Whole Blueberry Powder Modulates the Growth and Metastasis of MDA-MB-231 Triple Negative Breast Tumors in Nude Mice\textsuperscript{1–3}

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Abstract

Previous studies in our laboratory demonstrated that blueberry (BB) extract exhibited antitumor activity against MDA-MB-231 triple negative breast cancer (TNBC) cells and decreased metastatic potential in vitro. The current study tested 2 doses of whole BB powder, 5 and 10\% (wt:wt) in the diet, against MDA-MB-231 tumor growth in female nude mice. In this study, tumor volume was 75\% lower in mice fed the 5\% BB diet and 60\% lower in mice fed the 10\% BB diet than in control mice \((P = 0.05)\). Tumor cell proliferation (Ki-67) was lower in the 5 and 10\% BB-fed mice and cell death (Caspase 3) was greater in the 10\% BB-fed mice compared to control mice \((P \leq 0.05)\). Gene analysis of tumor tissues from the 5\% BB-fed mice revealed significantly altered expression of genes important to inflammation, cancer, and metastasis, specifically, Wnt signaling, thrombospondin-2, IL-13, and IFN-\gamma. To confirm effects on Wnt signaling, analysis of tumor tissues from 5\% BB-fed mice revealed lower \(\beta\)-catenin expression and glycogen synthase kinase-3\(\beta\) phosphorylation with greater expression of the \(\beta\)-catenin inhibitory protein adenomatous polyposis coli compared to controls. A second study tested the ability of the 5\% BB diet to inhibit MDA-MB-231-luc-D3H2LN metastasis in vivo. In this study, 5\% BB-fed mice developed 70\% fewer liver metastases \((P = 0.04)\) and 25\% fewer lymph node metastases \((P = 0.09)\) compared to control mice. This study demonstrates the oral antitumor and metastasis activity of whole BB powder against TNBC in mice. J. Nutr. 141: 1805–1812, 2011.

Introduction

TNBC\textsuperscript{4} is so named due to lack of expression of the ER, PR, and the HER2 protein (1). This type of cancer comprises \(\sim 10–15\%\) of all breast cancers (2) and has a poorer outcome than those breast tumors expressing ER/PR or the HER2 protein (3,4). Additionally, patients with TNBC have shorter relapse-free and overall survival (5), a higher likelihood of distant metastasis within the first 5\, years of diagnosis, larger tumors \((\geq 2\, cm)\), and a greater percentage of positive LN than patients with other subtypes of breast cancer (6). TNBC are also characterized by an aggressive clinical history with poor disease-free and overall survival (7,8). Currently, there is no defined, standard treatment strategy for prevention of recurrence for this disease other than traditional chemotherapy, to which it is highly resistant. Due to the lack of ER, PR, and HER2 expression, traditional targeted therapies are not an option. A limited amount of data exists on which to base targeted preventive measures for occurrence and recurrence of this disease. Thus, there is an urgent need to identify both vulnerable targets in these breast cancers and safe, effective dietary strategies for the prevention of primary disease, metastasis, and recurrence.

BB are rich in bioactive substances such as flavonoids (anthocyanins, flavan-3-ols, flavones, and flavonols) and proanthocyanidins and exhibit inhibitory effects such as induction of apoptosis, inhibition of cell proliferation, modulation of cell signaling, metastasis and effects on gene expression in a variety of cancer cell lines (9–12). In addition, BB possess potent antioxidant potential (13,14), which can be beneficial to the prevention of carcinogenesis and metastasis.

Tumor invasion and metastasis is a complex, multi-step process influenced by many factors such as the expression of key proteases, their inhibitory proteins, and the signaling pathways that control them. The bioactive substances in berries inhibit different components of this system. Proanthocyanidins inhibit \(\beta\)-glucuronidase, elastase, and hyaluronidase (15,16), which are enzymes important to degradation of the extracellular matrix. Anthocyanidins and proanthocyanidins have been shown to stabilize the extracellular matrix by stabilizing collagen, increasing collagen synthesis, and inhibiting collagenase (17). Studies have also shown that luteolin and quercetin inhibit MMP-2 and MMP-9 secretion (18) and cell migration (19).

