Published in final edited form as:

Cancer Prev Res (Phila). 2011 April ; 4(4): 568–581. doi:10.1158/1940-6207.CAPR-10-0140.

Effect of Chronic and Intermittent Calorie Restriction on Serum Adiponectin and Leptin and Mammary Tumorigenesis

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Abstract

The effect of chronic (CCR) and intermittent (ICR) caloric restriction on serum adiponectin and leptin levels was investigated in relation to mammary tumorigenesis. 10-wks old MMTV-TGF- α female mice were assigned to ad libitum-fed (AL) (AIN-93M diet), ICR (3-wks 50% caloric restriction, AIN-93M-mod diet, 2x protein, fat, vitamins, and minerals followed by 3-wks 100% AL consumption of AIN-93M), and CCR (calorie and nutrient intake matched for each 6-wks ICR cycle, ~75% of AL) groups. Mice were sacrificed at 79 (end of restriction) or 82 (end of refeeding) wks of age. Serum was obtained in cycles 1, 3, 5, 8, 11 and terminal. Mammary tumor incidence was 71.0%, 35.4%, and 9.1% for AL, CCR and ICR mice, respectively. Serum adiponectin levels were similar among groups with no impact of either CCR or ICR. Serum leptin level rose in AL mice with increasing age but was significantly reduced by long-term CCR and ICR. The ICR protocol was also associated with an elevated adiponectin:leptin ratio. In addition, ICR-Restricted mice had increased mammary tissue AdipoR1 expression and decreased leptin and ObRb expression compared to AL mice. Mammary fat pads from tumor-free ICR-mice had higher adiponectin expression than AL and CCR mice while all tumor-bearing mice had weak adiponectin signal in mammary fat pad. Although we did not demonstrate an association of either adiponectin or leptin with individual mice in relation to mammary tumorigenesis, we did find that reduced serum leptin and elevated adiponectin:leptin ratio were associated with the protective effect of intermittent calorie restriction.

Keywords

Breast cancer; Animal models of cancer; Diet and cancer; Adiponectin; Leptin; Intermittent Restriction/Refeeding

Introduction

Epidemiological and preclinical rodent studies suggest an important, but still controversial role of adipose tissue mass in mammary tumorigenesis (1–9). Factors produced directly in adipose tissue, adipokines, in particular leptin and adiponectin, are now recognized for their influence on breast cancer risk and mammary tumor biology (10–13). Their physiological and pathological relationships are largely in opposition to each other, as are their biological effects on mammary and breast cancer cells. For example, leptin stimulates the proliferation of ER-positive breast cancer cell lines (14–17) while results for ER-negative breast cancer cell lines are less consistent (17–19). In contrast studies using human breast cancer cell lines indicate that adiponectin inhibits proliferation of a number of ER-positive and ER-negative breast cancer cell lines (20–23). Also, leptin receptors (ObRb and ObR) (14,15,17,24); and

adiponectin receptors 1 (Adipo R1) and 2 (Adipo R2) as well as adiponectin (20,22,23,25) itself have been found to be expressed in a number of human breast cancer cell lines.

In addition to *in vitro* studies indicating that human breast cancer cells respond to these adipokines, leptin, adiponectin and their receptors have been identified in breast/mammary tumors and mammary tissues of humans and rodents (20,25–29). Analyses of human tissue biopsies revealed that leptin and its receptor are overexpressed in breast tumors compared to non-cancer breast epithelium and the expression of leptin is positively correlated with expression of the leptin receptor in breast carcinoma cells (26,27,30,31). With respect to adiponectin, Karaduman *et al* reported that adiponectin levels measured by ELISA were significantly higher in mammary tissue obtained from breast cancer patients than in this tissue obtained from healthy control subjects (29). In another study adiponectin mRNA expression level was significantly higher in mammary tissue adjacent to breast tumors compared to either breast tumor tissue or to control tissue from subjects without breast cancer, but AdipoR1 mRNA expression level in mammary tissue adjacent to the breast tumor was similar to the tumor itself. However, AdipoR1 level in a number of breast tumors was higher than in control mammary tissue obtained from an individual without breast cancer, while there were no differences in AdipoR2 expression levels among control, adjacent and tumor tissues (20). In the MMTV-TGF- α transgenic mouse which develops hormone responsive mammary tumors, ObRb was found to be expressed in both mammary fat pad and mammary tumors (28).

A number of studies have evaluated serum leptin and adiponectin levels in women with breast cancer. Results for leptin have been inconsistent (32–44). However, serum adiponectin levels have been reported to be lower in women with breast cancer compared to controls (20,36,38,43,45–47). In addition it was reported that lower serum adiponectin (47) and higher leptin (44) levels are associated with higher mammary tumor grade.

