Impact of Short-term Calorie Restriction on HER2-Overexpressing Breast Cancer

Magdalena Rainey

Honors Thesis
Department of Nutrition
Gillings School of Global Public Health
University of North Carolina at Chapel Hill

April 27, 2018

Approved by:

[Signatures]

Stephen D. Hursting, PhD, MPH (Advisor)
Laura A. Smith, MPH (Second Reader)
Abstract

Background: Among American women, 30% of all new cancer diagnoses are of breast cancer (BC) and ~25% of these cancers overproduce the growth-promoting tyrosine kinase receptor, HER2. Although there are targeted treatments available for HER2-overexpressing BC, this disease is associated with poorer prognosis and increased risk of recurrence, making identification of new treatment methods crucial. It is well-established that calorie restriction (CR), or chronic dietary energy restriction by ~30%, has significant tumor suppressive effects across cancer subtypes and has potential to be utilized as an adjunctive therapy. This study investigates the mechanisms by which CR decreases HER2-overexpressing BC progression.

Methods: To mimic CR in vitro, murine MMTV-neu cells that overexpress HER2 were treated with medias containing reduced serum (1%), reduced glucose (1mM), or reduced serum and reduced glucose (1%/1mM) compared to control media (10% serum, 25mM glucose). The impact of differential nutrient restriction on MMTV-neu regulatory mechanisms was characterized using MTT assays, anchorage independent colony formation assays, cell cycle analysis, and apoptosis assays. To investigate the mechanism of this alteration, western blotting analysis of proteins along the PI3K/Akt and Ras/Erk pathways was conducted. The efficacy of nutrient restriction as an adjunct therapy was explored by treating cells cultured in restricted medias with lapatinib, doxorubicin, and cyclophosphamide.

Results: Serum restriction significantly reduced cellular viability and colony formation of MMTV-neu cells and promoted increased cellular apoptosis, and G0/G1 cell cycle arrest. In MMTV-neu cells treated with serum restricted medias (1% and 1%/1mM), there was a significant decrease in activity of tumor-suppressor protein p21. Both p21 and phospho-21 expression was decreased with serum restriction, as well as the proportion of phospho-p21 relative to p21 (p<0.05). Serum and glucose restriction (1%/1mM) increased the sensitivity of MMTV-neu cells to treatment with lapatinib and cyclophosphamide, resulting in reduced cellular viability (p<0.05).

Conclusion: These results suggest that short-term CR, achieved specifically through serum restriction alone or in combination with glucose restriction, is associated with decreased phosphorylation and cytoplasmic localization of p21, which may be responsible for modulating anti-proliferative activities. This finding is significant because clinical studies have found that increased cytoplasmic p21 in HER2-overexpressing BC predicted reduced survival in patients at 5 years. Utilizing CR in conjunction with existing therapies may prevent or reverse cytoplasmic p21 localization in this cancer subtype, highlighting the importance of this investigation.
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>5</td>
</tr>
<tr>
<td>a. Obesity and HER2-Overexpressing Breast Cancer</td>
<td>5</td>
</tr>
<tr>
<td>b. HER2/neu Tumorigenesis</td>
<td>5</td>
</tr>
<tr>
<td>c. HER2/neu Signaling Mechanisms</td>
<td>6</td>
</tr>
<tr>
<td>d. HER2/neu Therapeutic Targets and Resistance</td>
<td>6</td>
</tr>
<tr>
<td>e. Calorie Restriction as an Adjunct Therapy</td>
<td>7</td>
</tr>
<tr>
<td>f. Impact of Dietary Energy Balance on HER2 Tumorigenesis</td>
<td>9</td>
</tr>
<tr>
<td>2. Goal and Hypotheses</td>
<td>10</td>
</tr>
<tr>
<td>3. Methods</td>
<td>12</td>
</tr>
<tr>
<td>4. Results</td>
<td>17</td>
</tr>
<tr>
<td>5. Discussion</td>
<td>27</td>
</tr>
<tr>
<td>6. Conclusion</td>
<td>34</td>
</tr>
<tr>
<td>7. References</td>
<td>35</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank Dr. Hursting for his guidance and encouragement over the past two years. The opportunity to be part of a lab that holds rigorous standards for undergraduates has allowed me to focus on independent research and develop a lifelong passion. I have Dr. Hursting, and his constant support to thank for this. My graduate student mentor, and close friend, Laura Smith, has taught me every research technique discussed in this paper, has encouraged me through months of challenging experiments, and has been patient through all of my shortcomings. She has taught me to care deeply about the pursuit of scientific investigation and the important implications that it holds. Thank you for bringing enthusiasm and perseverance to bench work.
Introduction

Obesity and HER2-overexpressing Breast Cancer

It is well-established that among women with obesity, there is an increased risk of developing invasive breast cancer as well having poorer clinical outcomes upon diagnosis (1-5). Obesity at time of breast cancer (BC) diagnosis is associated with resistance to therapy, increased risk of recurrence, and lower survival rates (6-8). Put in the context of the staggering obesity epidemic faced by the U.S. alongside an estimated 252,000 new invasive breast cancer cases annually, this association poses a significant public health concern (9,10).

Given the heterogeneity of this disease, associations between host and tumor characteristics must be studied according to molecular subtype of BC. One of the most aggressive forms, characterized by amplification and/or overexpression of the human epidermal growth factor receptor 2 (HER2) gene, accounts for ~20% of all BC diagnoses and is associated with greater tumor burden, higher rates of distal metastases, and worse overall survival rates (11,12). Development of therapies that specifically target the HER2/neu receptor have demonstrated clinical effectiveness, however, patients with obesity persistently suffer from worse outcomes which highlights the importance of elucidating the mechanisms behind this obesity cancer link as well as developing novel therapies to ameliorate the burden (13).

HER2/neu Tumorigenesis

Over-expression of the HER2 oncogene induces replication stress, leading to a DNA damage response and senescence, a non-proliferative but viable state. Alternatively, cells can acquire mutations that allow them to bypass this barrier to tumorigenesis, resulting in genomic instability and cancer progression (14-16). Through preclinical studies on MMTV-HER2/neu transgenic mice, which hyperactively express the HER2 oncogene, the Hursting lab has
identified an association between dietary energy balance and breast cancer progression. Mice fed a high-fat, obesogenic diet exhibit both an increased frequency and decreased latency of breast cancer development, compared to lean, calorie restricted mice (17).