Preliminary data from our laboratory showed that whole BB juice had an inhibitory effect on the proliferation and survival of TNBC cells in vitro. In addition, treatment with BB juice decreased

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\textsuperscript{1} Supported by U.S. Highbush Blueberry Council and NIH grant ES08258.
\textsuperscript{2} Author disclosures: L. S. Adams, N. Kanaya, S. Phung, Z. Liu, and S. Chen, no conflicts of interest.
\textsuperscript{3} Supplemental Figure 1 and Supplemental Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.
\textsuperscript{4} Abbreviations used: APC, adenomatous polyposis coli; BB, blueberry; ER, estrogen receptor; GSK, glycogen synthase kinase; LN, lymph node; LYZ, lysozyme; MMP, matrix metalloproteinase; PR, progesterone receptor; TNBC, triple negative breast cancer.
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the metastatic potential of MDA-MB-231 TNBC cells in vitro through reduced activity of MMP-9 and urokinase-type plasminogen activator while increasing the expression of plasminogen activator inhibitor-1 (12). In vivo, whole BB juice decreased MDA-MB-231 tumor size and cell proliferation and increased apoptosis. Immunohistochemical analysis of tumor tissues from BB-fed mice showed lower activation of the cell signaling proteins phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), and nuclear factor-κB (NF-κB), which are important to cancer cell survival and metastasis. To build on these previous findings, the current study measured to monitor differences between groups. Tumor size was extracted from 3 tumors of the control and 5% BB groups using the Trizol method following the procedures reported in our recent paper published in the Journal of Applied Oncology. RNA for microarray and RT-PCR analysis was removed, weighed, and sent for hematoxylin and eosin, Ki-67, and Caspase 3 histological staining through the City of Hope Pathology Core Facility. This cell line was cultured in Eagle’s MEM with 10% FBS in the presence of 0.10 U/L penicillin and 0.1 g/L streptomycin and incubated at 37°C with 95% air and 5% carbon dioxide and used in experiments during the linear phase of growth. MDA-MB-231-luc-D3H2LN luciferase-expressing cells were purchased from Caliper Life Sciences. This cell line was cultured in Eagle’s MEM with 10% FBS in the presence of 1 × 10^5 U/L penicillin and 0.1 g/L streptomycin. MDA-MB-231-luc-D3H2LN cells will metastasize to the LN, lungs, and liver upon orthotopic injection into immune-deficient mice, according to the manufacturer.

### Materials and Methods

**Mice and diets.** Female, 6- to 7-week-old, intact, BALB/c Nu-Nu, athymic mice were purchased from Charles River Laboratories. The mice were housed and maintained under standard conditions in the City of Hope Animal Facility. All experimental protocols were in accordance with the NIH guidelines for the ethical treatment of animals and approved by the City of Hope Research Animal Care Committee. Mice were randomly assigned to cohorts (n = 10 mice/COHORT) that received 1 of 3 pelleted diets for the duration of the experiment. Whole BB powder was used in this study to avoid the trauma of daily gavage required when using BB juice. The control diet (AIN-93G) and the AIN-93G diet modified to contain 5 or 10% freeze-dried whole BB powder (wt:wt) were produced and purchased from Research Diets. Sucrose and cellulose were decreased in the BB diets to adjust for the addition of the BB powder (Table 1). The nutrient content and total phenolic and anthocyanin content of the BB powder is provided in Supplemental Table 1. In addition, diets were isocaloric and nutritionally balanced for fat, protein, and carbohydrate content. The BB doses were based on previous studies by the Stoner (20) laboratory with black raspberry and a study by Wu et al. (21) using BB powder. The U.S. Highbush Blueberry Council provided the powder in sealed aluminum cans, which remained unopened until it was added to the diets. All mice consumed food and water ad libitum. The amount of food consumed and body weight were measured weekly to monitor differences between groups and the overall health of the mice.

