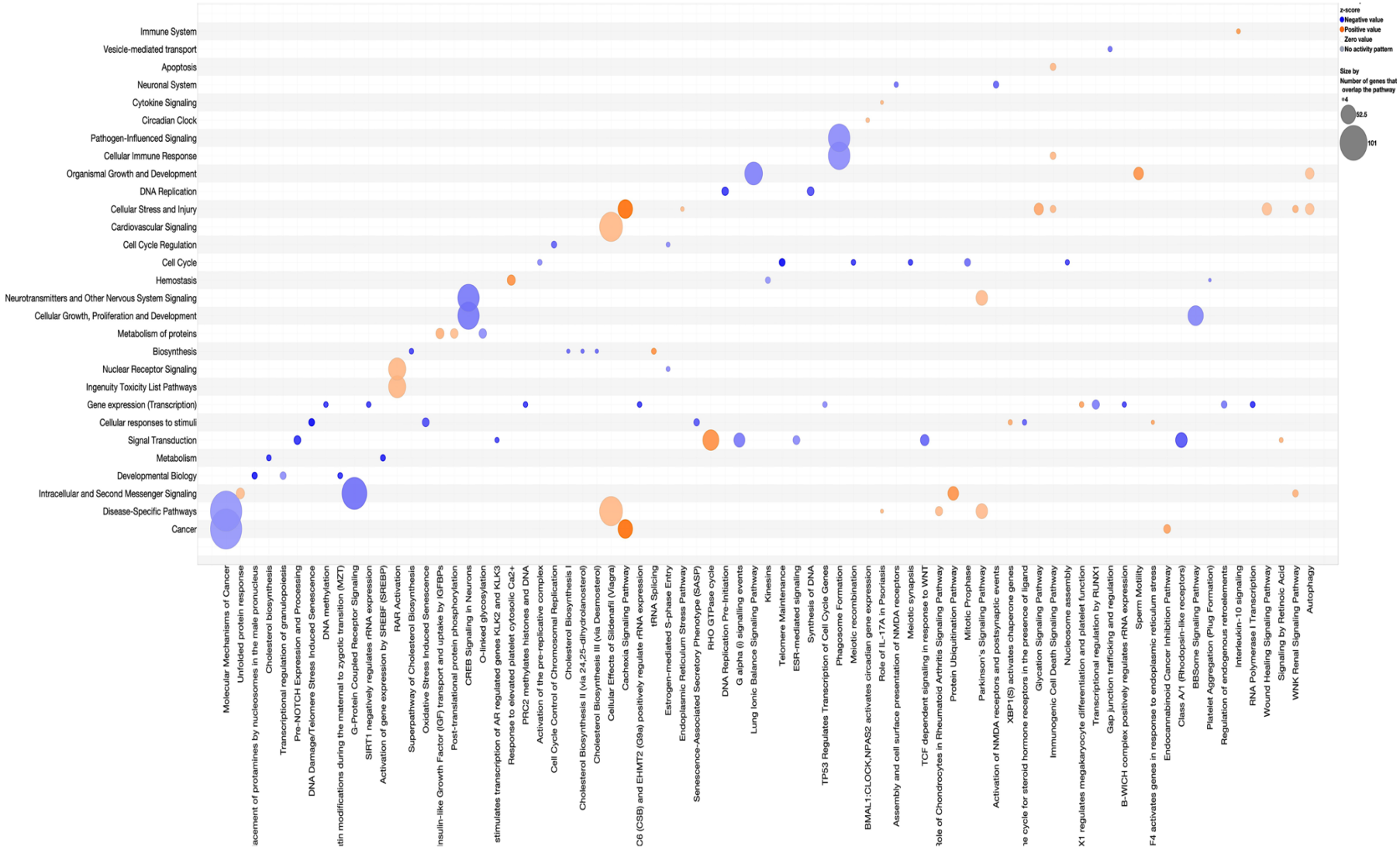


Figure 1. A Bubble volcano plot showing canonical pathways (X axis) and pathway categories (Y axis) impacted by the treatment of cultured MCF-7 cells with a combination of vitamin A and D (1:1) at the IC50 concentration. Multiple canonical pathways associated with cancer were significantly altered. Autophagy and apoptosis signaling pathways are significantly upregulated, while metastatic cancer signaling is significantly downregulated. The pathways impacted are depicted as bubbles, with the size of the bubble indicating the number of differentially expressed genes overlapping the pathway, and the colors of the bubbles are Z scores. The red/orange colors show significant upregulation, while bubbles depicted in blue show significant downregulation. Only differentially expressed genes having a Log2 fold change > ±1.5 and a false discovery rate (q) of < 0.01 were included.