HER2/neu Signaling Mechanisms

The HER2 gene is a member of the ErbB receptor tyrosine kinase family. Its associated protein, Neu, enhances several kinase signaling cascades upon forming a homo- or heterodimer with a ligand-bound epidermal growth factor receptors (EGFR, HER3, HER4). Dimerization results in phosphorylation of the intracellular domains of the receptor tyrosine kinases (RTK) and leads to recruitment of signaling molecules to initiate kinase pathways (18). These pathways can be inappropriately activated in cells that overexpress HER2/neu via autophosphorylation of specific tyrosine residues that stabilize signaling through EGFR. This bypasses regulatory mechanisms, increasing the oncogenic potential of EGFR and inducing cell transformation and tumorigenesis (19).

The most common HER2 signaling pathways include the phosphatidylinositol-3 kinase (PI3K)/Akt and RAS/Mitogen-activated protein kinase (MAPK) cascades (20). In the RAS/MAPK cascade, adaptor proteins bind phosphorylated HER2/neu and activate RAS, a protein kinase, that initiates a cascade to activate extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK interacts with kinases and transcription factors to promote cell growth, proliferation, and survival (21). The PI3K/Akt pathway is activated by HER2/RTK dimerization and intracellular phosphorylation. The heterodimer PI3K is recruited and activated to phosphorylate PIP2 to PIP3. PIP3 induces translocation of Akt to the plasma membrane where it regulates proteins involved in cell survival. Through these interactions, the PI3K/Akt activation is involved with inhibition of apoptotic pathways, and activation of survival pathways (22).
**HER2/neu Therapeutic Targets and Resistance**

The aberrant growth signals promoted by HER2 dimerization are effectively blocked with a regimen of therapies (23,24). At present, first-line HER2-positive BC protocol indicates use of adjuvant chemotherapy combined with one year of trastuzumab therapy, a humanized monoclonal antibody (mAb) directed against the extracellular domain of HER2 that blocks signaling ligand-independent HER2 activity(24,25). In advanced stages of this disease, lapatinib, a small molecule dual HER2/1 tyrosine kinase inhibitor is used to inhibit activation of the HER2/EGFR pathway by blocking auto-phosphorylation (23,26,27). The action of lapatinib has demonstrated effectiveness to induce cellular apoptosis while trastuzumab binding to the extracellular HER2 domain may function by sterically modulating the detection of phosphate moieties by cytoplasmic signal transduces (28).

While the advent of these therapies has dramatically improved overall survival, a significant number of patients are unresponsive from the start of treatment, and even more acquire resistance over the course of treatment (29,30). The proposed mechanisms of this conferred resistance to therapy includes impaired binding of antibody, upregulation of HER2/neu downstream signaling pathways, signaling through alternate pathways including IGF1R, c-MET, EGFR, and HER2, and failure to trigger immune-mediated destruction of cells (31). Potential therapies that reduce trastuzumab resistance include the antibody-drug conjugate, T-DM1, that targets HER2 and delivers a cytotoxic agent as well as inhibitors of the PI3K/Akt/mTOR pathway (32,33).

**Calorie Restriction as an Adjunctive Therapy**

Another approach that targets signaling molecules along the same PI3K/Akt/mTOR axis is the utilization of calorie restriction mimetics in conjunction with chemotherapy. This considers
the complex metabolic reprogramming that occurs in malignant cells driven by mutations in oncogenes and tumor suppressors (34-36). Long-term calorie restriction (CR), or dietary energy restriction by ~30%, has demonstrated tumor suppressive effects across cancer subtypes (37-40). This may be mediated in part by enhanced apoptosis within tumors, reduced angiogenesis, and alterations in key metabolites and systemic signaling pathways involving insulin-like growth factor (IGF)-1 and inflammatory markers (41). Recent studies have found that CR mimetics including intermittent / short-term fasting (total caloric restriction ranging from 24 hours to 6 days), produce similar metabolic benefits modulated by chronic CR, but may be more feasible in a clinical setting, potentiating its use as an adjunct therapy in combination with traditional chemotherapy (42). Compared to oncogene-driven cells, normal cells suspected to short-term CR enter a chemo-protective state characterized by decreased cellular division and increased repair pathways, termed the differential stress resistance (DSR) (42-44). This has significant implications for reducing the cytotoxicity associated with chemotherapy. Conversely, malignant cells are selectively sensitized to chemotherapeutics by short-term CR, called the differential stress-sensitization (DSS) where cancer cells are preferentially destroyed by chemotherapy (44). While the mechanism is poorly understood, it is likely related to the inability of cancer cells to adapt to an environment where nutrients are not available in excess (45). In vitro studies demonstrated that breast cancer cells cultured in medium supplemented with serum from fasted mice were more susceptible to the chemotherapeutic drugs cyclophosphamide (CP) and doxorubicin (DXR), which are commonly used in combination with trastuzumab for HER2-positive BC treatment (46). These findings were confirmed by in vivo work where mice placed on a short-term food starvation regimen (fasting 32 hours prior and 16 hours post-injection with water ad libitum) in combination with cisplatin chemotherapy had significantly reduced tumor
growth, modulated through hyperactivation of the ataxia-telangectasia mutated (ATM)/human homologue of Rad53 (Chk2)-dependent stress response pathway (47). Together, these findings demonstrate both the protective and anti-tumorigenic effect of CR mimetics in conjunction with chemotherapy and highlight the necessity of identifying the molecular targets that they modulate.

**Impact of Dietary Energy Balance on HER2 Tumorigenesis**

The Hursting laboratory aims to characterize the molecular mechanisms through which obesity-related metabolic changes drive tumorigenesis. A recent study that informed this current project modeled chronic CR by placing female MMTV-HER2/neu transgenic and wild type FVB mice on either a low-fat control regimen (CON, 10 kcal% fat), a 30% CR regimen relative to control, or a high-fat diet induced obesity (DIO, 60 kcal% fat) regimen. Compared to the CON and DIO groups, calorie restricted mice had decreased adiposity and obesity-related serum markers including IGF-1, insulin, leptin, as well as preserved ERα and ERβ expression, increased disease-free survival rates, and reduced tumor burden (37). These novel findings highlighted the need for further investigation of the mechanisms underlying these diet-gene interactions.
Goals and Hypotheses

This project aims to elucidate the mechanisms through which calorie restriction drives molecular and metabolic changes in MMTV-HER2/neu breast cancer cells.

