The Impact of Obesity Reversal on Breast Cancer-Promoting Chronic Inflammation: The Role of Diet Type and Gut Microbiota

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Abstract

Introduction: Obesity is growing epidemic and risk factor for the development of breast cancer. Studies conducted to date have not shown a consistent relationship between weight loss and the risk of cancer development however there has been an increasing focus on calorie restriction diets and intermittent energy restriction diets and their impact on breast cancer progression. We aimed to improve the understanding of how different energy restriction diets affect obesity-associated, tumor-promoting inflammation in breast cancer and the role of gut microbiota in this inflammation.

Methods: Female C57BL/6 mice were randomized onto control diet (n = 20) and diet induced obesity diet (n = 80). At week 15, the diet induced obesity (DIO) group was randomized to four different groups: mice that remained on DIO diet, a high-carb calorie restriction diet, a low-carb calorie restriction diet, and an intermittent energy restriction diet. At week 25, mice were injected with a mixture of E0771 cells. Three to four weeks later, a final sacrifice was performed. Serum and tissue samples were collected and analyzed to determine significant differences in body composition, tumor weight and size, serum hormone and adipokine levels, and local mRNA expression of cytokines and proteins.

Results: The obese group had significantly greater body weight, visceral white adipose tissue, and more severe tumor outcomes than the Control and the calorie restriction groups (High Carb Calorie Restriction, Low Carb Calorie Restriction, and Intermittent Energy Restriction). Additionally, the obese group had significant dysfunctional changes in serum hormones and adipokines and greater expression of pro-inflammatory cytokines than anti-inflammatory cytokine. The calorie restriction groups reversed this. Lastly, the obese group presented with a more permeable gut epithelium and lower expression of colonic occludin that maintains gut impermeability. This led to increased serum lipopolysaccharides in the obese group. There was little to no reversal of these changes in the calorie restriction groups.

Conclusion: Obesity induced significant systemic and local changes in the mice that resulted in more severe tumors. The calorie restriction diets were able to reverse these changes (except for changes in the gut epithelium and serum lipopolysaccharide levels) and had less severe mammary tumors.
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I would like to dedicate this manuscript to my mom and dad, both of whom have supported me in this endeavor and for my entire life. I would not be who I am today without them.
Introduction

Obesity

Obesity is growing epidemic and health concern in the United States (US). According to the Centers for Disease Control (CDC) more than one-third of US adults are obese (based off of population data collected between 2011 and 2012)\textsuperscript{1}. The National Institutes of Health (NIH) echoes these findings, reporting that more than two-thirds of US adults are either overweight or obese\textsuperscript{2}.

Multiple factors contribute to the development of obesity, including socioeconomic status, education level, age, sex, race/ethnicity, eating and physical activity habits, and genetic heritability. Obesity is implicated in an increased risk of cardiovascular disease, type II diabetes, and several types of cancers, including esophageal, pancreatic, colorectal, and breast\textsuperscript{3}. While the direct mechanisms for how obesity increases the risk of cancer development are still being studied, several possibilities have emerged, including dysregulated leptin and adiponectin secretion, high levels of insulin-like growth factor 1 (IGF-1), and hyperinsulinemia. High levels of IGF-1, a hormone that is anti-apoptotic and promotes cell growth\textsuperscript{4}, have been consistently seen in obese individuals\textsuperscript{4} and may account for their increased risk of tumor development. Additionally, insulin resistance and subsequent hyperinsulinemia, found in obese individuals, have been associated with increased inflammation, and growth\textsuperscript{5}; all of which can contribute to increased tumor development.

Cancer

The American Society of Clinical Oncology has projected that cancer will soon outpace heart disease as the leading killer of individuals in all age groups in the US\textsuperscript{6,7}. Cancers arise when there are genetic dysfunctions within cells that promote uncontrolled
cell growth. In women, who make up about half US population, breast cancer is the second most common type of cancer. One out of every eight women in the US is expected to develop invasive breast cancer during her lifetime. Although being overweight or obese is a risk factor for breast cancer development, the relationship between the two is complex and still under investigation.