Under normal circumstances higher serum leptin levels are associated with increased body weight and body fat in humans and rodents (8,28,48–54), while in contrast adiponectin levels are lower at higher body weights (49,50,55,56). Interventions which result in weight loss have been reported to “normalize” these circulating factors such that leptin is reduced and adiponectin increased. With increasing body weight the relative relationship of these two adipokines becomes more divergent. Thus, dependent upon body weight status tissues would be exposed to very different relative amounts of these two proteins. For example, we recently reported that mice with goldthioglucose-induced obesity had adiponectin:leptin ratio 10-fold less than did lean mice (57). There are two studies related to adiponectin:leptin ratio in women with breast cancer. Chen and coworkers found that women with breast cancer had increased ratio of leptin:adiponectin (conversely a decreased adiponectin:leptin ratio). In another report the adiponectin:leptin ratio was calculated using average values and was found to be 15 % higher in age-matched controls than in postmenopausal breast cancer subjects, although for premenopausal women the average adiponectin:leptin ratio was 30 % lower in age-matched controls than in women with breast cancer (43). These results suggest that the balance of adiponectin to leptin rather than either adiponectin or leptin levels alone may play important roles in the development of cancer.

In general these cited studies obtained blood samples at the time of breast cancer diagnosis thus the findings are not definitive for adiponectin and leptin involvement in breast tumor development, i.e, the serum changes may reflect physiological response to the presence of the tumor and/or the tumor itself produces the adipokines. In humans a long-term prospective study would be very difficult and expensive to undertake. Thus, the use of a relevant preclinical model would provide the opportunity to assess the role of serum adipokines in mammary tumorigenesis. We have utilized MMTV-TGF- α mice as a model

for human breast cancer as tumors develop primarily in the second year of life and are considered to be hormone responsive (58,59).

We have reported that calorie restriction prevents and delays tumor development in MMTV-TGF- α mice (54,60,61). An interesting aspect of these calorie restriction studies has been the consistent finding that when calories are restricted intermittently this protocol is more protective than the same degree of restriction implemented by chronic restriction. The aim of the present study was to determine if serum leptin and/or adiponectin play a role in the protective effect of calorie restriction in relationship to the manner in which calories are reduced. Longitudinal blood samples were obtained at specific ages over the course of the study as well as at the terminal end point. Thus, whether serum adipokine levels were able to predict mammary tumors could be addressed. Additionally, the expression of proteins associated with the signaling of leptin and adiponectin was measured in mammary tissue, mammary fat pad, and mammary tumors.

Material and Methods

Animals and Study Design

MMTV-TGF- α female mice overexpressing human TGF- α were produced at the Hormel Institute (Austin, MN, USA) and genotyped as previously described (62). At 8 weeks of age mice were randomized and assigned to one of the following dietary groups: AL (N=75), CCR (N=75), and ICR (N=75). They were housed individually and provided ad libitum access to water and powdered AIN-93M diet for two weeks to allow accommodation to the powdered diet. At 10 weeks of age mice began to consume their assigned diets. Those in the AL group continued to have free access to AIN-93M diet. Mice in the CCR group were given a diet formulated to be isocaloric with the AIN-93M diet with 25% increases in protein, vitamin, mineral and fat content. This diet was given at 75% of age-matched ad-libitum consumption. Mice in the ICR group were provided a modified AIN-93M diet with 2-fold increases in protein, vitamin, mineral and fat content, and fed at 50% of the consumption level of AL mice during each 3-weeks of restriction. Following each restriction period ICR mice were provided with AIN-93M diet at 100% of age-matched AL consumption for 3 weeks. The diet compositions have been previously described in detail (62,63). This approach results in all mice receiving the same absolute intakes of all nutrients except carbohydrate during each six week cycle. Further, the only difference between CCR and ICR mice is the manner in which the overall restriction of 25% is implemented. Over the course of the study food intakes and general condition of each mouse were determined daily. Each mouse was weighed weekly and at that time mice were palpated for mammary tumors. Once a tumor was detected, the mouse was closely monitored for signs of tumor-related distress. Mammary tumors were measured weekly with calipers. All mice were euthanized by CO₂ overdose when they reached the predetermined terminal age of either 79 weeks (end of restriction) or 82 weeks (end of refeeding) or when mammary tumor size exceeded 20 mm in length or weight loss exceeded 25% from the previous week. When results are presented specific to the ICR mice during restriction periods they are further classified as ICR-Restricted and during refeeding periods they are classified as ICR-Refed. The Hormel Institute Animal Facility is accredited by AAALAC. The University of Minnesota IACUC approved the study and procedures.

Tissue Sample collection and Histopathological analysis

At sacrifice, fat pads (mammary, retroperitoneal and parametrial), mammary tissues from the back of the neck and axillary areas, livers, mammary tumors and any abnormalities were removed and weighed. A sample of each tissue was placed in 10% neutral buffered formalin. The remaining tissues were stored at -70°C. Left mammary fat pads, mammary

tissues or mammary tumors and tissue samples that appeared abnormal were sent to the Department of Pathology and Laboratory Medicine of the Mayo Foundation (Rochester, MN, USA) for histopathological analyses to determine malignancy and/or disease status.