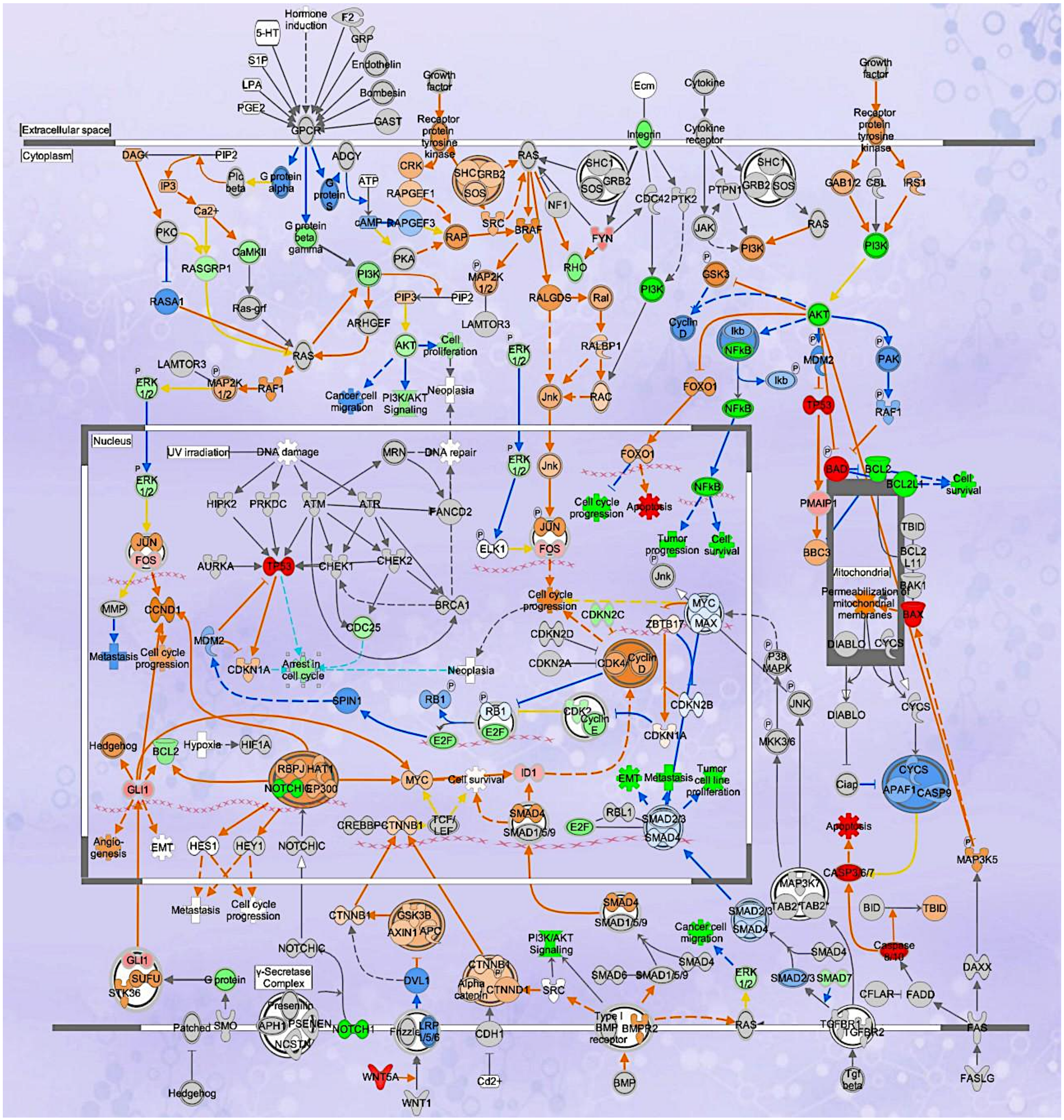


Figure 2. Ingenuity Pathway analyses of RNA-seq data generated from MCF-7 breast cancer cells treated with vitamin A and D3 at the IC50 concentration. The molecular mechanisms of cancer canonical pathways were significantly impacted by vitamin A and D treatment of cultured MCF- 7 cells. Treatment reduced Integrin expression, inhibited cell cycle progression by reducing gene expression of cyclin-CDK complexes, *cAMP*, and *PI3K/Akt*; induced apoptosis by upregulating the expression of caspase 3/7, caspase 8-9, and downregulating expression of *MAPK/ERK* and *PI3K/Akt* mRNAs. It also upregulated autophagy signaling through increased ceramide, ER stress, and downregulating *mTORC1/2*. The red/pink/orange colors represent genes and events that are significantly upregulated. Genes and events that are significantly downregulated are shown in green/blue. Of the 864 genes associated with molecular mechanisms in cancer, 101 were differentially expressed in treated cells. Only genes having a Log2 fold change > ±1.5 and a false discovery rate of < 0.01 were included in this figure.

Combinations of vitamins A and D3 reduce breast tumor load in MCF-7 xenografts in a dose and time-dependent manner: To determine the in vivo anti-tumor effects of this combination, we performed a dose escalation/comparison study in a postmenopausal athymic mouse MCF-7 xenograft model. Female athymic OVX mice with E2 implants developed breast cancer tumors after subcutaneous implantation of MCF-7 breast cancer cells. After four weeks, when the average tumor volume (TV) reached 100-200 mm^3 the mice were randomized into treatment groups. Just prior to randomization, the E2 implants were removed and substituted by daily subcutaneous injection of E2 (estradiol) at 100 pg/mouse for the entire treatment period to maintain the xenograft tumors and to provide a xenograft model that more closely mimics the physiological hormonal status of postmenopausal women. The mice were then randomized into groups (Table 1) and fed the control chow (Group 1) with low vitamin A and D, or chows containing Vit A 10,000IU and Vit D 5,000IU/Kg diet (Group 2); Vit A 25,000IU and Vit D 5,000IU (Group 3) or Vit A 25,000IU and Vit D 10,000IU/Kg diet (Group 4). Figure 3 shows that the weight of the mice injected with MCF-7 cells remained constant over the entire treatment period, with no significant weight loss or gain was observed in any of the groups.