**Aim 1. Determine the impact of nutrient stress on MMTV-HER2/neu BC cell characteristics.**

Short-term calorie restriction was mimicked *in vitro* to determine the sensitivity of BC cancer cells to nutrient stress. Under conditions of nutrient restriction, changes in cancer cell viability and proliferative activity as well as regulation of apoptosis and the cell cycle were measured. It is hypothesized that MMTV-HER2/neu BC cells will be unable to reprogram metabolic pathways in the absence of excess protein and growth factors mimicked by serum restriction, which is expected to reduce the viability of cells and alter growth and cell cycle maintenance patterns.

**Aim 2. Determine the efficacy of nutrient restriction as an adjunct therapy in conjunction with targeted and chemotherapies.**

MMTV/HER2-neu cells were cultured in nutrient restricted medias and treated with either the targeted anti-HER2 therapy, lapatinib, or standard chemotherapeutics, cyclophosphamide and doxorubicin, to determine the efficacy of short-term CR as an adjunct therapy. It is hypothesized that cancer cells cultured in serum restricted medias will have increased sensitivity, and thus reduced viability, in response to treatment with lapatinib, doxorubicin, and cyclophosphamide.

**Aim 3. Identify molecular targets altered by nutrient stress in MMTV-HER2/neu BC cells.**

MMTV-HER2/neu breast cancer cells cultured in nutrient restricted medias were assessed for changes in key proteins along the PI3K/Akt and RAS/ERK signal transduction pathway. Given the intersection of key metabolic and proliferative pathways, it is hypothesized
that proteins involved in the DNA damage response and cellular growth will be impacted by nutrient restriction including Akt, Erk, PTEN, PCNA, and p21.

**Aim 4. Determine the impact of energy balance on markers of DNA damage and senescence in WT and MMTV-HER2/neu transgenic mice.**

The molecular targets of short-term nutrient restriction determined through *in vitro* work were assessed in mammary tissue isolated from wild type and MMTV-HER2/neu transgenic mice exposed to chronic CR. By determining levels of key proteins and mRNA involved in DNA damage repair and senescence pathways in mammary tissue isolated from both cancer and epithelial cells, differences in response to chronic CR were determined. It is expected that tissue from MMTV-HER2/neu mice will have a reduction in total levels of p21 in ductal structures, given the significant reduction in cancer cells modeled *in vitro*. 
Methods

I. Short-term calorie restriction & MMTV-HER2/neu BC cell characteristics in vitro

Murine MMTV/HER2-neu breast cancer cells were maintained in DMEM medium supplemented with 10% bovine cattle serum (BCS) and 5mg/ml penicillin/streptomycin. To mimic calorie restriction in vitro, cells were treated with medias containing reduced serum (1% BCS), reduced glucose (1mM Glc), or reduced serum and reduced glucose (1% BCS/1mM Glc) compared to control media (10% BCS/25mM Glc) (48). Cells were maintained at 37°C in a 5% humidified chamber.

MTT assay

MMTV-HER2/neu cells were seeded (8x10^3 cells/well) in a 96-well plate overnight, serum and glucose restricted for 24 hours, then treated with calorie restricted medias (CON, 1% BCS, 1mM Glc, 1% BCS/1mM Glc) for the remaining 24 hours. Cells stained with solution containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 hours, media aspirated, and replaced with sodium dodecyl sulfate (SDS). Plate placed on shaker for 5 minutes and UV spectroscopy used to measure absorbance at 570 and 690 nm via the Cytation 5 Cell Imagining reader (BioTek). Percent viability determined by the ratio of absorbance in each treatment group relative to control. Differences between groups assessed using one-way ANOVA using Turkey’s multiple comparisons tests with p<0.05 considered significant.

Colony Formation Assay

MMTV-HER2/neu cells were seeded (500 cells/well) in a 6-well agarose gel plate in triplicate corresponding to each treatment group (CON, 1% BCS, 1mM Glc, 1%BCS/1mM Glc). Cells were grown and monitored for 14 days, until cells treated with control media formed colonies.
visible colonies. Wells were imaged using EVOS FL Auto Imaging System. Number of colonies classified by size quantified.

**Apoptosis Assay**

MMTV-HER2/neu cells were seeded (2.5x10^4 cells/well) in a 6-well plate overnight, serum and glucose starved for 24 hours, then treated with calorie restricted medias (CON, 1% BCS, 1mM Glc, 1% BCS/1mM Glc) for the remaining 48 hours. Cells prepared with the FITC Annexin V/Dead Cell Apoptosis Kit for Flow Cytometry (ThermoFisher) following manufacturer’s protocol. Phosphatidylserine (PS), a phospholipid that is translocated to the outer surface of the membrane upon induction of pro-apoptotic signaling, is efficiently bound by Annexin V, which is conjugated to fluorescein isothiocyanate (FITC), a green fluorophore, for detection via flow cytometry (49). Dead cells stained with propidium iodine (PI). Samples processed through the Accuri 6 BD (Biosciences) and 30,000 events were recorded at 488nm laser excitation and emission at 530 nm and 600 nm were recorded. PI fluorescence intensity plotted against FITC fluorescence intensity and groups of cells categorized; live cells with minimal fluorescence, apoptotic cells with green fluorescence, dead cells with both red and green fluorescence.

**Cell Cycle Analysis**

MMTV-HER2/neu cells were seeded (2.5x10^4 cells/well) in a 6-well plate overnight and treated with either complete or calorie restricted medias (CON, 1% BCS, 1mM Glc, 1% BCS/1mM Glc) for 24 hours. Cells were fixed, permeabilized, and stained with propidium iodide (PI) for flow cytometric analysis of cell cycle according to manufacturer’s instructions (Abcam). Single cell suspension run through the CyAn ADP High-Performance Flow Cytometer
Magdalena Rainey

(Beckman Coulter) and emission of PI recorded using 488nM laser excitation using. Forward scatter and side scatter measured on 10,000 cells and plots gated to exclude cellular debris (50).