Assessment of Serum Leptin and Adiponectin Concentrations

Blood samples were collected from the orbital sinus from all mice at the terminal points and over the study (cycles 1, 3, 5, 8, and 11 from three cohorts corresponding to the first, second and third weeks of restriction and refeeding of ICR mice). For each mouse two samples were obtained per cycle such that for cohort one samples were obtained corresponding to the first week of restriction and the first week of refeeding (week 1 and 4 of the cycle), for cohort two after two weeks of restriction and two weeks of refeeding (week 2 and 4 of the cycle) and cohort three after three weeks of restriction and three weeks of refeeding (week 3 and 6 of the cycle). Serum samples were stored in -20°C until used. Leptin was measured using the Mouse Leptin ELISA kit (EZML-82K, Linco Research, St. Charles, MO). Serum Adiponectin levels were determined using a Mouse Adiponectin ELISA kit (EZML-60K, Linco Research, St. Charles, MO). Adiponectin/leptin ratio was calculated for each mouse by dividing adiponectin serum concentration by leptin concentration.

Western Blot Analysis

Tissue samples (mammary tissue from tumor-free mice, mammary tumors, and mammary fat pad) were homogenized in extraction buffer with protease inhibitors. Total protein was extracted using a Total Protein Extraction Kit (Chemicon International, Temecula, CA) and quantified by the Bradford assay. Extracted proteins were electrophoresed on 4%-15% polyacrylamide gradient gels and then blocked in Tris-Base solution containing 5% milk concentrate and 1% Tween-20 and then transferred to a Immobilon membranes (Millipore, Bedford, MA) and probed using primary antibodies for leptin (Ob) (Santa Cruz Biotechnology, Santa Cruz, CA), leptin long (ObRb) and total (ObR) (Santa Cruz Biotechnology, Santa Cruz, CA) forms of the leptin receptors and for adiponectin (Pro Sci Incorporated, Poway, CA), adiponectin receptor 1 (Adipo R1) (Santa Cruz Biotechnology, Santa Cruz, CA), and adiponectin receptor 2 (Adipo R2) (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-rabbit (for Ob, ObR, ObRb, and adiponectin) (Cell Signaling, Danvers, MA) or anti-goat (for Adipo R1 and AdipoR2) (Santa Cruz Biotechnology, Santa Cruz, CA) immunoglobulin G (IgG) were used as a secondary antibody. Antibody-bound proteins were detected by enhanced chemifluorescence (ECF substrate; Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed with the Storm 840 Machine Imaging System (Molecular Dynamics, Sunnyvale, CA, USA). Standard molecular weight markers were run simultaneously for comparing molecular weights of the visualized proteins. The intensity of Western blot bands was quantified by densitometric analysis using the *ImageJ* program. Results were expressed as the ratio of intensity of the protein of interest to that of β -actin (Delta Biolabs LLC, Gillingham, CA) from the same sample.

Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Serum data were analyzed by ANOVA followed by Neuman-Keuls test or t-test. Mammary tumor incidence was analyzed by the "Chi-square" test and two-group log-rank test. Graph Pad Prism version 4 was used for statistical analyses (GraphPad Software, //www.graphpad.com/).

Results

Mammary Tumor Incidence, Body and Tissue Weights and Terminal Serum Measurements

The data presented here are part of an extensive study of serum factors potentially involved in mammary tumor prevention resulting from different modes of calorie restriction. In our first paper (61) we focused on the IGF-I axis and we also fully described tumor characteristics for the AL, CCR and ICR groups. The mammary tumor incidence was 71.0%, 35.4%, and 9.1% for AL, CCR and ICR mice ($P < 0.0001$), respectively and all groups were significantly different from each other (Figure 1A). In addition the age at which mammary tumors were detected (Figure 1B) was extended from 61 weeks of age (± 1.1) in AL mice to 69 (± 1.6) and 67 (± 4.2) weeks of age in CCR and ICR mice respectively although only the CCR value was significantly different from AL mice ($P < 0.005$).

While in our previous paper we also completely described body weight changes, here we do present final body weights (Figure 2Aa) as well as mammary (Figure 2Ab) and internal fat pads weights (Figure 2Ac). It can be seen that AL mice were the heaviest compared to all other groups, followed by CCR ($P < 0.001$) and ICR-Refed ($P < 0.001$) with similar weights while ICR-restricted mice weights were the lightest ($P < 0.001$). In addition, ICR-Restricted mice had significantly lower body and fat pad weights than CCR ($P < 0.01$) and ICR-Refed ($P < 0.05$) mice. There were no significant differences between ICR-Refed and CCR mice for any of these measurements. During each weight loss/regain cycle, food intake for both restricted groups was 22.9–28.7% lower than that of AL mice (not shown) and food intakes of the CCR and ICR groups as per the experimental design were almost identical during each food restriction/refeeding cycle (data not shown).