The tumor volume continued to grow in the control group (Group 1, n = 10) over the treatment period, reaching a mean volume of ~124 mm^3 by the end of the study. In the treatment groups, the mean tumor volumes were significantly lower over the treatment period as determined by one-way ANOVA followed by Dunnett's multiple comparison test (Figures 4A and B). As compared with control MCF-7 xenograft mice, treatment with 10,000 IU Vitamin A and 5,000 IU vitamin D/Kg diet (Group 2) significantly reduced tumor volume by a total of 19.6% ($p = 0.039$; 95% CI 0.7943 to 38.42) over the total treatment period; by increasing the vitamin A dose to 25,000 IU vitamin A and maintaining the vitamin D dose at 5,000 IU/Kg diet (Group 3), we observed a significant reduction in tumor volume ~ 45.35% ($p<0.0001$; 95% CI 26.53 to 64.16); and finally treatment with vitamin A 25,000 IU and increasing the vitamin D dose to 10,000 IU/kg diet (Group 4) significantly reduced tumor volume by >70% ($p<0.0001$; 95% CI 51.53 to 89.16) as seen in Figure 4B. However, using the more robust two-tailed Student T test, comparisons between the control group and each of the treatment groups showed that only the group 4 mice treated with vitamin A 25,000 IU and vitamin D 10,000 IU had a significant reduction in tumor volume by the end of the treatment period (Figure 5A and B). No significant results were observed in either Groups 2 or 3 using a two-tailed Student t test as compared with controls (Figure 6 A and B). The results show that by increasing either the vitamin A and/or the vitamin D dose within the combination, the tumor volume trended downward in all groups, with the higher doses having a significant impact on tumor volume over time. These results suggest that both vitamins A and D, together are acting together to reduce tumor volume in MCF-7 xenografts.