Breast cancer results from unregulated cell growth within the breast tissue, and the most common form is ductal carcinoma (cancer within the ducts of breast tissue). There are four major molecular subtypes of breast cancer: luminal A, luminal B, triple negative, and HER2 type. The triple negative subtype tends to be more aggressive than luminal A and B and has a poor prognosis. This subtype is also negative in three different receptor types: estrogen receptor, progesterone receptor, and HER2.

Inflammation may be an important mechanism linking obesity to cancer development. In obesity, increased adipose tissue can lead to secretion of increased levels of adipokines, cytokines, and chemo-attractants that promote growth and inflammation. Inflammatory cytokines like interleukin 6 (IL-6), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) are overexpressed in the adipose tissue of obese individuals. These cytokines can also be found in the local tumor environment and have been shown to drive acute inflammation, promote cell growth, and prevent apoptosis. Increased inflammation in the local tumor environment or changes in other tissue types that promote inflammation are potential mechanisms by which obesity can increase breast cancer development.

**Gut and Microenvironment**
In addition to other systemic changes, obesity is known to induce changes in the gut microenvironment and increased permeability of gut epithelium\textsuperscript{14}. Although the role of these microorganisms is not completely understood, the gut microbiota are thought to provide additional metabolic functions including: fermentation of complex polysaccharides, provision of additional energy-containing metabolites for the host to utilize, metabolism of bile acids, and metabolism of other molecules\textsuperscript{15} in the gut in cooperation with the host via “crosstalk”. This complex relationship between the gut microbiota and the host shows their interdependence. Therefore, severe systemic changes in the host, such as those that occur during obesity, can lead to changes in gut integrity and the gut microenvironment\textsuperscript{16}.

It is known that gut microbiota change in response to the state of the host, whether they are lean or obese\textsuperscript{17}. Transplantation of gut microbiota from an obese mouse into a germ-free mouse results in significant weight gain in comparison to germ-free mice transplanted with microbiota from a lean mouse\textsuperscript{18}. This demonstrates that an obesogenic diet can induce changes in the gut microenvironment that further promote weight gain.

In obese individuals, the epithelial barrier function is impaired and allows for the translocation of certain molecules, such as lipopolysaccharides (LPS), into blood. LPS has been shown to induce inflammation and promote tumor growth\textsuperscript{19,20} and could contribute to tumor-promoting inflammation in obese individuals. While the exact mechanism for how LPS diffuses into the circulation and drives tumor promotion is still under investigation, it has been shown that LPS can elicit a strong immune response and can be important in mediating chronic inflammation\textsuperscript{20}. This increased intestinal
permeability and increased serum LPS may contribute to systemic inflammation in obesity that promotes tumor development.

**Interventions to Combat Obesity**

Studies conducted to date have not shown a consistent relationship between weight loss and the risk of cancer development. While some studies support that intentional weight loss does reduce the incidence of obesity-related cancers\(^\text{21,22,23}\), others have shown no benefit. The International Agency for Research on Cancer (IARC) reported that avoiding weight gain has been shown to prevent cancer risk, but there was insufficient evidence to support the hypothesis that intentional weight loss reduces cancer risk\(^\text{24}\). Determining a direct association between intentional weight loss and cancer risk is difficult since few individuals maintain significant weight loss for a long period of time. Therefore, protective benefits of intentional weight loss in large populations are hard to study. However, calorie restriction (CR), a method for reducing weight, has been found to be protective against cancer development in mice\(^\text{25,26}\).