Here we used serum and tissues from these mice with the focus on serum adiponectin and leptin levels in relationship to mammary tumor development as well as assessment of protein expression of these adipokines and their receptors in mammary tissues and tumors. At euthanasia AL mice had serum leptin levels (Figure 2Ba) significantly higher than CCR ($P < 0.01$), ICR-Refed ($P < 0.001$), and ICR-Restricted ($P < 0.001$) mice. CCR mice had significantly higher serum leptin values than ICR-Refed ($P < 0.05$) and ICR-Restricted ($P < 0.01$) mice. There were no significant differences for serum leptin levels between ICR-Refed and ICR-Restricted mice. There was no statistical difference ($P > 0.05$) for terminal adiponectin concentration among the groups (Figure 2Bb). We also calculated serum adiponectin:leptin ratios and found that at euthanasia ICR-Restricted mice had an adiponectin:leptin ratio significantly higher than CCR ($P < 0.01$), ICR-Refed ($P < 0.01$) and AL ($P < 0.01$) mice. There were no significant differences in adiponectin:leptin ratio between ICR-Refed, CCR and AL mice (Figure 2Bc). Leptin (Figure 2Ca) and adiponectin (Figure 2Cb) serum concentrations were not significantly different for AL, CCR, ICR-Restricted and ICR-Refed mice that eventually developed mammary tumors compared to those that did not. Terminal adiponectin:leptin ratio of ICR-Restricted mice with mammary tumors was significantly higher ($P < 0.05$) than ICR-Restricted mice without tumors, however AL, CCR and ICR-Refed mice exhibited no differences in adiponectin:leptin ratio between tumor-bearing and tumor-free mice (Figure 2Cc).

Correlation between weight parameters and terminal serum leptin, adiponectin or adiponectin:leptin ratio are presented in Table 1. At euthanasia serum leptin concentration of all mice was positively correlated with terminal body weights, total fat pad weights and mammary fat pad weights. In contrast, terminal serum adiponectin concentration was not correlated with terminal body weights, total fat pad weights and mammary fat pad weights. It was found however, the terminal adiponectin:leptin ratio was negatively correlated with body weights, total fat pad weights and mammary fat pad weights.

Adiponectin, AdipoR1, AdipoR2 and Leptin, ObR, ObRb protein expression in Mammary Tissue and Tumors; Adiponectin and Leptin protein expression in Mammary Fat Pads

A summary of the results of expression of proteins of interest for mammary tissues, tumors and fat pads is presented in Table 2 and representative western blots are shown in Figure 3. Mammary tissue protein expression of adiponectin was not different among the groups. AdipoR1 expression was somewhat higher in mammary tissue obtained from ICR mice but only the ICR-Restricted mice value was significantly different ($P<0.05$) compared to that of AL mice. There was not a significant difference in AdipoR2 expression among any of the groups. Expression of leptin was lower in all calorie restricted mice compared to AL mice but was only significant for CCR ($P<0.05$) and ICR-Restricted ($P<0.05$) mice, and there was no significant difference in leptin expression among ICR-Refed, CCR and ICR-Restricted mice. ObR expression was significantly lower in ICR-Refed mice ($P<0.05$) compared to AL mice but there was not a significant difference in ObR expression in mammary tissue among AL, CCR, and ICR-Restricted mice and also among ICR-Restricted, CCR, ICR-Refed groups. ObRb expression was significantly lower in ICR-Restricted mice ($P<0.05$) compared to AL mice and values from all calorie restricted mice were similar.

In mammary tumors expression levels of adiponectin, AdipoR1 and leptin were not significantly different among the groups. AdipoR2 expression was significantly higher in mammary tumors from ICR-Restricted mice ($P<0.05$) compared to tumors from AL, CCR and ICR-Refed mice. ObR and ObRb proteins were not detected in mammary tumors from CCR and ICR-Refed mice and were expressed in only one of three tumors from ICR-Restricted mice. For AL mice all of the tumors expressed ObRb but only two of five tumors expressed ObR.

In mammary fat pads of mice without mammary tumor adiponectin expression tended to be higher for both ICR-Restricted ($P<0.05$) and ICR-Refed mice compared to AL and CCR mice although the ICR-Refed value was not significantly different from these two groups. In contrast, adiponectin protein expression was only detected in mammary fat pad of one ICR-Refed mouse with no expression in the other three groups of tumor-bearing mice. Leptin expression in mammary fat pads for both tumor-free and tumor-bearing mice was not statistically different either within or among the groups.