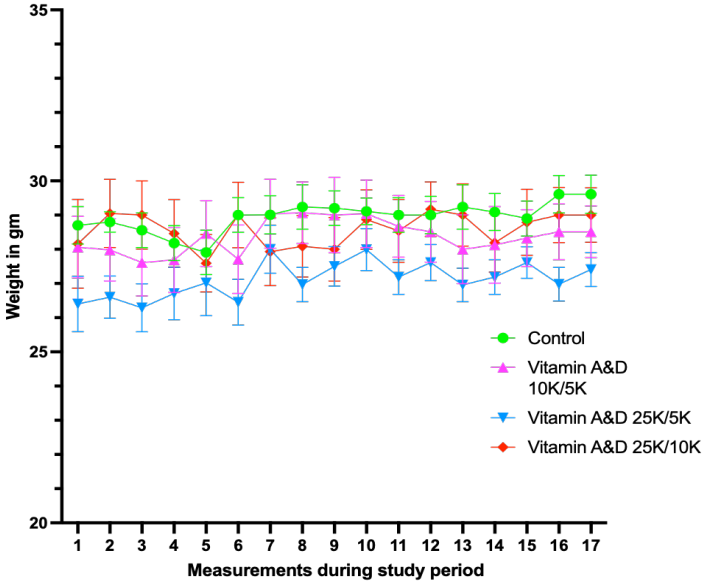
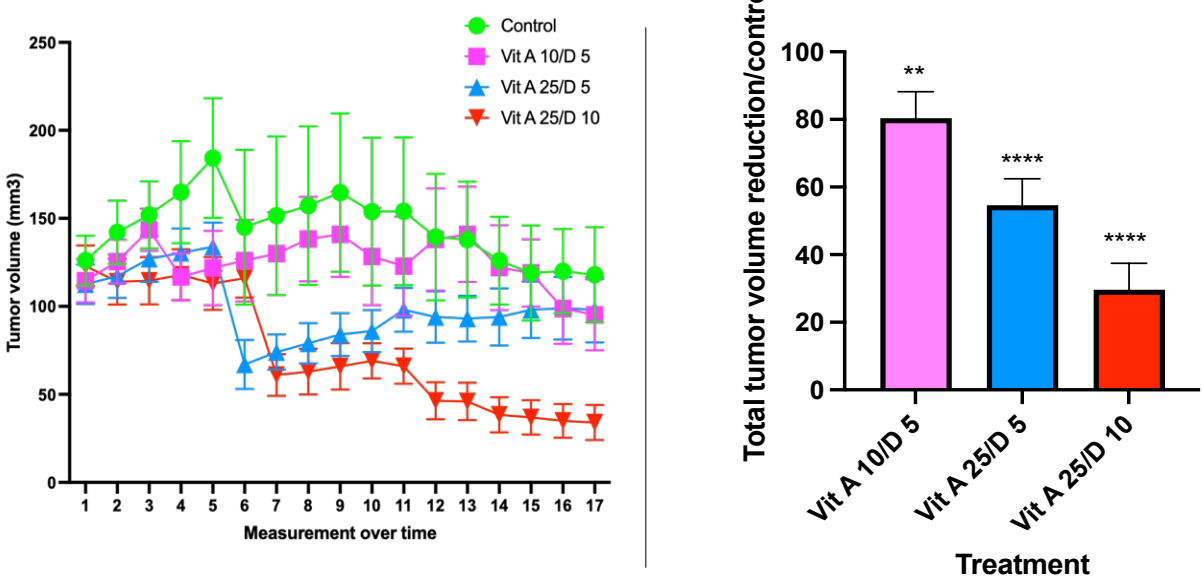
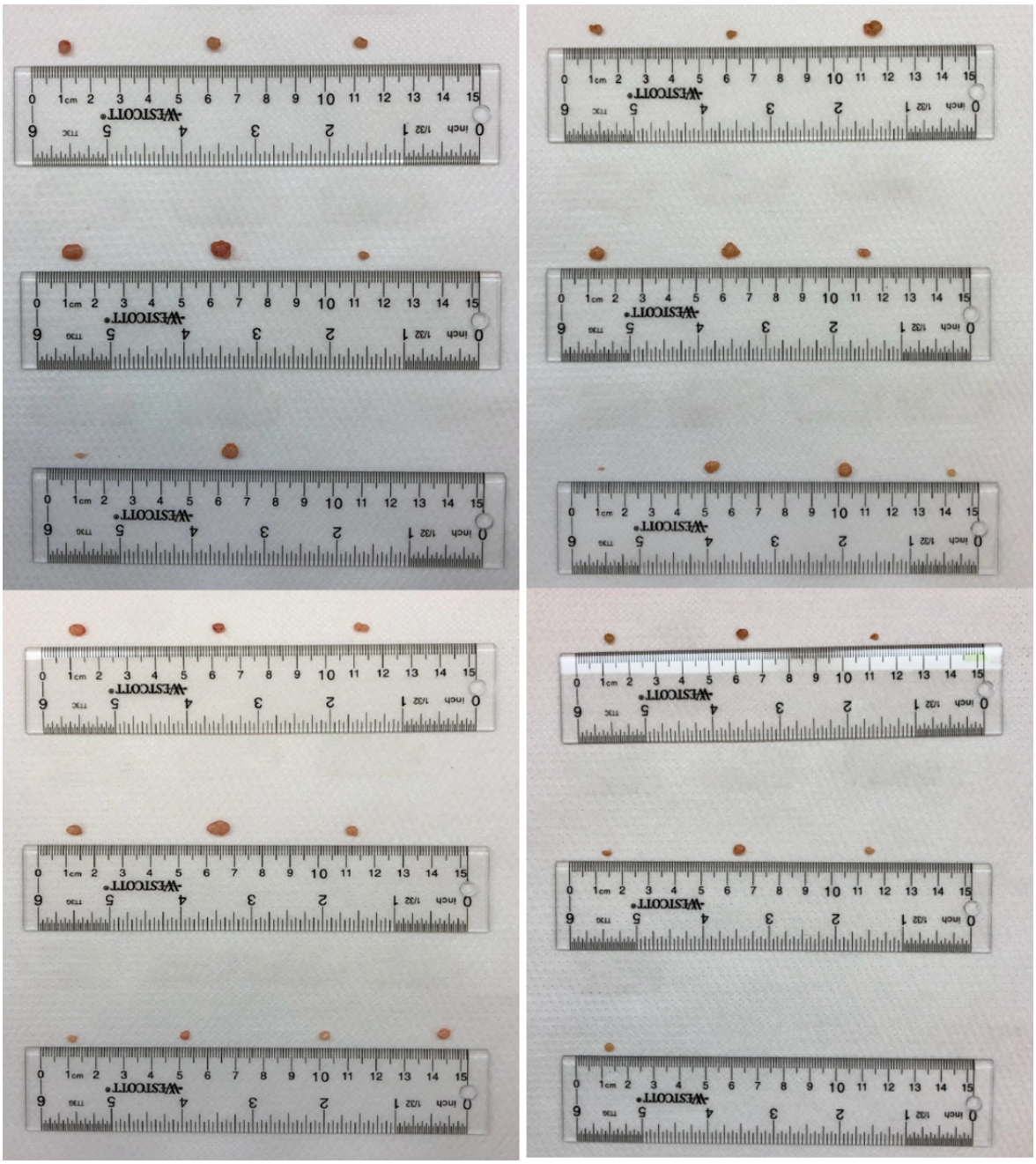


Figure 3. Body weights of female OVX athymic mice with MCF-7 tumors over the treatment period. Body weights were averaged by group. The mice were removed from the cage for body weight measurements and detailed clinical observations recorded twice weekly post dose initiation. The error bar represents the standard error of the mean (SEM). The graph depicts the mean plus SEM of the weights of mice in each group (n = 10/arm) over the study period. No significant difference in body weight was observed for the mice in the treatment groups versus the control group.