Diet and exercise interventions have been proposed and developed to combat and reverse obesity by primarily restricting calories. Such diet interventions/diet plans include: the Atkins diet, the Zone diet, the Ornish diet, the South Beach diet, and many others. These diets differ primarily in macronutrient distribution. However, a consistent limitation with all diet interventions is long-term adherence. An individual may lose weight initially, but due to the rigorous nature of the diet plan and whether it properly meets the needs of the individual’s behavioral, cultural, and other personal beliefs, long-term adherence may not be actualized. Therefore, the individual is likely to regain the weight they lost and potentially can gain more than their starting weight\(^\text{27,28}\).
The diet plans mentioned above are considered to be chronic or daily CR diets. While these daily CR diets have been shown to reverse the systemic perturbations induced by obesity\textsuperscript{29}, there has been an increasing focus on intermittent energy-restriction (IER) diets, where the individual has repeated sessions of severe CR interspersed with low-to-moderate CR. This type of plan has been proposed as an alternative option to chronic CR since it is more flexible and may lead to better health outcome, such as a reduction in the risk of several types of cancers including breast\textsuperscript{30}. Harvie et al (2013) compared an IER Mediterranean type diet with a daily energy restricted (DER) Mediterranean type diet in adult women with an increased risk of breast cancer but no prior breast cancer. IER diets required a 70\% CR two days per week while the diet on the remaining five days met their estimated energy requirements. Both the IER and DER diets restricted energy intake by 25\% on average. The authors found that the IER diet resulted in improved insulin sensitivity, reduced insulin resistance, and improved fat loss when compared to the DER group. This study and several others have shown that IER diets have superior short-term effects on metabolic factors when compared to DER diets and can be an alternative to more popular dieting plans\textsuperscript{30,31,32}, but long-term studies would be needed to determine their impact on breast cancer risk.
Goal and Hypotheses

Goal:

To improve the understanding of how different energy restriction diets affect obesity-associated, tumor-promoting inflammation in breast cancer and the role of gut microbiota in this inflammation.

- Aim 1: Determine the impact of different energy diets on body weights, body composition, and serum metabolic hormones.
- Aim 2: Determine the impact of different energy diets on tissue inflammation markers by measuring serum marker levels and localized, pro-inflammatory gene expression in mammary fat pads.
- Aim 3: Characterize the impact of these diets on gut microbiota composition.

Hypotheses:

- Aim 1: Compared to control, DIO will promote greater levels of serum metabolic hormones (but lower levels of adiponectin). LCCR, HCCR, and IER will reverse the effects of DIO to some degree.
- Aim 2: Compared to control, DIO will promote greater levels of tissue inflammation markers (but lower levels of IL-10). LCCR and IER will show greater reversal of DIO effects when compared to HCCR.
- Aim 3: Compared to control, DIO will promote significant changes, reducing diversity of divisions of the gut bacteria. LCCR and IER will show moderate changes with a more diverse microenvironment compared to HCCR.
Methods

Female C57BL/6 mice were randomized to receive a control diet (10% kcal from fat, n=20) or diet-induced obesity (DIO) diet (60% kcal from fat, n=80) and housed two per cage. After 15 weeks on the control or DIO diet, the control mice continued on the same diet, and the DIO mice were randomized to continue on the DIO regimen (n=20) or change to one of three CR diets (n=20/group): high carb 30% calorie restriction (HCCR), low carb 30% calorie restriction (LCCR), or intermittent energy calorie restriction (IER) for a further 10 weeks (resulting in a total of five groups: control, DIO, HCCR, LCCR, IER). Five mice/group were sacrificed at week 25 of the study (interim sac), while the remaining mice in each group (n=15) were injected with a solution of E0771 mammary tumor cells into the 4th mammary fat pad. These mice were monitored for tumor progression at least three days/week and tumor sizes were estimated using a caliper biweekly. Approximately 3-4 weeks after injection of E0771 cells, mice were sacrificed by CO₂ euthanasia plus cervical dislocation. At necropsy, mammary tumors, liver tissue, lung tissue, brain tissue, colon, blood, visceral white adipose tissue (VWAT), 4th and 9th mammary fat pads (MFP), and potential tumor metastases from the body cavity were collected. Weekly body weights and food intake were measured and recorded for the duration of the study. Stool was collected every five weeks through the end of study.