**Cell culture.** MDA-MB-231 cells were obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 containing 10% FBS in the presence of 0.10 U/L penicillin and 0.1 g/L streptomycin and incubated at 37°C with 95% air and 5% carbon dioxide and used in experiments during the linear phase of growth. MDA-MB-231-luc-D3H2LN luciferase-expressing cells were purchased from Caliper Life Sciences. This cell line was cultured in Eagle’s MEM with 10% FBS in the presence of 1 × 10^5 U/L penicillin and 0.1 g/L streptomycin. MDA-MB-231-luc-D3H2LN cells will metastasize to the LN, lungs, and liver upon orthotopic injection into immune-deficient mice, according to the manufacturer.

**Experimental protocol (microarray).** After 2 wk of receiving the experimental diets, mice were s.c. injected in the hind flank with MDA-MB-231 cells mixed with an equal volume of Matrigel (BD Biosciences) at a concentration of 2 × 10^6 cells/injection site. Body weights were monitored weekly as an indicator of overall health and diet intake was measured to monitor differences between groups. Tumor size was measured weekly using calipers and volume calculated by the formula \((4/3\pi r_1^2 \times r_2)\), where \(r_1\) is the smaller radius and \(r_2\) is the larger radius. Six weeks postinjection, mice were euthanized and tumors were removed, weighed, and sent for hematoxylin and eosin, Ki-67, and Caspase 3 histological staining through the City of Hope Pathology Department Core Facility. RNA for microarray and RT-PCR analysis was extracted from 3 tumors of the control and 5% BB groups using the Trizol method following the procedures reported in our recent paper published in the Journal of Applied Oncology. The nutrient content and total phenolic and anthocyanin content of the BB powder is provided in Supplemental Table 1. In addition, diets were isocaloric and nutritionally balanced for fat, protein, and carbohydrate content. The BB doses were based on previous studies by the Stoner (20) laboratory with black raspberry and a study by Wu et al. (21) using BB powder. The U.S. Highbush Blueberry Council provided the powder in sealed aluminum cans, which remained unopened until it was added to the diets. All mice consumed food and water ad libitum. The amount of food consumed and body weight were measured weekly to monitor differences between groups and the overall health of the mice.

**Microarray analysis.** Synthesis and labeling of cRNA targets, hybridization of GeneChips, and signal detection were carried out by the Microarray Core Facility at the City of Hope using the Affymetrix GeneChip Human Gene1.0 ST array (Affymetrix) as previously reported (22). Ki-67 and Caspase 3 staining was analyzed through the City of Hope Pathology Core Facility. This cell line was cultured in Eagle’s MEM with 10% FBS in the presence of 0.10 U/L penicillin and 0.1 g/L streptomycin. MDA-MB-231-luc-D3H2LN cells will metastasize to the LN, lungs, and liver upon orthotopic injection into immune-deficient mice, according to the manufacturer.