A B

Group 1: Control, Vitamin A & D 5/1K IU/Kg diet **C** **Group 2:** Vitamin A & D 10/5K IU/Kg diet



Group 3: Vitamin A & D 25/5 IU/Kg diet **Group 4:** Vitamin A & D 25/10K IU/Kg diet

Figure 4 A-C. A. Graphs of tumor volume reductions in MCF-7 xenografts in OVX female athymic mice. MCF-7 breast cancer xenografts were established by subcutaneous injection of MCF-7 cells suspended 1:1 in sterile media: Matrigel into the right rear flank of each mouse. The mice were removed from the cage and digital caliper tumor measurements recorded twice weekly post dose initiation. **B.** One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test of total tumor volumes in all treatment groups as compared with control groups over the entire study period in GraphPad/Prism Version 10.3 (San Diego, CA). **P< 0.01, ****P<0.0001. **C.** Photographs of tumors from each group visually showing a decline in tumor size/volume in each of the treatment groups. Mice with spontaneous tumor remission (two in group 1 and three in group 4 were not included in the final analysis).

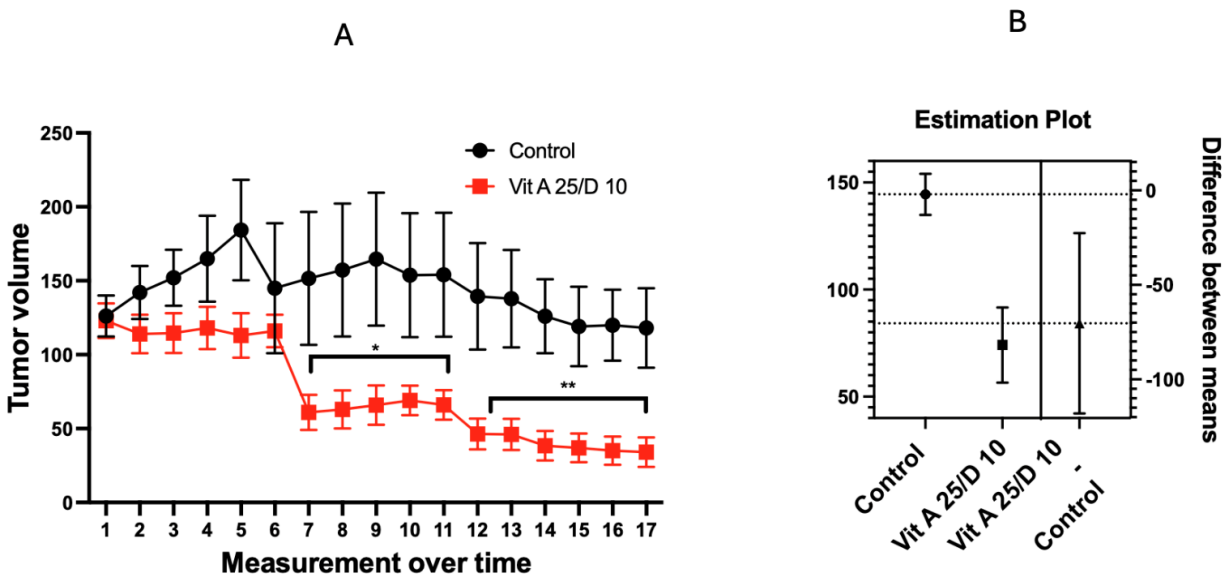


Figure 5A and B. A. Graphs of tumor volume reductions in MCF-7 xenografts in OVX female athymic mice in Group 4 treated with vitamin A 25,000 IU/Kg diet and vitamin D 10,000 IU/Kg diet versus control mice. Statistical analyses were performed using a two-tailed unpaired Student T test in GraphPad/Prism Version 10.3 (San Diego, CA). Only Group 4 showed a significant difference in the unpaired two-tailed T test at week 4 of treatment. **B.** An estimation plot showing the 95% confidence interval. *p<0.05; **p<0.01.