Diet type:

Table 1 describes the composition of the diets. The HCCR and LCCR diets restricted daily calories by 30% compared to ad libitum control. The IER diet restricted calories by 70% for two nonconsecutive days per week and 14% for the remaining five
days. The average daily kilocalorie intake and macronutrient distribution for IER was equivalent to LCCR.

<table>
<thead>
<tr>
<th>Diet Type</th>
<th>Percent Carbohydrate Composition</th>
<th>Percent Fat Composition</th>
<th>Percent Protein Composition</th>
<th>Percent Calorie Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70</td>
<td>10</td>
<td>20</td>
<td>0</td>
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<tr>
<td>DIO</td>
<td>35</td>
<td>45</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>HCCR</td>
<td>57</td>
<td>14</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>LCCR</td>
<td>41</td>
<td>30</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>IER</td>
<td>41</td>
<td>30</td>
<td>29</td>
<td>70 (2 days), 14 (5 days)</td>
</tr>
</tbody>
</table>

Table 1: Percent kCal distribution of diets

Weight and Tumor analysis:

Body composition/percent fat was determined using quantitative magnetic resonance. VWAT was weighed after removal during necropsy. Mammary tumors were weighed after removal and the dimensions measured in order to determine tumor volume.

Stool analysis:

Stool was collected in cryovials and stored at -80º C. Stool will be analyzed by 16S rRNA gene sequencing to determine gut microbiota diversity.

Serum analyses:

Blood was collected in 0.5 mL microcentrifuge tubes from mice at the time of sacrifice. After allowing the samples to clot for at least 30 minutes at room temperature, samples were centrifuged and sera collected and stored at -80º C. Serum levels of
hormones, adipokines, and cytokines were analyzed by Bio-Plex Multiplex Immunoassay (Bio-Rad), as per the manufacturer’s protocol for these assays.

mRNA expression levels (occludin, tight junction proteins in murine colon tissue):

During necropsy, colon tissue was harvested and frozen at -80°C. RNA was isolated from colon tissue using TRIzol reagent (ThermoFisher), and cDNA was synthesized via High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative mRNA levels of occludin and ZO-1 (tight junction protein 1) were quantified by real-time qPCR using Taqman™ Gene Expression Assays and a ViiA™ 7 Real-Time PCR System (Applied Biosystems). Relative mRNA levels of IL-6, IL-1β, IL-10, and TNF-α were measured in the 9th MFP, distal to tumor, using the same methods.

Serum Endotoxin

A Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific) was used to quantify serum endotoxin levels.

Statistical analysis:

A one-way ANOVA test (using the program GraphPad Prism 6) was used to determine the significant differences between the five groups, at a significance level of p < 0.05.
Results

Body Weight, Percent Body Fat, and VWAT Analysis:

The DIO group weighed significantly more than Control and the three CR groups (all p values < 0.0001). Mean body weights in the CR groups were significantly less than Control (all p values < 0.0001). However, there was no difference in weight between the three CR groups (Figure 1).

The DIO group had the greatest percentage of body fat (mean body fat percentage = 52.02%) and was significantly greater than Control (mean body fat percentage = 24.73%) and the three CR groups (mean HCCR = 13.91%, mean LCCR = 15.91%, mean IER = 11.49%) (all p values less than 0.0001). The HCCR, LCCR, and IER had significantly less body fat than Control (p = 0.0002, p = 0.0029, p < 0.0001, respectively). However, the three CR groups did not differ significantly in body fat percentage from each other (Figure 2a).

Following the trend for weight and body fat percentage, the DIO group’s VWAT weighed significantly more than Control (p < 0.0001), as well as the three CR groups (all p values < 0.0001). Control did not differ significantly from the three CR groups (Figure 2b).
Figure 1. Weekly body weights for all groups up to week 27. Different letters indicate significant differences, p < 0.05.

2a. Figure 2. Body fat percentage (a) and VWAT weight (b) at time of necropsy. Different letters indicate significant differences, p < 0.05.