II. MMTV-HER2/neu Targeted Therapy and Adjunctive CR in vitro:

Chemotherapy treatment and MTT assay

MMTV-HER2/neu cells were seeded (2.5x10³ cells/well) in a 96-well plate and grown in complete media for 24 hours, then treated with nutrient restricted medias (CON, 1% BCS, 1mM Glc, 1% BCS/1mM Glc) for 48 hours. Following the first 24 hours of nutrient restriction, cells were treated with sub-lethal doses of chemotherapies typically used in HER2-positive BC treatment regimens. Drugs were placed in media at dosages corresponding to determined IC₅₀ values: 5μM lapatinib, 5μM doxorubicin, or 500μM cyclophosphamide (51-53).

III. Molecular targets altered by nutrient stress in MMTV-HER2/neu BC cells

Cellular Extraction and Western Blotting

MMTV-HER2/neu cells were cultured in 100mm dishes at densities to reach 70% confluency after 72 hours. Once adhered, cells were treated with serum free media for 24 hours and corresponding calorie restricted medias for the remaining 24 hours. Whole cell lysates were created using RIPA lysis buffer (Sigma Aldrich) supplemented with protease inhibitor cocktail (Sigma Aldrich). Cells scraped from dish and incubated on ice for 20 minutes. Cellular debris separated via centrifugation at 14000 RPM for 15 minutes at 4°C and the protein-containing supernatant collected. Protein concentration determined via Bradford Assay with a Bradford protein quantification reagent. Equal concentrations of each protein lysate were denatured by boiling for 5 minutes in solution containing 2X Loading Buffer (Biorad) and 5% B-mercaptoethanol. Protein separated via SDS-PAGE and transferred to nitrocellulose membrane for western immunoblotting. Membranes blocked at room temperature with gentle rocking for 1
hour in Tris-buffered saline containing 0.1% Tween (TBST) and 5% Bovine Serum Albumin. Membranes were blocked with primary antibody overnight at 4°C, washed with TBST, and blocked in secondary antibody for 1 hour at room temperature. Chemiluminescent detection utilized; ECL binding substrate (Biorad) placed on membrane and imaged with the ChemiDoc XRS+ System (Biorad).

IV. Animal tissue histology analysis

IHC of mammary glands with anti-p21 antibody

Whole mount slides of mammary tissue collected from female MMTV-HER2/neu transgenic and wild type FVB mice from a previous dietary intervention study were analyzed (37). Mice received either a low-fat control regimen (CON, 10 kcal% fat), a 30% calorie restricted (CR) regimen relative to control, or a high-fat diet induced obesity (DIO, 60 kcal% fat) regimen. Samples taken from both transgenic and WT mice at baseline (0 months) and 5 months on diet that were formalin-fixed and paraffin-embedded were analyzed via immunohistochemistry (IHC) staining using an anti-p21 antibody. Mammary glands were analyzed for positive nuclear and cytoplasmic p21 staining, quantified as ratio of positive staining relative to total structures.

qRT-PCR Analysis of Mammary Tissue

Total RNA was extracted from flash-frozen mammary tissue collected from wild type FVB mice at 5 months in the control and calorie restricted diet group using the RNeasy Lipid Tissue Mini Kit (Qiagen) following manufacturer’s protocol. RNA concentration and quality determined using the NanoDrop (Thermo Fisher Scientific) spectrometer. RNA suspended in High Capacity RNA-to-cDNA 5X Master Mix (Applied Biosystems) and reverse-transcribed in the Simpliamp Thermal Cycler (Thermo Fisher Scientific). cDNAs were assayed in triplicate.
utilizing TaqMan gene probes for CDKN1A, CDKN2A, IL6, and TP53. PCR reactions conducted by the ViiA 7 Real-Time PCR System (Applied Biosciences). Gene expression data were normalized against β-actin and analyzed using the ΔΔCt method. Statistical analysis compared ΔCt values in control and calorie restricted groups using unpaired T-tests (54).
Results

I. Nutrient stress alters MMTV-HER2/neu breast cancer cell characteristics in vitro

Upon culturing MMTV-HER2/neu cells in nutrient restricted medias, it was determined that serum restriction, not glucose restriction alone, impacts cellular viability, proliferation, regulation of apoptosis, and cell cycle maintenance. First, viability was assessed via MTT assay which measures the concentration of metabolically active cells by quantifying the amount of reagent that is reduced to a molecular form that is detectable through spectrophotometry. Following 24 hours of nutrient restriction, MMTV-HER2/neu cells cultured in reduced serum medias (1%BCS, 1%BCS/1mM Glc) had significantly decreased viability compared to cells cultured in complete media (p=0.02; p=0.03) (Figure 1). These results indicate that MMTV-HER2/neu BC cells are sensitive to serum restriction, not to glucose restriction alone. This confirms the hypothesis that under conditions were protein and growth factors are limiting, breast cancer cells are unable to undergo sufficient reprogramming to sustain increased metabolic activity.

Next, the impact of nutrient restriction on cancer cell growth patterns and potential tumor characteristics were assessed by measuring colony formation. MMTV-HER2/neu cells grown for 14 days in complete or nutrient restricted medias demonstrated differences in anchorage independent colony formation (Figure 2). Whole plate images were analyzed and the total number of colonies formed, grouped by size, were assessed. It was determined that cells cultured in serum restricted media had a reduced total number of colonies formed, as well as a reduced size of colonies present compared to control. There were no significant differences in the proportion of colonies formed among cell cultured in glucose restricted media. Interestingly, the dual serum and glucose restricted group had a total colony number comparable to control, but a
much larger proportion of the colonies were smaller in size than control. These results indicate that serum restriction has potential to alter the tumorigenic potential of HER2/neu breast cancer cells, confirming the hypothesis that nutrient restriction alters cell growth patterns.