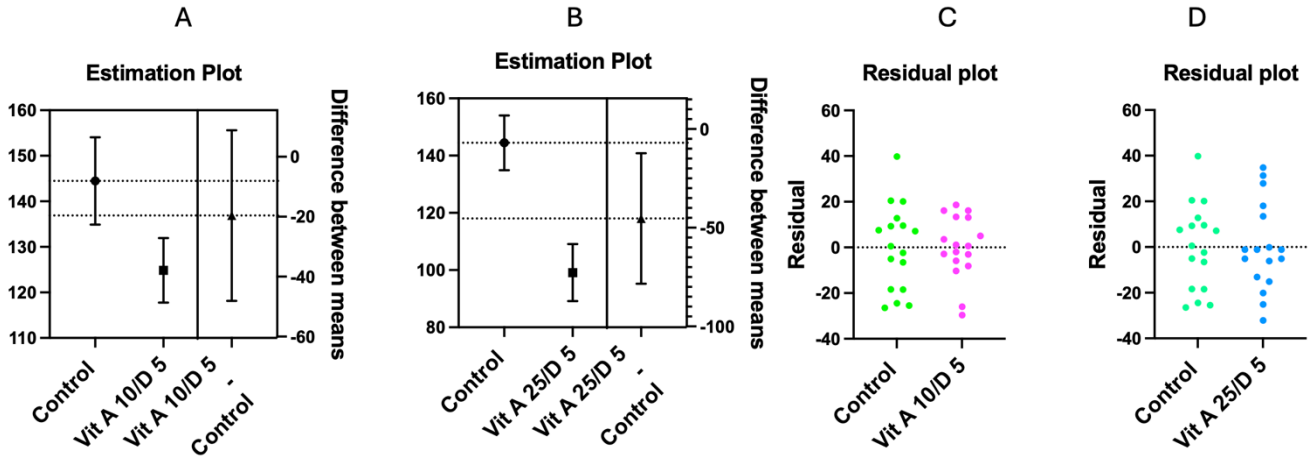


Figure 6A-D. Statistical analysis with estimation and residual plots from an unpaired Student T test. **A-B.** Although the one-way ANOVA showed a significant reduction in total tumor volume over the study period, the more robust unpaired two-tailed Student T test showed that the data for both treatment Group 2 (vitamin A 10,000 IU and vitamin D 5,000IU/Kg/diet and Group 3 (vitamin A 25,000IU and vitamin D 5,000 IU/Kg diet) were not significant. **C-D.** The residual plots a displaying the residuals of the 17 measurements on the vertical axis (Y) and the independent variable on the horizontal axis (X). The points are randomly dispersed, concluding that a linear model was appropriate. The results also show the trend in tumor volume reduction in both treatment groups.

DISCUSSION

Over the past 20 years, scientific and medical investigations have shown that functional foods and their bioactive compounds reduce the risk of numerous chronic diseases, including cancer, and improve immune function [8, 10-12, 26-27]. Such research is highly significant for public health as much of the population in the U.S. and other countries do not receive the daily recommended doses of micronutrients due to poor diets. In the U.S. alone, it is estimated that ~95% of the population do not meet the daily requirements for vitamin D and ~43% do not meet the daily vitamin A requirements [28]. Very importantly, both elderly women and women of color fall short of the

recommended dietary intakes and score low for both vitamins [28, 29-31]. These deficiencies likely contribute to the increasing rates of cancer, obesity, heart disease and other chronic diseases worldwide [32-36]. In the U.S., breast and skin cancers are the top two diagnosed cancers in women, and both have been associated with low serum levels of vitamin A and D [34-36]. Similarly in China, breast cancer ranked first among causes of cancer deaths in young women aged 15-44 years, and there is an increased trend in mortality rates [37]. Breast cancer rates are rising in Sub-Saharan Africa, with the region having the highest age-adjusted mortality rate globally. In Nigeria, the incidence stands at 69.1 cases per 100,000 people, while the age-adjusted mortality rate is 25.5 per 100,000, making breast cancer a major contributor to cancer-related fatalities among women in the country [39]. Thus, functional food research is essential not only for understanding the role that natural bioactive compounds play in disease risk, but also for the development of novel therapies that will reduce the incidence, as well as offer low costing treatments for breast cancer in all countries.

Over the last five years, our research has focused on the hypothesis that nutrient combinations may be more potent in combating cancer by affecting a broader range of signaling pathways [17-19]. Given that vitamins A and D, both fat-soluble, and often coexist in functional food products, we have been exploring the effects of this combination across different types of cancer [17-18, 40-41]. Previously, we have reported that the treatment of cultured breast cancer cell lines with all trans-retinoic acid (ATRA), D2, or D3 alone inhibited the growth of all breast cancer cell lines, but were synergistic when used in combination in the estrogen dependent (ER+) human MCF-7 breast cancer cell line [17]. When MCF-7 cells were treated with combinations of ATRA+D2+D3, the IC50 was significantly reduced, indicating synergism [17]. Interestingly, the IC50 of this combination in MCF-7 cells was similar to that of 5-flurouracil [17]. Treatment of MCF-7 cells with the combination of ATRA+D2+D3 induced apoptosis, increased caspase 3/7 activities, as well as altered the expression of genes associated with the activation of autophagy and apoptosis, such as *Bax, PTEN* and *p53* [17]. Furthermore, this combination downregulated estrogen-signaling pathways in MCF-7 cells, suggesting antiestrogenic effects [17]. Thus, our previous data suggested that the combination of vitamin A and D3 had multiple mechanisms of action in MCF-7 breast cancer cells.