Tumor progression and invasion, final tumor volume, and final tumor weight:

The DIO group progressed significantly faster compared to Control and the CR groups and the DIO tumors were estimated to be significantly larger when palpated at three weeks after tumor injection (p < 0.0001). Control was significantly larger than
HCCR (p = 0.0414) and IER (p = 0.0268), but not different from LCCR at this time point. However, the three CR groups were not significantly different from each other (Figure 3).

At the time of necropsy, incidence of tumor body wall invasion was assessed. Control had a similar incidence of tumor body wall invasion (64.286%) compared to DIO (60%). However, the CR groups had a much lower incidence of tumor body wall invasion (HCCR: 14.286%, LCCR: 6.667%, IER: 6.667%) than both Control and DIO (data not shown).

![Final Tumor Volume Week 3 In Vivo](image)

Figure 3. Tumor growth since injection of E0771 cells at week 25. Different letters indicate significant differences, p < 0.05.

DIO, on average, had significantly greater tumor volumes and weight than Control (p = 0.0002 and p < 0.0001, respectively) and the three CR groups (all p values < 0.0001). Tumor volume and weight in the Control group were significantly different from HCCR (p = 0.0326 and p = 0.0092, respectively) and IER (p = 0.0233 and p = 0.0106, respectively) but not from LCCR. The three CR groups did not differ from each other in final tumor volume or weight (Figure 4).
Figure 4: Final tumor volume (a) and final tumor weight (b) at time of necropsy.
Different letters indicate significant differences, p < 0.05.

Serum IL-6, Hormones and Adipokines analysis:

DIO had higher serum levels of IL-6 than Control (p = 0.0007), HCCR (p = 0.0026), LCCR (p = 0.0086), and IER (p = 0.0157). Control did not have significantly different IL-6 levels than the three CR groups and nor did the three CR groups differ from each other (Figure 5).

Figure 5: Serum IL-6 levels. Different letters indicate significant differences, p < 0.05.
Control, DIO, and HCCR serum adiponectin levels did not differ significantly from each other. However, Control differed from LCCR (p = 0.0292) and IER (p = 0.0061). DIO did not significantly differ from LCCR but had significantly lower adiponectin levels than IER (p = 0.0292). There were no significant differences between the three CR groups (Figure 6a).

DIO serum leptin levels were significantly greater than Control and the CR groups (all p value < 0.0001). However, Control and the three CR groups did not significantly differ from each other in serum leptin (Figure 6b).

Figure 6: Serum adiponectin levels (a) and serum leptin levels (b). Different letters indicate significant differences, p < 0.05.

DIO serum insulin levels were significantly greater than Control (p = 0.0221), HCCR (p = 0.0201), LCCR (p = 0.0019), and IER (p = 0.008). However, Control did not significantly differ from the three CR groups (Figure 7a). Additionally, DIO serum IGF-1 levels were significantly greater than Control (p = 0.0057) and the CR groups (p value <
However, Control and the three CR groups did not significantly differ from each other in serum IGF-1 (Figure 7b).

![Figure 7a: Serum insulin levels](image)

![Figure 7b: Serum IGF-1 levels](image)

Figure 7: Serum insulin levels (a) and serum IGF-1 levels (b). Different letters indicate significant differences, p < 0.05.

DIO had significantly greater serum levels of resistin than HCCR (p = 0.0132), LCCR (p = 0.0051), and IER (p = 0.0030), however DIO did not differ significantly from Control. Additionally, Control levels were not significantly different from the three CR groups in serum resistin (data not shown).

DIO GIP levels were significantly greater than Control (p = 0.0012), HCCR (p = 0.0171), LCCR (p = 0.0036), and IER (p = 0.0123) groups. The three CR groups did not significantly differ from each other and did not differ from Control in serum GIP levels (Figure 8)
Figure 8: Serum GIP levels. Different letters indicate significant differences, \( p < 0.05 \).