To further examine changes in growth patterns, alterations in cell cycle regulation were measured in response to nutrient restriction. Apoptosis was measured using Annexin V/fluorescein isothiocyanate (FITC) staining and processed via flow cytometry. Upon induction of apoptotic signaling, phosphatidylserine is translocated to the outer surface of the plasma membrane, which is efficiently bound by Annexin V conjugated to FITC for signal detection. Apoptotic cells experience increased Annexin V staining relative to live cells, and are represented as a cluster of in the upper right quadrant of the fluorescence graph (figure 3). Dead cells, stained with propidium iodine are represented in the third quadrant of the graph, and live in the first quadrant. It was determined that nutrient restriction induces apoptosis in MMTV-HER2/neu cells. There was a significant increase in cellular apoptosis among cells cultured in reduced serum medias (1%BCS and 1%BCS/1mM Glc) compared to control (Figure 3). Cells subject to glucose restriction alone also experience a moderate shift in increased apoptosis. Additionally, there was a significant increase in the number of dead cells in both serum restricted groups. This further confirms the role of nutrient restriction in altering patters of cell cycle maintenance and its potential to induce apoptosis in cancer cells.

The impact of nutrient restriction on cell cycle regulation was further explored by utilizing propidium iodine staining to quantify the relative number of cells in each phase of the cell cycle. The proportion of propidium iodine staining correlates with the amount of DNA present in the cell where two copies of each chromosome (2N) characterizes non-dividing cells in either the G1 phase and G0, or senescent cells lacking replicative potential. Cells actively
replicating DNA (2N-4N) are in the S phase. Following DNA replication, cells enter G2 or M phase (4N) (50). Sharp peaks furthest left are characteristic of the G1/G0 phase, and shifting right peaks correspond to the S and G2/M phases respectively (figure 4A). Cells cultured in serum restricted medias experience G0/G1 arrest compared to control, which may be associated with increased cellular senescence. A greater proportion of glucose restricted cells were present in the G2/M phase compared to control and serum restricted cells. Arrest in the G2/M phase is characteristic of increased DNA damage which prompts cells inhibit the final steps of cell division.

![Graph](image)

**Figure 1.** Following 24 hours of nutrient restriction, MMTV-HER2/neu cells cultured in reduced serum medias had significantly decreased viability compared to control (p=0.02; p=0.03).

![Images](image)

**Figure 2.** MMTV-HER2/neu cells grown for 14 days in complete (CON) or nutrient restricted (1% BCS, 1mM Glc, 1% BCS/1mM Glc) medias demonstrated differences in anchorage independent colony formation.
Figure 3. MMTV-HER2/neu cells cultured in serum restricted medias for 48 hours have an increased proportion of apoptotic cells (quadrant 2) and dead cells (quadrant 3) compared to control.

Figure 4. Cell cycle analysis of MMTV-HER2/neu cells demonstrates that serum restriction induces G0/G1 arrest compared to control while glucose restriction increases the proportion of cells present in the G2/M phase compared to control and serum restricted cells.
II. Nutrient restriction sensitizes MMTV-HER2/neu cells to targeted therapy in vitro

Finally, the efficacy of nutrient restriction as an adjunct therapy was assessed. MMTV-HER2/neu cells cultured in nutrient restricted medias and treated with lapatinib (LP), doxorubicin (DXR), or cyclophosphamide (CP) and MTT assays were used to quantify cellular viability. It was determined that breast cancer cells cultured in serum restricted medias (1% BCS and 1% BCS/1mM Glc) and treated with lapatinib experienced decreased viability compared to cells cultured in control medias (p=0.030, p=0.016) (figure 8A). This provides evidence that serum restriction sensitizes HER2/neu BC cells to lapatinib treatment. Under the same nutrient restriction regimen, breast cancer cells treated with chemotherapies that inhibit DNA replication, DXR and CP, do not show a clear trend in changes in viability in response to therapy. DXR inhibits DNA synthesis by inhibiting DNA double helix ligation and thereby halting replication (55). CP also inhibits replication, by crosslinking DNA strands at guanine bases, forming inappropriate linkages, and inducing strand breakage and apoptosis (56). (B) Contrasting trends associated with lapatinib treatment, cells cultured in serum restricted medias and treated with DXR had increased viability compared to cells cultured in control media. Cells cultured in dual serum and glucose restricted media (1% BCS/1mM Glc) were significantly more viable compared to cells cultured in control media treated with equivalent drug doses (p=0.015). (C) Oppositely, cancer cells cultured in dual nutrient restricted media demonstrated reduced viability compared to control in response to treatment with CP (p=0.048). Contrasting trends in the response of cancer cells to chemotherapy following nutrient restriction may be attributed to differences in mechanism of action of drugs.
Figure 8. Cellular viability assessed via MTT assays of MMTV-HER2/neu cells cultured in nutrient restricted medias and treated with lapatinib (LP), doxorubicin (DXR), or cyclophosphamide (CP). (A) Breast cancer cells cultured in serum restricted medias (1% BCS and 1% BCS/ 1mM Glc) and treated with lapatinib experienced decreased viability compared to cells cultured in control medias (p=0.030, p=0.016). (B) Cells cultured in dual serum and glucose restricted media (1% BCS/ 1mM Glc) and treated with DXR were significantly more viable compared to control (p=0.015). (C) Cells cultured in dual nutrient restricted media demonstrated reduced viability compared to control in response to treatment with CP (p=0.048).

III. Nutrient stress alters expression of molecular targets along the PI3K/Akt signaling pathway

Having characterized key alterations in MMTV-HER2/neu growth and maintenance patterns in response to nutrient restriction, the mechanisms that drive these changes were explored. Whole cell lysates of cells cultured in nutrient restricted medias were isolated and probed for specific proteins via western blotting. Antibodies for proteins that act as signaling molecules along the PI3K/Akt signaling pathway and that are known to modulate cell senescence were used to identify potential molecular targets of nutrient restriction.