Breast cancer cells survive and cause metastatic disease by the activation or dysregulation of multiple signaling pathways that are associated with autophagy and apoptosis [42-43]. In this work, RNA-seq analysis of MCF-7 cells treated with vitamin A and D combinations showed significant alterations in the transcriptome, and gene expression was significantly altered in pathways favoring both apoptosis and autophagy. Activation of one such pathway, the phosphoinositide 3- kinase (*PI3K*)/protein kinase B (*Akt*)/mammalian target of rapamycin (*mTOR*) signaling pathway, has been reported to reduce both autophagy and apoptosis, as well as initiate the epithelial mesenchymal transition in breast cancer cells, leading to tumor invasion [42]. Activation of *P13K/Akt/mTOR* signaling also increases nuclear factor−κB (NF-κβ), a pro-inflammatory transcription factor, thereby reducing the transcription of pro-apoptotic proteins in the Bcl-2 family, inhibiting apoptosis and promoting survival, leading to metastatic disease [42]. Re-analysis of our existing RNA-seq data using IPA showed that treatment of cultured MCF-7 cells with a combination of vitamin A and D significantly down-regulated the transcription of multiple genes in the *P13K/Akt/mTOR* pathway, including *ITBG* (integrin), *P13K, Akt, mTOR*, and *NF-κ*β. These data suggest that this signaling pathway is likely involved in the mechanism of vitamin A and D3 in MCF-7 cells. Furthermore, the vitamin A and D combination significantly downregulated integrin mRNA expression in MCF-7 cells. Integrins are

transmembrane proteins that enable epithelial cell adhesion to the basement membrane (BM) to form epithelial tissues, and mediate signal transduction between epithelial cells and their environment [reviewed in 43-44]. The survival of epithelial cells requires integrin-mediated adhesion to the BM, and apoptosis occurs if these cells disconnect from the BM. Integrins also play a crucial role in apoptosis, cell growth, signaling and migration, and aberrant activation of integrins promotes tumor formation, proliferation and metastatic disease [44]. Activation of integrins also increases cancer cell survival cell by upregulating the expression of *Bcl-2*, *PI3K-Akt* and *NF-κβ*, and down-regulating *p53* mRNA expression [43-44]. Here, we show that the combination of vitamins A and D downregulated integrin, *Bcl-2*, *PI3K-Akt* and *NF-κ*β mRNA expression, and upregulated p53 expression, suggesting that down-regulation of integrin expression may play a significant role in how combinations of vitamins A and D exert their effects on MCF-7 cells.

Since the effects of combinations of vitamins A and D in breast cancer xenografts had not previously been reported, we investigated the antitumor effects of increasing doses of vitamin A and D combinations in a postmenopausal MCF-7 breast cancer xenograft mouse model. The human MCF-7 breast cancer cell line from ATCC was initially developed from tissues isolated from a postmenopausal woman with metastatic infiltrating ductal carcinoma [45]. Since over 70% of breast cancers are hormone-dependent, the MCF-7 xenograft model is frequently employed in breast cancer research. This cell line is hormone-dependent and replicates many clinical characteristics, especially in the context of treating postmenopausal women with hormone receptor-positive breast cancer [45-46]. Given that most breast cancers occur after menopause, the use of ovariectomized female athymic (immune compromised) mice has been the gold standard in cancer research, as it allows for the levels of estrogen to be controlled [47- 48]. In this study, increasing doses of vitamin A or D (within the combination) reduced tumor volume as compared with control mice as analyzed by one-way ANOVA. However, only the dose of vitamin A 25,000 IU/Kg diet/day and vitamin D 10,000 IU/Kg diet/day was significantly effective in reducing tumor load (>70%) when analyzed using the two-tailed T test. No significant toxicity or metastatic disease were observed in any of the treatment groups over the study period. While previous in vivo studies have reported that increasing doses of calcitriol (a synthetic vitamin D analogue) or retinoic acid derivatives alone significantly reduced tumor volume in breast cancer xenograft models [49-53], prior to this study there are no reports of the anti-tumor effects of vitamin A and D combinations in MCF-7 xenografts in mice.

During the study we noted two major issues associated with vitamin A and D treatment of breast cancer xenografts. First, the time to tumor reduction for the breast cancer model was almost three-fold longer than we what observed for a colon cancer xenograft model [40]. Secondly, a dose-dependent reduction in MCF-7 tumor volume was observed in mice with increasing doses of either vitamin A or D (within the combination), with the highest dose (25,000 IU vitamin A and 10,000 IU of vitamin D/Kg diet) being statistically significant using a two-tailed Student T test**.** It is possible that these issues may be due to the time it takes for these compounds to achieve effective concentrations in breast tissues, or there may be alterations in compound metabolism. Although the MCF-7 xenograft model in OVX athymic mice has some limitations, such as requiring estrogens to maintain tumor growth, historically as an animal model of postmenopausal breast cancer it is has provided excellent results in determining if compounds have estrogenic effects, as well as in anticancer drug studies (including tamoxifen), where the results have translated well into clinical outcomes [54-55].

In terms of public health, epidemiological studies

suggest that there is an association between the ingestion of vitamins A and D and cancer-related morbidity and mortality [34-36, 56]. A meta-analysis by Hossein et al., [56] reported that low vitamin D intake and deficiency were inversely associated with breast cancer incidence. Recently, Munoz and Grant [57] analyzed epidemiological cancer incidence and 25(OH)D-cancer levels and suggested that the incidence of cancer would significantly decline if vitamin D levels were closer to 80 ng/mL versus 10 ng/mL, indicating that higher levels of vitamin D are needed to observe anti-cancer effects. They further reported that the clinical data supporting vitamin D in cancer has been poor due to the poor quality and execution of the clinical trials [57]. Furthermore, a recent meta-meta-analysis published by Arayici et al., [58] reviewed the data (including meta-analyses) associated with vitamin D ingestion and the serum levels of 25-hydroxyvitamin-D on cancer incidence and mortality. They concluded that higher vitamin D levels may have significant benefits in reducing cancer incidence and mortality [58].