Mammary Fat Pad cytokine analysis:

DIO had significantly greater IL-6 cytokine expression in the 9\textsuperscript{th} MFP (distal to tumor) when compared to Control (\( p = 0.0204 \)), HCCR (\( p = 0.0018 \)), LCCR (\( p = 0.0129 \)), and IER (\( p = 0.0009 \)). However, there was no significant difference in IL-6 gene expression between Control and the three CR groups (Figure 9a).

DIO had significantly greater TNF-\( \alpha \) expression in the 9\textsuperscript{th} MFP (distal to tumor) when compared to HCCR (\( p = 0.0002 \)), LCCR (\( p = 0.004 \)), and IER (\( p = 0.0021 \)). However, DIO and Control TNF-\( \alpha \) levels did not significantly differ from each other and nor did Control differ significantly from the three CR groups. The three CR groups did not differ from each other in relative TNF-\( \alpha \) expression (Figure 9b).

DIO had significantly greater IL-1\( \beta \) expression in the 9\textsuperscript{th} MFP (distal to tumor) when compared to Control (\( p = 0.0112 \)), HCCR (\( p = 0.0001 \)), and IER (\( p = 0.0002 \)). However there was no difference in DIO and LCCR IL-1\( \beta \) expression. As well, Control did not differ significantly from the three CR groups and nor did the CR groups differ significantly from each other (Figure 9c).
DIO had significantly lower IL-10 cytokine expression in the 9th MFP (distal to tumor) when compared to Control ($p = 0.0055$) and HCCR ($p = 0.0193$). There was no significant difference in IL-10 gene expression between Control and the three CR groups. DIO did not significantly differ in IL-10 expression from LCCR, and IER. Lastly, the three CR groups did not differ from each other in relative IL-10 expression (Figure 9d).

Figures 9. Relative IL-6 (a), TNF-α (b), IL-1β (c), and IL-10 (d) expression in the 9th mammary fat pad. Different letters indicate significant differences, $p < 0.05$. 
Tight Junction Proteins, Occludin, and Serum LPS:

There were no significant differences between the five groups in the relative gene expression of ZO-1 in the colon (data not shown).

Control had greater occludin expression in the colon than DIO (p = 0.0258), HCCR (p = 0.0002), and IER (p = 0.0081). Control did not differ significantly from LCCR and nor did DIO differ significantly from HCCR and IER. The three CR groups did not significantly differ from each other in relative occluding expression (Figure 10a).

Control serum LPS levels were significantly lower than DIO (p < 0.0001), HCCR (p = 0.0033), and IER (p = 0.0331). However, there was no significant difference in LPS levels between Control and LCCR. DIO LPS levels were significantly greater than LCCR (p = 0.0032) but were not significantly different from HCCR, or IER. The three CR groups did not differ from each other in serum LPS (Figure 10b).

![Figure 10](image)

Figure 10: Relative mRNA occludin expression (a) in colon tissue and serum LPS (b). Different letters indicate significant differences, p < 0.05.
Discussion

The purpose of this study was to determine the effects of obesity and obesity-reversing energy restricted diets on breast cancer development and the gut microenvironment. Our study replicated some known diet effects. For example, our DIO model exhibited known characteristics of obesity\(^\text{33}\), such as: significantly greater body weights, greater body fat percentages, greater average VWAT weight, higher serum GIP, hyperinsulinemia, and hyperleptinemia. Additionally, our CR groups displayed known CR characteristics, such as a reduction in mammary tumor growth in rodent models\(^\text{34,35}\). HCCR has previously been shown to reduce body weight and serum levels of metabolic hormones such as leptin, insulin, and IGF-1\(^\text{36}\). LCCR has also been shown to reduce body weight, serum insulin levels, and serum IGF-1 levels, and increase serum adiponectin levels\(^\text{36,37}\). Lastly, IER diets have also been shown to reduce serum insulin levels and body fat\(^\text{30}\). Our study design not only replicated known diet effects but also allowed for concurrent comparison of the effects of several diet interventions, in one novel study design.