It was determined that MMTV-HER2/neu cells cultured in serum restricted medias experienced decreased expression of the tumor suppressor protein, p21 and phospho-p21, compared to control (p=0.03; p=0.03) (figure 5A,B). Phosphorylated p21 at Thr145 is associated with cytoplasmic localization of the protein, while the unphosphorylated form remains in the nucleus to regulate the cell cycle (57). Upstream of p21, nutrient restriction did not impact expression of protein kinase B (Akt), which plays in integral role in cellular survival pathways.
by inhibiting apoptosis and inducing protein synthesis (figure 5B). Combination of serum and glucose restriction resulted in a modest increase in expression of the active form of the protein, p-Akt, relative to control (p=0.03) (figure 5E). Another key regulator of cell proliferation, the mitogen activated kinases, Erk and its active phosphorylated form, p-Erk were not significantly altered by nutrient restriction (figure 5C,F). These results indicate that p21 may be an important molecular target of nutrient restriction.

Figure 5. (A, D) MMTV-HER2/neu cells cultured in serum restricted medias experienced decreased expression of the tumor suppressor protein, p21 and phospho-p21, compared to control
(p=0.03; p=0.03). (B) Short-term CR did not impact expression of protein kinase B (Akt). (E) Combination of serum and glucose restriction resulted in a modest increase in p-Akt expression compared to control (p=0.03). (E, F) Expression of Erk and p-Erk, mitogen activated kinases, are not significantly altered by nutrient restriction. (G) Western blotting demonstrating expression levels of proteins of interest relative to the housekeeping proteins B-actin and GAPDH.

IV. Dietary energy balance modules p21 localization in vivo

In order to confirm that molecular targets of nutrient restriction identified in vitro are similarly regulated by dietary energy balance in vivo, mammary tissue isolated from wildtype FVB mice and transgenic MMTV-HER2/neu mice on either high a low-fat control regimen (CON, 10 kcal% fat), a 30% calorie restricted (CR) regimen relative to control, or a high-fat diet induced obesity (DIO, 60 kcal% fat) regimen was examined. Mammary tissue was assessed for presence of p21 in ductal structures using IHC staining to determine if there were differences in protein localization. In the transgenic mouse model, lean mice on a CR diet regimen for 5 months had less cytoplasmic p21 staining compared to CON and DIO mice and similar trends were mirrored in FVB tissue, although not to the same extent (figure 6). MMTV-HER2/neu mice on a CR regimen had significantly reduced cytoplasmic p21 levels compared to CON and DIO mice (p=0.03; p=0.01), this correlates with the reduced phospho-p21 expression demonstrated in vitro under nutrient restricted conditions. Lean FVB mice on a CR diet regimen had reduced ductal p21 levels compared to mice on a DIO regimen (p=0.03) and lean MMTV-HER2/neu mice had significantly reduced ductal p21 compared to CON mice (p=0.045). The most significant trend to note from these results is the ratio of ductal p21 to cytoplasmic p21, which is highest in the transgenic calorie restricted mice. These demonstrates that dietary energy balance regulates localization of p21 in an MMTV-HER2/neu model in vivo.

(Talk about how this lets us understand chanNext, RNA from wildtype mammary tissue was examined to determine the impact of calorie restriction on regulation of key molecular
targets in non-transformed tissue. Utilizing qRT-PCR, expression patterns for genes involved in senescent pathways were examined in mammary tissue isolated from lean FVB wildtype mice fed a CR diet and normal-weight FVB mice on a CON diet for 5 months. FVB tissue used as a model for normal, non-transformed mammary epithelium. Comparing CR to CON, there were no significant differences in gene expression patterns for CDKN1A, CDKN2A, or IL6 (p=0.31, p=0.92, p=0.44). CDKN1A encodes the tumor suppressor, p21, which induces senescence and downregulates H2AX expression through a p21-CDK-Rb pathway (58). CDK2A encodes tumor suppressor, p16, which participates in the initiation and maintenance of cellular senescence and tumor suppression (59). The IL6 gene encodes Interleukin 6 (IL6), a cytokine secreted by senescent cells (60). Only expression of T53, which encodes the protein p53, was reduced in CR mammary tissue compared to CON (p=0.0035). P53 plays a dual role in cell cycle control where under conditions of mild stress it activates cellular repair mechanisms, but under conditions of severe stress, this protein activates apoptotic and senescent pathways (61).
Figure 6. IHC staining for p21 in the nucleus and cytoplasm of mammary ducts isolated from wildtype FVB mice (E) and from transgenic MMTV-HER2/neu mice (F). (C) MMTV-HER2/neu mice on a CR regimen had significantly reduced cytoplasmic p21 levels compared to CON and DIO mice (p=0.03; p=0.01). (B) Lean FVB mice on a CR diet regimen had reduced ductal p21 levels compared to mice on a DIO regimen (p=0.03). (D) Lean MMTV-HER2/neu mice had significantly reduced ductal p21 compared to CON mice (p=0.045).

Figure 7. RNA isolated from FVB analyzed utilizing qRT-PCR to compare differences in expression patterns between diet groups. There were no significant differences in gene expression patterns for CDKN1A, CDKN2A, or IL6 (p=0.31, p=0.92, p=0.44). Expression of T53 was reduced in CR mammary tissue compared to CON (p=0.0035).
Discussion

This study determined that short-term calorie restriction mimetics impact the viability, growth patterns, induction of apoptosis, and maintenance of cell cycle in MMTV-HER2/neu breast cancer cells. It was determined that the cells experience a differential response under conditions of serum restriction compared to glucose, where substantial changes in cell maintenance patterns were demonstrated in response to serum restriction. It was also found that short-term restriction also alters protein levels of key intermediates along signaling pathways upregulated in HER2/neu-overexpressing breast cancer. In particular, this study identified tumor suppressor protein p21 as a potential target and provided pre-clinical evidence for the use of nutrient restriction as an adjunct therapy.

First, by mimicking short-term caloric restriction in vitro, it was determined that MMTV-HER2/neu breast cancer cells have decreased metabolic activity under conditions of serum restriction compared to control (1%BCS p=0.02, 1%BCS/1mM Glc p=0.03). MTT assay that measured cellular viability of nutrient-restricted BC cells confirmed the hypothesis that cells are sensitive to an environment where proteins and growth factors in serum are not available in excess and respond by decreasing metabolic activity. This finding coincides with the work of Lamming et. al. which found that intermittent fasting and acute protein restriction inhibits tumor growth via decreased phosphorylation of mechanistic target of rapamycin complex 1 (mTORC1) in a xenograft mouse model of breast cancer (62). The IGF/mTOR pathway, which is activated by insulin and amino acids, plays an important role in stimulating growth and proliferation, and is also upregulated under conditions of diet-induced obesity (63). Further, human studies have demonstrated the importance of modest protein restriction in a chronic CR regimen in order to modulate anti-cancer effects associated with decreased IGF-1 levels (64). This highlights the
importance of examining the impact of caloric restriction with respect to macronutrient composition of dietary regimen *in vivo* or corresponding mimetic *in vitro*.