Several studies have reported that elevated serum levels of vitamin A are also inversely linked to the risk of breast cancer [35]. In a comprehensive review, Kim et al. analyzed 150 prospective cohort studies and nested case-control studies to determine the relationship between blood concentrations of retinol and carotenoids and breast cancer risk [35]. Results of this analysis showed that higher serum levels and doses of retinol (and derivatives) reduced the risk of breast cancer in both pre- and postmenopausal women [35]. Furthermore, high serum or plasma levels of α- and β- carotene (carotenoid precursors to vitamin A) were also reported to significantly reduce the risk of metastatic breast disease, as well as improve breast survival rates [34-36]. Some studies however, showed no effect or little effect of vitamin A derivatives on breast cancer, suggesting that the clinical data maybe conflicted. However, Kim et al., [35] indicated that this may be due to the limitations of the clinical studies, citing poor study design and protocols, length of the study, low doses administered and the heterogeneity of breast cancer [35]. Data from our MCF-7 xenograft breast cancer study supports the use of higher doses of vitamins A and D and further suggest that the treatment period may need to be extended to see effects.

CONCLUSIONS

While previous published studies have shown that either vitamin A or vitamin D alone reduced tumor growth in xenograft studies, no previous study had reported that the combination of vitamins A and D reduced tumor load and had synergistic effects in breast cancer xenografts. Our results show that by increasing either the vitamin A and/or the vitamin D dose within the combination, the tumor volume trended downward in all groups, with the higher doses having a significant impact on tumor volume over time. These results suggest that both vitamins A and D, together are acting together to reduce tumor load in MCF-7 xenografts with no observed toxicity or significant impact on body weight. Thus, the effects of the combination were both time- and dose-dependent. In terms of public health, these data are significant as they support the results from published meta-analyses indicating that that higher serum levels and doses of both vitamins may be needed to reduce morbidity and mortality associated with breast cancer. Thus, future clinical trials should focus on treatment with a combination of these vitamins at higher doses and for longer periods of time. In terms of mechanisms of action, transcriptomic analysis of MCF-7 cells treated with a vitamin A and D combination induced differential gene expression that significantly overlapped with the molecular mechanisms of cancer canonical signaling pathway, that included both apoptosis and autophagy. These results support our hypothesis that the fat-soluble vitamins A and D have synergistic effects in breast cancer due to multiple mechanisms of action and are worthy of

further clinical investigation.

List of abbreviations: Akt: protein kinase B, ANOVA: analysis of variance, ATRA: all trans- retinoic acid, BC: breast cancer, Bcl-2: B-cell lymphoma 2, Bax: bcl-2-like protein 4, D2: ergocalciferol, D3: cholecalciferol, DEG: differential expressed genes, ER: estrogen receptor, FC: Fold change, FDR: false discovery rate; IPA: Ingenuity Pathway Analysis, ITBG: integrin, MCF- 7: Michigan Cancer Foundation-7, mTOR: mammalian target of rapamycin, NF-κB: nuclear factor kappa beta, P53: p53 tumor suppressor, PTEN: phosphatase and tensin homolog, PI3K: Phosphoinositide 3-kinase

Authors' contributions: GBM, ZA, MMC and NAR were responsible for the research design, animal protocols, IACUC approvals, funding and conducted the research. TOL, SP and NAR grew, treated and harvested the breast cancer cells, isolated RNA, and analyzed data. NSL and ZA performed the RNA purification, mRNA-seq; PNK and MMC performed the bioinformatics and statistical analyses. GBM performed data analysis and IPA analysis. All authors were involved in writing and editing the manuscript.

Competing interests: The authors state that there are no conflicts of interest.

Acknowledgements and Funding: We would like to acknowledge our deep appreciation for the generous educational sponsorship from the First Analysis Institute of Integrative Studies (GBM) and research grants from the Regenstein Foundation (GBM); a Postdoctoral fellowship award from the Schlumberger Foundation (TOL/GBM); and a Raman Post-Doctoral Fellowship by the University Grants Commission, Govt. of India to (NAR/GBM). PK and MMC were supported partly by the University of Illinois at Chicago (UIC) Center for Clinical and Translational Science (CCTS) through Grant UL1TR002003. We would also like to gratefully acknowledge F. Oliver Nicklin at the First Analysis Institute of Integrative Studies for the theoretical conferences, as well as his support with hypothesis generation and the direction of the work. We would like to thank Dr. Qing Ai at Research Diets, Inc**,** New Brunswick, NJ for her helpful discussions concerning the rodent diet and diet preparation, and Jackson Laboratories, Sacramento CA for their work on the athymic xenograft mouse model. The contents are primarily the responsibility of the authors and do not necessarily represent the official views of the funding agencies.

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