**In vivo metastasis assay.** The study was carried out using 6- to 7-week-old intact, female, BALB/c Nu-Nu, athymic mice. Mice were divided into 2 experimental groups of 10 mice. Two weeks prior to injection with tumor cells, mice were started on 1 of 2 diets: control (AIN-96G) or AIN-96G + 5% BB powder (wt:wt). We chose the 5% BB diet for further study, because the 10% BB diet did not confer any advantage in decreasing tumor volume. After 2 wk of receiving the experimental diets, luciferase-expressing MDA-MB-231-luc-D3H2LN cells suspended in matrigel (2 × 10^6 cells/injection site) were injected into the mammary fat pad of the mice. Mice were imaged just prior to cell injection to obtain a baseline reading and then weekly starting at wk 2 posttumor cell injection using the IVIS imaging system. For imaging, mice were anesthetized using isoflurane and then injected with D-Luciferin Firefly Luciferase reagent (Invitrogen) was used for total RNA isolation. The same RNA was used as assay that was used in the microarray analysis to ensure consistency in the data. The 2 most upregulated genes in the microarray analysis were verified, because the changes between the control and 5% BB groups were the most evident. An iScript cDNA Synthesis kit (Bio-Rad) and SYBR Green Supermix were used for cDNA preparation and to carry out the PCR reaction. PCR primers for LY2 were as follows: 5′CTCATTTGTTGCGGCTTG3′ and TGCTTCTGTCTCCAGCATTG3′; thrombospondin 2 (THBS): 5′TGCTGCGTTTGGACTACATC3′ and TTGGAGACAGATCTCGAGAATG3′; and human β-actin (used as an internal control): 5′AGAAGGAGAATCAGCTGCCCTGGCAC3′ and 5′CCTGCTTCGTGATCCACCACATCTGC3′. Reactions were run in triplicate on the iCycler iQ5 Real-Time PCR Detection system (Bio-Rad) and the results were analyzed with the software provided.

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### Table 1: Formulations and nutrient composition of experimental diets

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<th>D10012G</th>
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<td>70.3</td>
<td>40.03</td>
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<td>31.0</td>
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<td>70.0</td>
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<td>t-Butyldihydroquinone</td>
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<tr>
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<td>101.82</td>
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</table>

1 Prepared by Research Diets.
2 BB, Blueberry.
3 Composed according to (61).
4 Composed according to (61).
potassium salt (Caliper Life Sciences) at a concentration of 150 mg/kg. Three weeks postinjection, imaging included shielding the primary tumor with black fabric to minimize the bioluminescence from the primary tumor so that metastatic regions could be observed. The front limbs were secured with tape to expose the axillary/brachial LN areas. Five weeks postinjection, mice were euthanized via CO2 asphyxiation and tumors were removed, weighed, and sent for β-catenin histological staining at the City of Hope Pathology Department Core Facility. β-Catenin staining intensity was graded as 1 (weak), 2 (moderate), or 3 (strong). A total of 15 fields (3 tumors, 5 fields/tumor) were analyzed per group. To confirm metastases, organs (liver, lungs, and axillary/brachial LN) were placed in Petri dishes containing 0.30 μL luciferin, and imaged for luminescent signals.

Western blotting. Protein was isolated from tumor specimens from 3 separate mice per experimental group (control and 5% BB) using T-PER Tissue Protein Extraction Reagent (Thermo Scientific) according to the manufacturer’s protocol. Briefly, tumor specimens were weighed and homogenized in T-PER reagent containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich) using the T25 Basic tissue homogenizer (IKAWorks). Samples were centrifuged at 10,000 × g for 5 min and the supernatant was run on a 10% acrylamide gel, transferred to a nitrocellulose membrane, and probed with antibodies to phospho-GSK-3β, APC, or α-actin (as a loading control) (Cell Signaling Technology). Bands were visualized via chemiluminescence using HRP-conjugated secondary antibodies and quantified using Biorad Quantity One software.

Statistical analysis. Statistical analysis of data (with the exception of microarray data) was done using either Student’s t test to compare 2 means, 1-way ANOVA plus Dunnett’s Multiple Comparison Test to compare more than one mean to a control, or 2-way ANOVA plus Bonferroni post test to compare multiple means over time; metastasis frequency was analyzed as a contingency table by using GraphPad Prism 4 software. Differences were considered significant at P ≤ 0.05.

Results

MDA-MB-231 tumor volume. Food intake and weight gain did not differ between experimental groups (data not shown). Tumor volume was affected by diet (P = 0.0001) and time (P = 0.0001). It was lower than in controls in the 5% BB-fed group at wk 5 and 6 and in the 10% BB-fed group at wk 6 (Fig. 1A). Cell proliferation (Ki-67 expression) was lower in the 5% (P = 0.01) and 10% (P ≤ 0.05) BB-fed groups (Fig. 1B) and apoptosis (Caspase 3 expression) was greater in the 10% BB group compared to control mice (P ≤ 0.05) (Fig. 1C).