In addition to reduced viability demonstrated with serum restriction, this study also found that MMTV-HER2/neu cells grown in serum restricted medias experienced decreased anchorage independent colony formation. The ability of a cell to grow and divide independently of a solid surface is a hallmark of carcinogenesis and is used to determine if cells have undergone malignant transformation. This contrasts non-transformed cells, which undergo apoptosis under conditions where there is no surface to adhere to (65). These results demonstrate that serum restriction limits the growth of MMTV-HER2/neu cells *in vitro*.

Alterations in cell viability and growth can be attributed to differences in cell cycle regulation impacted by nutrient stress. It was found that short-term CR induces cellular apoptosis and necrosis in BC cells cultured in serum restricted medias. Likewise, Lee, et. al. found that breast cancer cells exposed to intermittent fasting (IF) conditions, a CR mimetic, had increased markers of DNA damage and cellular apoptosis. This group proposed that reduction of IGF-1 as a result of IF removed the block on apoptosis that this signaling molecule confers, and therefore has potential to sensitize BC cells to nutrient stress (46). Similar results were found *in vivo* by Saleh et. al. that modeled triple-negative-breast cancer and found that mice on a CR diet regimen had increased markers of apoptosis in tumor tissue, which was linked to downregulation of the IGF-1 signaling pathway (66). These findings highlight the potential of CR with protein restriction or IF to reduce IGF-1 levels and induce cellular apoptosis.

Further, examination of cell cycle regulation demonstrated that serum restriction causes an accumulation of cells in the G0/G1 phase, and a moderate decrease of cells in the S phase compared to control. Serum starvation may result in cell cycle arrest through stress induction in
cancer cells that are unable to adapt to the loss of external growth factors by reprogramming their metabolism (67). This may be modulating stress-induced senescence, which occurs independent of telomere shortening. This explanation is limited in that multiple tests are necessary to confirm cellular senescence including examination of morphological changes, activation of tumor suppressor pathways, and increased lysosomal B-D-galactosidase activity (14). It was also demonstrated that a greater proportion of glucose restricted cells were present in the G2/M phase compared to control and serum restricted cells. Arrest in the G2/M phase is characteristic of increased DNA damage which prompts cells to inhibit the final steps of cell division.

Together, these results indicate that MMTV-HER2/neu cells are sensitive to changes in the availability of serum-related nutrients and biological factors. This provides evidence for the mechanism through which caloric restriction impacts patterns of growth and progression of these cancer cells in vivo.

Having defined then impact of nutrient restriction on cell cycle regulation, it was posited that these mechanisms would act synergistically with chemotherapies to increase the sensitivity of breast cancer cells to drug treatment. Several rodent \textit{in vivo} studies have demonstrated that short-term food starvation sensitizes cancer cells to chemotherapies in mouse models of lung, breast, and pancreatic cancer (39,40,47). However, this relationship has only been explored for HER2/neu breast cancer \textit{in vitro}, highlighting the importance of this work. This study demonstrated that HER2/neu cells cultured in serum restricted medias had increased sensitivity to treatment with the tyrosine kinase inhibitor, lapatinib (LP). This finding is in agreement with the work of Caffa et. al. which demonstrated that four-day starvation of the HER2-overexpressing human cell line, SKBR3, acted synergistically with lapatinib. This finding was rationalized with the hypothesis that inhibition of MAPK signaling by nutrient restriction blocks
the compensatory mechanisms along the PI3K/Akt pathway that limit the efficacy of TKIs and confer therapeutic resistance (68). These findings support the development an in vivo model of HER2-overexpressing BC combing short-term fasting with lapatinib treatment, or other targeted therapy. Further exploration is needed to determine which genes are differently expressed in response to short-term fasts in oncogene driven breast cancer. A major limitation of this current work is the lack of mechanistic relationship between serum starvation and lapatinib sensitivity in HER2/neu cells. Future work will explore differences in expression of protein and RNA along the MAPK signaling pathway. Additionally, if an in vivo model to test this hypothesis were to be developed, several cycles of chemotherapy with short-term starvation would need to be incorporated to explore whether or not cancer cells are able to acquire metabolic alterations to evade downregulation of proliferative pathways under nutrient restricted conditions.

There were less clear trends found when nutrient restriction was combined with non-specific chemotherapy treatment in HER2/neu BC cells. Nutrient restricted cells treated with doxorubicin (DXR) had increased viability compared to control, opposing the proposed hypothesis. While greater exploration of the mechanism underlying this relationship is necessary, including cell cycle analysis and apoptosis assays, it is plausible that reduction of mTORC1 activity in response to nutrient restriction results in decreased p70S6 kinase (S6K) activation, which negatively regulates IRS-1 to block activation of Akt. Under conditions where mTORC1 is reduced, Akt activity increases as a compensatory mechanism to promote growth (69). Therefore, metabolic activity may be elevated to compensate for this nutrient restricted environment.

However, nutrient restriction demonstrated a synergistic effect with cyclophosphamide (CP) treatment. The mechanism of CP results in increased apoptosis of cells, which corresponds
to trends demonstrated by nutrient restriction alone, which may modulate the synergism. Future work is needed to elucidate this relationship including apoptosis assays and cell cycle analysis to confirm this hypothesis. These findings are in agreement with several studies which demonstrated that modeled stress-sensitization in different cancer cell lines, including a metastatic triple negative cell line that experienced increased oxidative stress, caspase 3 cleavage, and apoptosis in response to short-term starvation with chemotherapy treatment (46).