Longitudinal Adipokines Serum Levels

One of the goals of the present investigation was to prospectively evaluate serum concentrations of the adipokines, leptin and adiponectin. Blood samples were obtained in cycles 1, 3, 5, 8, and 11. During each cycle cohorts of mice had blood samples obtained at weeks 1 and 4, 2 and 5 and 3 and 6. For AL and CCR mice there were no differences in values obtained over the six week periods and these results were combined. Data for AL and CCR mice are presented separately for those which eventually developed mammary tumors from those that did not by the time the study was terminated. For ICR mice results were combined within each three weeks of restriction or three weeks of refeeding and presented separately for restriction and refeeding periods as well as for mice which eventually developed mammary tumors compared to those that did not.

As shown in Figure 4Aa serum adiponectin concentrations were not significantly different for AL mice that eventually developed mammary tumors compared to those that did not. While serum adiponectin level of tumor-free CCR mice appeared to increase to some degree between Cycle 1 and 3, tumor-bearing CCR mice continued to have a rise in adiponectin values until Cycle 8 (Figure 4Ab). Interestingly, tumor-bearing mice compared to those without mammary tumors from both CCR [Cycle 1 ($P<0.03$), Cycle 5 ($P<0.04$) and Cycle 8 ($P<0.05$)] and ICR [ICR-Restricted (Cycle 3 ($P<0.03$), ICR-Refed (Cycle 1 ($P<0.03$), Cycle

3 ($P<0.04$)] groups (Figures 4Ac and 4Ad) tended to have higher adiponectin levels prior to when tumors were eventually palpated in Cycle 10. In Cycle 11 CCR mice with tumors had similar adiponectin levels as did tumor-free CCR mice, while ICR-Restricted tumor-bearing mice continued to have significantly higher adiponectin values than mice without mammary tumors ($P<0.04$).

Prospective serum leptin concentrations for AL (Figure 4Ba) and CCR (Figure 4Bb) mice with or without mammary tumors increased from Cycle 1 to Cycle 8. In Cycle 11 values were slightly decreased in both AL groups and in tumor-free CCR mice compared to earlier time points, whereas the level of leptin for CCR mice with mammary tumors continued to increase. Interestingly, at all time points AL mice without mammary tumors had higher serum leptin levels than AL mice with mammary tumors with values significantly different in Cycle 1 ($P<0.001$), Cycle 5 ($P<0.05$), Cycle 8 ($P<0.05$) and Cycle 11 ($P<0.05$). In contrast to the AL group, CCR mice which developed mammary tumors had higher leptin values than mice which did not develop mammary tumors (statistical significance in Cycle 3 ($P<0.02$), Cycle 5 ($P<0.05$) and Cycle 11 ($P<0.0001$)). There is one more important difference between AL and CCR groups across the study (except mice with mammary tumors in Cycle 1), i.e., leptin concentrations for AL mice were consistently significantly ($P<0.001$) higher than for CCR mice. The mean value of leptin concentration for AL group was 1.76 ± 0.43 ng/ml in Cycle 1 and increased to 28.29 ± 3.08 ng/ml in Cycle 11, whereas CCR mice increased leptin concentration from 1.34 ± 0.34 ng/ml in Cycle 1 to only 6.84 ± 0.89 ng/ml in Cycle 11. Higher values of leptin for AL compared with CCR mice included those with and without mammary tumors.

Serum leptin concentrations of ICR mice were reduced by restriction compared to refeeding for most of the cycles whether or not mammary tumors developed (Figures 4Bc and 4Bd) although only four ICR mice did so. Leptin values were significantly different between restriction and refeeding for ICR mice in Cycle 5 ($P<0.02$), Cycle 8 ($P<0.05$) and Cycle 11 ($P<0.05$) for tumor-free mice and in Cycle 1 ($P<0.03$), Cycle 8 ($P<0.05$) and Cycle 11 ($P<0.05$) for tumor-bearing mice. With respect to the concentration of leptin in relation to the development of mammary tumors, in the first half of study (Cycles 1 and 3) ICR mice that eventually developed mammary tumors had higher leptin concentrations in the corresponding restriction or refeeding period than mice that did not develop tumors. In contrast to the early time-points, later in the study in Cycle 8 prior to tumor detection by palpation in Cycle 10 and later when tumors were growing in Cycle 11 in both restriction (Figure 4Bc) and refeeding (Figure 4Bd) periods mice that did not develop mammary tumors had significantly ($P<0.05$) higher leptin levels than those later confirmed to be tumor-bearing.

Results for the adiponectin:leptin ratio over the course of the study are presented in Figure 4Ca–4Cd. AL mice had the highest adiponectin:leptin ratio in Cycle 1 regardless of eventual tumor status (Figure 4Ca). Values of adiponectin:leptin ratio for AL mice declined with increasing age. At all ages the adiponectin:leptin ratio was significantly higher in AL mice which developed mammary tumors than in AL mice which remained tumor-free ($P<0.03$ in Cycle 1, $P<0.0001$ in Cycle 3, $P<0.0001$ in Cycle 5, $P<0.02$ in Cycle 5, $P<0.0003$ in Cycle 11). In contrast, there was no dramatic age-related change of the adiponectin:leptin ratio for CCR mice (Figure 4Cb), and there was little consistent difference between CCR mice which developed mammary tumors and those that did not, except in Cycle 8 when CCR tumor-bearing mice had significantly higher ($P<0.05$) adiponectin:leptin ratio than did mice without mammary tumor (Figure 4Cb). The adiponectin:leptin ratio of ICR mice depended upon the feeding regimen, i.e., regardless of whether or not they developed mammary tumors the adiponectin:leptin ratio was in almost all cases significantly higher in restriction than in refeeding periods (Figure 4Cc and 4Cd). As to differences of adiponectin:leptin ratio