Validation of microarray results by real-time PCR. Microarray analysis results were validated by real-time PCR using primers for the 2 most highly upregulated genes: THBS2 and LYZ in tumor samples. Expression of both genes was greater in tumors from 5% BB-fed mice compared to controls (P ≤ 0.05), validating the microarray analysis results (Supplemental Fig. 1).

Analysis of BB-responsive genes. The most relevant gene networks significantly altered by 5% BB powder ingestion identified by IPA in our data set are shown in Table 2. The top networks affected by BB ingestion in this study include: inflammatory disease, cancer, cell morphology (28 focus genes), reproductive system development and function, developmental disorder, genetic disorder (25 focus genes), inflammatory response, cell-to-cell signaling and interaction, hematological system development and function (22 focus genes), cell cycle, cancer, gastrointestinal disease (20 focus genes) and inflammatory response, cellular movement, and hematological system development and function (14 focus genes), IL-13 and IFNγ, both upregulated, are 2 central molecules in the top network regulated by 5% BB ingestion. The proteins encoded by these genes play important roles in tumor growth.

Genes were also grouped by biological function. Of the biological functions modulated by 5% BB ingestion, several are of prime interest to this investigation (Supplemental Table 2). Under diseases and disorders, those genes important to inflammatory response (58 genes) and inflammatory disease (69 genes) were altered by 5% BB. Under molecular and cellular functions, those genes important to cell-to-cell signaling and interaction (69 genes), cellular movement (66 genes) were altered. Under physiological system development and function, immune cell trafficking (48 genes) and humoral immune response (23 genes) were altered by 5% BB. These groupings show several important biological systems important to cancer that were affected by BB ingestion in our study.

Specific genes of interest that were significantly upregulated in BB-fed mice included: wingless-type MMTV integration site family

Symbol (Entrez Gene ID, log ratio)
ALOX15 (246, -0.70); ALOX5AP (241, 0.815); ARG1 (383, 0.922); Arginase (group); Collagen Alpha 1 (group); CYBB (1536, 1.262); Eotaxin (group); ESM1 (11082, 0.837); F13A1 (2162, 1.111); Fc Gamma Receptor (group); FGFR1A (2209, -1.044); GBP5 (16335, 1.217); GRNR1 (2796, -0.678); GRPRCSB (51704, -0.626); HBA1 (3039, 0.932); HBD (3045, 0.645); HB01 (3047, 1.208); Hemoglobin (complex); HLA-DOA (3111, -0.756); IFIT5 (24138, 0.707); IFNG (3458, 0.787); IL13 (3596, 0.866); IL13RA2 (3598, -0.674); INHBA (3624, 0.611); KCNJ15 (3737, 0.626); KIR2DS1 (3806, 0.678); LTB4R (1241, -1.118); MHC Class II (complex); NPY2R (4887, -0.748); PCK ALP/PHA/BETA (family); PLEX (5341, 1.159); GRP (534198, -0.744); SEPP1 (6414, 1.369); SLAMF1 (6504, -0.733); UBD (10537, -0.813).


Symbol (Entrez Gene ID, log ratio)
C3AR1 (719, 0.856); CD86 (942, 0.964); CD180 (4064, 0.707); CLEC7A (64581, 0.912); CLIP2 (7461, 0.707); HLA-DOA (3111, -0.756); IFIT5 (24138, 0.707); IFNG (3458, 0.787); IL13 (3596, 0.866); IL13RA2 (3598, -0.674); INHBA (3624, 0.611); KCNJ15 (3737, 0.626); KIR2DS1 (3806, 0.678); LTB4R (1241, -1.118); MHC Class II (complex); NPY2R (4887, -0.748); PCK ALP/PHA/BETA (family); PLEX (5341, 1.159); GRP (534198, -0.744); SEPP1 (6414, 1.369); SLAMF1 (6504, -0.733); UBD (10537, -0.813).