One limitation of these experiments was a lack of comparison between cancer cells and normal epithelial cells in response to chemotherapy treatment. Literature by the Longo laboratory has suggested that cells experience a differential stress response whereby normal cells enter a quiescent, chemo-protective state in response to nutrient stress, unlike cancer cells that maintain proliferative activities and are rendered more susceptible to chemotherapy. Future work will repeat glucose and serum restriction experiments in MCF10A, a human breast epithelia cell line to determine if this hypothesis is valid under these cell culture conditions.

Next, protein levels of key intermediates along the PI3K/Akt and Ras/Erk pathways were assessed following short-term calorie restriction to determine where along these axes nutrient stress impact cellular growth and proliferation patterns. Examination of intermediates along key signaling pathways revealed alternations in protein level of p-Akt and p-Erk. It is plausible that restriction of glucose modulates increased activation of Akt due to the role this molecule plays in regulation of glucose transporters. Under conditions where intracellular close levels are diminished in response to restriction, activation of Akt has potential to increase transcription of glucose transporters to alleviate the stress. Further, trending increases of the active form of Erk, p-Erk, which is involved in cell proliferation pathways was found under conditions of serum
restriction. This may indicate a compensatory mechanism for cells to maintain proliferative activities under conditions of reduced protein and growth factor availability.

Interestingly, it was discovered that serum restriction targets tumor suppressor protein p21 along the PI3K/Akt signaling pathway. MMTV-HER2/neu cells cultured in serum restricted medias experienced decreased expression of the tumor suppressor protein, p21 and phospho-p21, compared to control (p=0.03; p=0.03). These results coincide with the observed cell cycle arrest that occurs in response to serum restriction. The unphosphorylated form of p21 is concentrated in the nucleus where it acts as a cyclin dependent kinase inhibitor (CKI) and induces cell cycle arrest in response to DNA damage through p53 activation. It achieves this by binding PCNA, which is necessary for DNA replication (70). Conversely, proliferative activities of p21 are promoted when it is re-localized to the cytoplasm. This is achieved by phosphorylation at threonine 145 (T145) by Akt, a key regulator of proliferation and survival in the P13K signaling pathway downstream of HER2. T145 phosphorylation disrupts hydrogen bonding patterns between p21 and PCNA, destabilizing this complex, and preventing cell cycle inhibition. Additionally, cytoplasmic subcellular localization prevents any binding interaction with PCNA. Further, cytoplasmic p21 is able to resist apoptosis by binding and inhibiting ASK1 and procaspase 3, pro-apoptotic regulators (70). Reduction in phospho-p21 achieved by nutrient restriction may provide a mechanistic explanation for increased apoptosis and G0/G1 cell cycle arrest observed in vitro. Interpretation of these results is limited in that T145 phosphorylation is not a definitive marker of subcellular localization.

Further research is needed to examine the precise mechanisms by which serum restriction reduces levels of p21, and how this may impact regulation of key tumorigenic and senescent pathways. Based on previous work by Yaglom, et. al., it was determined that nuclear p21 plays a
role in reducing expression of a major DNA repair histone H2AX via inhibition of CDK4 phosphorylation activity which activates Rb upstream of H2AX. H2AX is involved in double strand break repair, and when its levels are decreased, it is posited that cancer cells have increased sensitivity to chemotherapeutic regimens that target these repair mechanisms (56). The findings of this study that serum restriction decreases expression of p21 may indicate an important mechanistic driver of the anti-tumorigenic effect of conferred by calorie restriction in MMTV-HER2/neu transgenic mice.

Ongoing work is being completed where HER2/neu cells are transiently transfected with plasmids that overexpress p21 in either the nucleus or the cytoplasm. We hypothesize that in cells that constitutively overexpress p21 in the cytoplasm, serum restriction will not mediate changes in cell growth and proliferation patterns that are normally conferred by serum restriction. Additionally, experiments are ongoing where cells are transfected with plasmids that express a short-hairpin RNA that constitutively destroys p21 mRNA, preventing its expression. This experiment will provide information on whether or not p21 is the major molecular target of nutrient restriction. We hypothesize that in cells that that have p21 reduced, there anti-proliferative alterations modulated by nutrient restriction under wildtype conditions will not occur.

Finally, we examined preserved mammary tissue from mice stained for p21 to determine if the mechanisms demonstrated by short-term CR in vitro were similarly impacted by long-term CR in vivo. This study found that lean MMTV-HER2/neu transgenic mice on a caloric restriction diet had significantly decreased cytoplasmic p21 levels compared to normal mice fed a control diet and obese mice fed a DIO diet. Importantly, the ratio of cytoplasmic p21 to nuclear p21 was reduced in the calorie restricted mice. This provides evidence for the hypothesis that
calorie restriction regulates p21 localization. Further, this coincides with in vitro data demonstrating reduction in cytoplasmic p21 in response to nutrient restriction.

**Conclusion**

This study provide evidence that short-term calorie restriction drives alterations in MMTV-HER2/neu breast cancer cell signaling pathways and subsequently in cell growth and maintenance patterns. HER2/neu breast cancer cells experience a differential response to glucose and nutrient restriction and have increased sensitivity to the therapies lapatinib and doxorubicin under conditions of serum restriction. Restriction of serum alters expression of tumor suppressor protein p21 in vitro and decreases the proportion of cytoplasmic to nuclear p21 in vivo, highlighting potential subcellular localization of this key protein.
References Cited

20. Paplomata E, O'Regan R. The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. Therapeutic advances in medical oncology 2014;6:154-66
33. Wilks ST. Potential of overcoming resistance to HER2-targeted therapies through the PI3K/Akt/mTOR pathway. Breast (Edinburgh, Scotland) 2015;24:548-55
40. Brandhorst S, Wei M, Hwang S, Morgan TE, Longo VD. Short-term calorie and protein restriction provide partial protection from chemotoxicity but do not delay glioma progression. Experimental gerontology 2013;48:1120-8
41. O'Flanagan CH, Smith LA, McDonell SB, Hursting SD. When less may be more: calorie restriction and response to cancer therapy. BMC medicine 2017;15:106
42. Lee C, Safdie FM, Raffaghello L, Wei M, Madia F, Parrella E, et al. Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index. Cancer research 2010;70:1564-72
54. Yuan JS, Reed A, Chen F, Stewart CN, Jr. Statistical analysis of real-time PCR data. BMC Bioinformatics 2006;7:85