Overall, we observed that obesity resulted in dysfunctional perturbations in serum metabolic hormone levels and enhanced severity of tumor outcomes. Significant reversal of DIO effects on serum metabolic hormones and adipokines, such as IGF-1, leptin, GIP, resistin, and insulin, was achieved with all three CR diets. The CR groups improved Control and DIO’s serum adiponectin levels by increasing them. The three CR groups also reduced tumor volume, tumor weight, and tumor invasiveness. Our results also showed that DIO induced a pro-inflammatory local environment in the MFP by stimulating increased expression of pro-inflammatory cytokines\(^\text{38,39}\) (i.e. IL-6, TNF-\(\alpha\), IL-
1β) and decreased expression of the anti-inflammatory cytokine IL-10\(^{40,41}\). The three CR diets appear to have reversed this inflammation to some degree. Since there were few significant differences amongst the three CR groups in the reversal of hormonal secretion and inflammation, we concluded that macronutrient composition of the weight loss diet may not be important.

We also observed that DIO induced significant changes in the gut such as a reduction in the mRNA expression of occludin. However, the three CR diets did not reverse this change. Conversely, there were no significant differences in ZO-1 (tight junction protein) mRNA expression between any of the groups. Occludin is critical for the regulation of tight junction proteins, for maintenance of the integrity of these proteins, and for preventing paracellular permeability of the gut epithelium. It has been noted with increasing evidence that absence of occludin in gut epithelium does not prevent the formation of tight junctions proteins but does increase permeability\(^{42,43}\). With our observations, we concluded that the effects of obesity on occludin expression may not be easily reversed by weight loss.

Serum LPS levels were higher in those groups that had lower expression of occludin (i.e. DIO, HCCR, LCCR, IER), providing further support that occludin is necessary for maintaining gut integrity and for preventing the translocation of LPS from the gut lumen to blood. Despite the evidence that supports LPS as strong initiator for inflammation, in this study, increased serum LPS in the CR groups did not appear to promote increased systemic or local MFP inflammation. This suggests that elevated serum LPS levels may not play a major role in promoting systemic or local inflammation, which can enhance breast cancer development.
In our model, the dysfunctional hormone levels could be a potential mechanism for how obesity enhances tumor development. Since GIP is known to be an “obesity hormone”, facilitating the accumulation of triacylglycerol into adipose tissue\textsuperscript{44,45}, this additional adipose seen with increased GIP levels could also promote a pro-inflammatory environment characteristic in obesity and cancer. The dysregulation of IGF-1 secretion seen under obesogenic conditions has been found to increase the risk and development of breast cancer\textsuperscript{46}, especially in pre-menopausal women. The increased secretion of IGF-1 observed in this study, together with the inflammatory environment induced by obesity may lead to tumor promotion. Therefore, CR diets may be more effective in reversing systemic hormonal perturbations, and may result in a greater/more effective reduction in the severity of breast cancer than control diets.

The increased local MFP inflammation seen in our study may be another mechanism by which obesity enhances the severity of tumor development. The increased IL-6, TNF-α, and IL-1β and decreased IL-10 observed in our study indicates the tumor-promoting effects of obesity. While the reversal of these cytokine levels by CR diets may result in a more effective reduction of breast cancer severity.

Although sample analysis is still ongoing, from our data thus far we have shown that CR is effective in reversing systemic hormonal changes and local inflammation induced by obesity. However, the CR diets may not be effective in reversing obesity-induced changes in the gut because of the persistant lower occludin levels and higher serum LPS. Further analysis and rRNA sequencing of the stool collected is needed to clarify the extent of gut microenvironment changes induced by diet type. Additional
studies are needed to determine whether there is a relationship between CR diet composition, breast cancer development, and the gut microenvironment

**Conclusion**

Based on our results, we can conclude that calorie restricted diets, of varying macronutrient composition and schedule, effectively reverse the mammary tumor promoting effects of chronic obesity.
WORKS CITED