Symbol (Entrez Gene ID, log ratio)
APOBEC3B (9562, 0.220); AZU1 (666, -0.274); BDKRB1 (623, -0.861); CD276 (80381, -0.145); CDA (587, -0.762); CEACAM8 (1088, -0.711); CEACAM9 (1084, -0.158); CFTR (1080, 0.845); CHST4 (10164, 1.238); CLEC7A (8218, 0.544); CSTF (8722, -0.699); GALNT1 (8463, 0.887); IL8 (3576, 0.568); IL1B (21790, 0.025); IL17C (21799, -0.094); IL11F (27179, -0.416); IL11F (56300, -0.575); Ingrain alpha (group); LPA (4018, -0.611); LPAR2 (9170, -0.614); LPAR3 (23566, -0.247); Mannose (9702, -0.341); NPR1 (9284, -1.177); NXF5 (55998, -0.195); NXX1 (29107, 0.067); PDZK1 (3651, -0.136); PIRB (29990, -0.704); PMP2 (5375, -0.675); POT1 (25913, 0.702); SNX4 (8723, 0.602); STEAP4 (79889, 0.671); SVL (6840, -0.470); TGF (7124, 0.565); TNP2 (79831, 0.284).

Discussion

This study illustrated that BB powder intake at 5 and 10% of the diet was nontoxic and did not affect food intake, weight, or overall health of the mice. In addition, the decreased tumor volume in the BB groups showed that BB intake suppressed the growth of triple negative breast tumors in mice. Interestingly, tumor volume was lower in the 5% BB diet than the 10% BB diet, although this difference was not significant (no dose response observed). This is consistent with a study by Wu et al. (21), who found that a 5% BB diet fed to pregnant Sprague-Dawley rats had significant, positive effects on breast maturation markers in progeny, whereas the 10% diet did not. This suggests that there may be an optimal level of BB intake.

The decrease in Ki-67 in both BB groups compared to controls and the significant increase in apoptosis (Caspa3e) in the 10% BB group suggests that the decreased tumor volume was likely due to decreased proliferation of the tumor cells in both BB groups and an increase in apoptosis in the 10% group. These data are in agreement with previous studies in our laboratory using BB extract in the same xenograft model (12). For microarray analysis, we utilized tumor specimens from mice fed the 5% BB diet, because there was no apparent advantage to 10% BB intake with regard to tumor volume reduction. The results suggest several mechanisms for the effects of BB intake on TNBC growth. BB intake affected gene networks important to the inflammatory response, which is not surprising given the established antioxidant action of BB phytochemicals. The oxidation radical absorbance capacity value of whole BB is given the established antioxidant action of BB phytochemicals. Important to the inflammatory response, which is not surprising given the established antioxidant action of BB phytochemicals. Inflammation is an important mediator of the promotion and progression of cancer and proinflammatory conditions within tumor tissues are related to malignant progression and metastasis (24). Inflammatory cells in primary tumors can also induce angiogenesis, contributing to blood vessel growth and subsequent metastasis; nonsteroidal, antiinflammatory drugs inhibit this process (25). Therefore, the antiinflammatory effects of BB intake likely contributed to its observed antitumorigenic and antimetastatic activity.

In addition, BB intake affected specific gene networks involved in cell-to-cell signaling and interaction, which are important to...
both cell survival and migratory behavior. BB also modulated genes involved in the cell cycle and cell movement, cancer, cell morphology, and cell-to-cell interaction. These results are in agreement with our previously published in vitro results (12).