related to tumor status in ICR group, in the first half of study ICR-Restricted but not ICR-Refed mice that later developed mammary tumors had lower adiponectin:leptin ratio [(Cycle 1 ($P<0.01$), Cycle 3 ($P<0.05$)] than mice that did not develop tumors. In contrast to the early time-points, later in the study at Cycles 8 ($P<0.05$) and 11 both ICR-Restricted and ICR-Refed mice with mammary tumors had significantly higher ($P<0.01$) adiponectin:leptin ratio than mice without mammary tumors.

When longitudinal data of adiponectin:leptin ratio were analyzed only for mice which developed mammary tumors, in Cycle 1 AL mice had the highest ratio followed by CCR, ICR-Restricted and ICR-Refed mice, however differences were not significant ($P=0.92$). In Cycles 3, 5, and 8 prior to mammary tumor detection AL mice had the lowest adiponectin:leptin ratio versus ICR-Restricted values and the difference reached significance in Cycle 8 ($P<0.01$). Interestingly, in Cycle 11, when detected tumors were growing adiponectin:leptin ratio of ICR-Restricted mice continued to increase and was significantly higher than AL ($P<0.01$), CCR($P<0.01$) and ICR-Refed mice ($P<0.01$).

Discussion

There is no universal and effective preventive strategy for breast cancer which is the most common neoplasia among women worldwide including the United States (64,65). Increased energy balance may be responsible for approximately one-third of human mammary tumors (66). Thus modifying total energy intake and/or expenditure may be one of the key elements of breast cancer prevention. In fact chronic calorie restriction has been consistently reported to prevent or delay mammary tumorigenesis in animal models (67–76). The consequence of multiple periods of calorie restriction followed by refeeding, i.e., intermittent calorie restriction, has been less well studied. However, several previous studies indicated a protective effect on prevention of spontaneous tumors (77,78). Further, we have consistently found that intermittent restriction prevents and delays mammary tumor development to a greater extent compared to chronic calorie restriction matched for the same nutrient and calorie intake in two transgenic mouse strains (60–62,79). Interestingly, it has been reported that women with a history of anorexia nervosa and presumably had bouts of calorie restriction followed by refeeding had a reduced incidence of breast cancer (80).

Our recent efforts have focused on identifying factors associated with mammary tumor development which may be modified by the intermittent calorie restriction protocol. We recently published detailed results for the impact of this intervention protocol on the IGF-I axis (61). Here, we have expanded our investigation to explore the effects of these two calorie restriction protocols on adipokine serum levels and their signaling pathways within target tissues. Our specific focus is on adiponectin and leptin.

We found at the termination of the study that in adult female mice serum adiponectin levels were similar among all groups with no impact of either long-term chronic or intermittent calorie restriction (Figure 2Bb). We also obtained serum samples over the course of the study and found that adiponectin serum levels of AL and CCR mice were slightly increased with aging. For ICR mice at some time points adiponectin concentration depended upon the dietary regimen and was somewhat higher in restriction than in refeeding. Reports in the literature for the effects of dietary intervention on serum adiponectin vary. In humans, calorie restriction and/or weight loss have been linked to increased serum adiponectin concentration in some studies (81–84), but not in others (85, 86). There are only a few reports of the influence of intermittent calorie restriction on serum adiponectin levels. Wan *et al* (87) reported that intermittent fasting of Wistar male rats resulted in increased levels of circulating adiponectin compared to control rats. However it was also reported that different levels of refeeding following alternate day calorie restriction for four weeks did not affect

adiponectin levels of C57BL/6J male mice (88), although it increased adiponectin concentration by 62–86% for females (89). We have previously reported that neither chronic nor intermittent calorie restriction altered adiponectin in a cross-sectional study of female MMTV-TGF- α mice on the C57BL6 background (54). Similar results have been found for C57BL6 TRAMP male mice in both a cross-sectional and a long-term study (63, 90). We also found no effect of goldthioglucose-induced obesity on adiponectin levels (57) and a recent online publication reported no effect of high-fat feeding or sucrose ingestion in water on serum adiponectin levels (91). Thus, it remains to be determined if diet composition or other factors such as physical activity may interact with calorie restriction to affect serum adiponectin levels.