The number one gene network affected by BB ingestion was inflammatory disease, cancer and cell morphology, and the 2 central molecules in this network are IL-13 and IFNγ. IFNγ is an immunostimulatory cytokine that also displays antiproliferative and tumoricidal properties (26–30). IL-13 is a cytokine that inhibits the production of proinflammatory cytokines and regulates cell proliferation, apoptosis, and differentiation (31,32), specifically in breast tumor cell lines (33,34). LTB4R was also included in this network. This gene was significantly downregulated in BB-fed mice compared to controls. This receptor is involved in eicosanoid signaling (35), inflammatory response (36), signal transduction (37), and cell adhesion and movement (38). Interestingly, LTB4R upregulates MMP 2 and 9 (39) and MMP9 activity was decreased by BB treatment in our previous in vitro experiments. LTB4R also affects metastasis through the activation of NF-κB signaling, which was reduced by BB treatment in our previous study (40). Therefore, a likely mechanism for the reduction of metastatic potential by BB is through a decrease in the expression of LTB4R. These genes and the network to which they belong are essential to the mechanisms we suspect are behind the effects of BB on the growth and metastasis of breast cancer.

THBS2 was the top upregulated gene in the 5% BB group. THBS2 mediates cell-to-cell and cell-to-matrix interactions (41–43). This protein functions as a potent inhibitor of tumor growth, migration, and angiogenesis (44–47). THBS2 modulates the activity of MMP2 (48), PI3K, and ERK (49), all proteins that are integral to cancer cell survival and metastasis. This result supports our previous in vitro data showing lower MMP2 and PI3K activity in BB-treated cells (50).

SFRP4 was significantly upregulated in the 5% BB-treated mice. The protein encoded by this gene is involved in the
with our previous data, we conclude that BB ingestion affects
regulation of apoptosis, cell growth, and differentiation (51) and
is commonly downregulated in breast cancer (52) and missing in
the MCF-7 breast cancer cell line (52). SFRP4 proteins are
inhibitors of Wnt/β-catenin signaling (53), which is involved in
metastasis signaling, neoplasia, and cancer. β-Catenin signaling
results in the expression of target genes that induce cancer cell
proliferation and metastasis, such as Cyclin D-1 and MMP-9.

In the absence of Wnt proteins, β-catenin is bound within an
inhibitory complex composed of APC, Axin, GSK-3β, and
casein kinase In. GSK-3β and casein kinase In phosphorylate
β-catenin, which is subsequently ubiquitinated and degraded by
the proteasome. In response to the presence of Wnt proteins,
GSK-3β is phosphorylated by AKT. The phosphorylated form
of GSK-3β is inactive and therefore cannot inhibit β-catenin.

β-Catenin expression and GSK-3β phosphorylation was
lower and APC expression was greater in tumors from BB-fed
mice compared to controls. Because GSK-3β is deactivated by
AKT, this is consistent with our previous data that showed BB
decreased AKT activity in vitro and in vivo. Other groups have
reported the inhibitory activity of phytochemicals on Wnt
signaling in breast tissues. Genistein reduced Wnt signaling in
mammary tumors of rats (54), curcumin inhibited the expression
of β-catenin in MCF-7 and MDA-MB-231 cells (55), EGCG
blocked Wnt signaling and the invasion of breast cancer cell lines
in vitro (56), and BB punch (a mixture of food extracts that
included blueberries) reduced GSK-3β in prostate xenograft
tumors in mice (57).

Through real-time imaging, we tracked primary tumor
growth and metastasis in mice over the duration of the study.
The frequency of liver and LN metastasis in BB-fed mice was
lower compared to control mice. Although the decreased metas-
tasis to the LN was not significant (P = 0.09), the study did show a
tendency for inhibition. The frequencies of liver compared to
LN metastasis did not differ in the control mice; therefore, the
differential inhibition by BB is interesting. This may be because
the liver is a more distant site from the primary tumor than the
LN. However, metastasis does not require circulation through the
lymph system. Primary tumors may spread through blood
vessels or through lymph vessels independently, and positive LN
are regularly detected in the absence of hematogenous metas-
tasis. Different patterns of metastasis may be due to the separate
mechanisms behind hemangiogenesis and lymphangiogenesis
(25). Therefore, BB may inhibit hematogenous metastasis to the
liver more effectively than lymphatic metastasis to the LN. It is
also possible that because the liver processes BB phytochemicals,
it was exposed to a more concentrated dose of antitumor phyto-
chemicals. Further studies into these hypotheses are required to
answer these questions.

The major active ingredients in BB are thought to be the
anthocyanins. A recent study using an 8% BB diet analyzed the
absorption, metabolism, and tissue distribution of anthocyanins
in Sprague-Dawley rats. The authors of this study reported that
anthocyanins from blueberries are bioavailable based on their
detection in urine and feces of rats after 4 and 8 wk of ingestion.
In addition, anthocyanins were metabolized by the intestinal
microflora, primarily into phenolic acids. Hippuric acid, the
main product of anthocyanin metabolism, was detected in the
urine of rats after 4 wk of the BB diet. Therefore, hippuric acid is
a possible biomarker of anthocyanin intake in animal and
human trials (58).

This study demonstrates that the oral intake of whole BB
powder effectively decreased MDA-MB-231-derived triple nega-
tive breast tumor growth and metastasis in mice. Taken together
with our previous data, we conclude that BB ingestion affects
tumor growth and metastasis through the mediation of key pro-
cesses such as inflammation, cell signaling, survival, and migration
through modulation of signaling pathways such as PI3K, AKT, NF-kB and β-catenin.

Importantly, the dosage used in these studies was nontoxic
and may be considered physiologic, because the human equiv-
alent dose of the 5% BB diet (based on body surface area) (59) is
300 g (10.6 oz.) of fresh BB (~2 cups/d). Future clinical trials
using whole BB powder are planned that will aid in the
determination of a suitable human dose. It is our hope that the
knowledge gained from this study may aid in the design of future
dietary strategies for the prevention of TNBC.

Acknowledgments
L.A. and S.C. designed research; L.A., N.K., and S.P. conducted
research; Z.L. analyzed microarray data; and L.A. wrote the
manuscript and had primary responsibility for final content. All
authors read and approved the final manuscript.

Literature Cited
1. Reis-Filho JS, Tutt AN. Triple negative tumours: a critical review.
2. Cleator S, Heller W, Coombes RC. Triple-negative breast cancer:
 tumor subtypes in independent gene expression data sets. Proc Natl
Acad Sci USA. 2003;100:8418–23.
4. Abd El-Rehim DM, Ball G, Pinder SE, Rakha E, Paish C, Robertson JF,
Macmillan D, Blamey RW, Ellis IO. High-throughput protein expres-
sion analysis using tissue microarray technology of a large well-
documented series of tumors identifies biologically distinct classes of breast
cancer confirming recent cDNA expression analyses. Int J Cancer. 2005;
5. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA,
6. Dent R, Trudeau M, Pritchard KI, Hanna WM, Sawka CA,
Lickley LA, Rawlinson E, Sun P, Narod SA. Triple-negative breast
7. van de Rijn M, Perou CM, Tibshirani R, Haas P, Kallioniemi O,
of cytokeratin 17 and 5 identifies a group of breast carcinomas with
8. Foulkes WD, Brunet JS, Stefansson IM, Straume O, Chappuis PO,
implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-
microvascular-proliferation+) phenotype of BRCA1-related breast can-
LL. Anticarcinogenic activity of strawberry, blueberry, and raspberry
Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry
extracts inhibit growth and stimulate apoptosis of human
11. Boivin D, Blanchette M, Barrette S, Moghrabi A, Beliveau R. Inhibition
of cancer cell proliferation and suppression of TNF-induced activation
phytochemicals inhibit growth and metastatic potential of MDA-MB-
231 breast cancer cells through modulation of the phosphatidylinosito-
13. Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P,
Klimis-Zacas D, Margaritis M, Lamari FN. Effect of polyphenol-rich
blueberry extract on cognitive performance of mice, on brain
antioxidant markers and acetylcholinesterase activity. Behav Brain