In contrast to adiponectin, serum leptin levels were significantly reduced by long-term chronic calorie restriction. Further, serum leptin levels of the mice on the intermittent calorie restriction protocol were reduced after restriction as well as after refeeding compared to both AL and CCR mice. Over the course of the study, serum leptin levels rose in AL mice with increasing age while this response was muted by calorie restriction. These findings are consistent with human studies (92–99).

Our interest in assessing these two adipokines in relationship with mammary tumorigenesis was based on a number of previous studies. *In vitro* experiments by ourselves and others have shown that leptin enhances proliferation of human breast cancer cell lines (14,15,17,18). In contrast, addition of adiponectin reduces human breast cancer cell proliferation (20,22,23,25). Human studies have not indicated a consistent relationship of serum leptin levels with breast cancer (as recently summarized by Grossmann *et al.* (100)) However, serum adiponectin levels have been found to be reduced in women with breast cancer compared to those without the disease (33,34,37,45). In most studies the focus was on either leptin or adiponectin alone, but in one study where both proteins were measured it was found that a low adiponectin:leptin ratio was associated with breast cancer (36). Based on these findings we conducted *in vitro* studies evaluating the impact of different adiponectin:leptin ratios on human breast cancer cell proliferation and the results support that this relationship may be more important in determining the overall consequence of how these two proteins affect tumor development rather than their absolute amounts (101). Here, although we did not demonstrate that individually either leptin or adiponectin was associated with mammary tumor development either during the early stages of tumorigenesis or once mammary tumors were detected, we clearly show that the intermittent calorie restriction protocol is associated with an elevated ratio of adiponectin:leptin as well as with reduced mammary tumor incidence. This is consistent with recent results from a cross-sectional study using this same mouse strain (54), as well as with our findings that intermittent calorie restriction delays prostate tumor detection and death in the TRAMP mouse model of prostate cancer and this too was associated with an elevated adiponectin:leptin ratio (63).

Circulating adipokines such as leptin and adiponectin exert their biological action on target cells not only by endocrine mechanism but also through paracrine and autocrine pathways (Reviewed by Vona-Davis (102)). Leptin acts by binding to its receptor, ObR, which is structurally related to the cytokine receptor family. The ObR gene can be alternatively spliced into several isoforms (103) with the isoform designated ObRb considered the active signaling protein. Previously, it was reported that ObR was overexpressed in breast tumor tissue compared to non-cancer breast epithelium and also the expression of leptin was found to be positively correlated with expression of its receptor, suggesting that leptin acts on mammary tumor cells via an autocrine pathway (26). In addition, the association between high intratumoral leptin receptor mRNA level and poor breast cancer prognosis was shown in the presence of high, but not low serum and intratumoral leptin levels (104). In the present study, we determined expression of leptin in mammary tumors, mammary tissue and

mammary fat pad, and also expression of total leptin receptor ObR and its long isoform ObRb in mammary tumors and mammary gland. Our data support that caloric restriction affected leptin and leptin receptor expression in mammary tissue. CCR and ICR-Restricted mice had significantly decreased leptin protein expression in mammary tissue vs the AL group. In addition, ICR-Restricted mice had significantly lower ObRb expression compared with AL mice.

The mammary fat pad is a microenvironment for breast tissue and locally produced adipokines can play important roles in mammary tumorigenesis. While we did not find significant differences in leptin protein expression in mammary fat pads among the dietary groups, earlier we showed that chronic and intermittent calorie restriction significantly decreased ObR and ObRb mRNA expression in mammary fat pad in comparison to the AL diet (60). In addition, Gallardo *et al* reported that long-term 20–25% calorie restriction promoted a change in the distribution of ObRa between internal and plasma membranes in isolated rat adipocytes, increasing its presence at the cell surface (105). Interestingly, Sucajus-Szilc *et al.* reported that leptin gene expression in rat hypothalamus is down-regulated by prolonged food restriction as in white adipose tissue, but refeeding after long-term food restriction increased serum leptin concentration and leptin gene expression in white adipose tissue but had no effect on hypothalamic leptin gene expression (106) indicating tissue specificity.

Interestingly, we determined that CCR and ICR-Restricted mice had significantly decreased leptin protein expression in mammary tissue compared to the AL group, however mammary tumors as well as mammary fat pads showed no difference in leptin protein expression and previously we reported that neither the ICR nor CCR protocols changed ObR and ObRb mRNA expression in mammary tumors (60). There are no published data about changes of leptin protein expression under long-term calorie restriction. In adipose tissue mRNA level for leptin has been reported to decrease in response to short-term fasting or severe chronic restriction (107). However the effect of long term intermittent or chronic calorie restriction on leptin protein expression in fat tissue, and particular in mammary fat, is unknown. We assume that leptin may have a role in the early phase of mammary tumor development. Analysis of mammary tissues, tumors and fat pads from our cross-sectional study (54) will provide details about leptin protein expression in target tissues at earlier stages of mammary tumor development.

We also determined expression levels of adiponectin and its two receptors, AdipoR1 and AdipoR2 (108) in target tissues. Adiponectin and its receptors have been shown to be expressed in healthy breast tissue and also in mammary malignancy (20,25,29,109). Previously, it was reported that breast tissue adiponectin levels measured by ELISA in patients with breast cancer were significantly increased in comparison to controls although there was no association between tumor stage and tumor size (29). Korner *et al.* (20) showed that adiponectin mRNA expression level was significantly higher in mammary tissue adjacent to breast tumors compared to either breast tumor tissue or to control tissue from subjects without breast cancer. In the same study AdipoR1 mRNA expression but not AdipoR2 was higher in breast tumors compared to control mammary tissue from cancer-free subjects. Jarde *et al.* (109) used immunohistochemical staining to assess adiponectin receptor expression in breast tissues. In contrast to Korner's study they showed that difference in AdipoR1 expression in breast cancer tumors and normal adjacent tissues was not statistically significant, however AdipoR2 expression was more pronounced in malignant cells than in patients' normal tissue. In our cross-sectional study (Dogan, S, and Cleary, MP unpublished) expression level of AdipoR2 was similar in mammary tumor and control tissues, but protein expression levels of AdipoR1 were significantly lower in mammary tumors compared to control mammary tissue obtained from 74 week old MMTV-

TGF- α mice. Here we found that in ICR-Restricted mice there was increased AdipoR1 expression in mammary tissues and increased AdipoR2 expression in mammary tumors. We also showed that caloric restriction did not affect adiponectin protein expression in mammary tissues, while in mammary tumors it was decreased in both CCR and ICR-Restricted mice in comparison to the AL group, however the differences was not significant. Integration of our data with previously published studies indicates an involvement of adiponectin receptors in breast tumorigenesis, but the relevance of these observations is still unclear.

Previously, it was reported that two months of 16% calorie restriction enhanced adiponectin expression in visceral fat of young but not senescent rats (110,111). In senescent rats a long-term fairly severe calorie reduction (40%) was need for mild induction of adiponectin expression in visceral fat. In our study, 25% chronic calorie reduction did not change adiponectin protein expression in mammary fat pad of tumor-free mice, but 50% restriction significantly increased adiponectin expression in mammary fat of ICR-Restricted mice compared with AL and CCR mice. It is important to note that tumor-bearing mice had a very low signal of adiponectin expression in mammary fat pad with strong leptin protein expression. Unbalance of these two adipokines in mammary tumor environment could be a critical in process of tumorigenesis.

In summary, we demonstrated that two modes of calorie restriction had different influences on serum leptin levels and on the adiponectin:leptin ratio. Mice subjected to chronic calorie restriction had reduced serum leptin level and increased adiponectin:leptin ratio compared to ad libitum fed mice while ICR mice had fluctuations of leptin concentration and adiponectin:leptin ratio. However it should be noted that three weeks of the same calorie and nutrient intake as that of ad libitum fed mice during refeeding did not restore values of ICR mice to levels of either AL and CCR mice. Serum leptin levels were influenced by age while there was very little effect on adiponectin. We also provided evidence for a strong association of serum leptin concentration as well as adiponectin:leptin ratio with fat mass and body weight. Some effects of the calorie restriction protocols were found on tissue expression levels of leptin and adiponectin receptors, although no consistent effects were noted. However, in this and other studies we have found that the a high adiponectin:leptin ratio is associated with reduced tumorigenesis in ICR mice. Thus, although it appears that adiponectin and leptin are involved in mammary tumorigenesis there are still many aspects of the relationship to be determined.

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Figure 1.

Tumor incidence and latency of MMTV-TGF- α Female Mice. **A.** Tumor incidence of MMTV-TGF- α Female Mice. $\chi^2 = 24.64$, 2 degrees of freedom, $P < 0.0001$. AL (n=49/69), CCR (n=23/65), (n=6/66). **B.** Tumor latency of TGF- α Female Mice. ANOVA $P < 0.005$. AL (n=49), CCR (n=23), ICR (n=6). *, †, ‡ Columns with different superscript symbol are significantly different.

Figure 2.

Terminal Body, Fat Pad Weights and Leptin, Adiponectin Serum Levels and Ratio of Adiponectin:Leptin of MMTV-TGF- α Female Mice. **A.** Final Body and Fat Pad Weights of MMTV-TGF- α Female Mice. **Aa**, **Ab** and **Ac**, AL (n=69), CCR (n=66), ICR-Refed (n=29) and ICR-Restricted (n=30) mice. **Aa**. Terminal Body Weight. ANOVA $P < 0.05$. **Ab**. Mammary Fat Pad Weight (combined right and left mammary fat pads. ANOVA $P < 0.05$. **Ac**. Internal Fat Pad Weight (combined parametrial and retroperitoneal fat pads). ANOVA $P < 0.05$. **B.** Terminal Leptin and Adiponectin Serum Level and Ratio of Adiponectin:Leptin of TGF- α Female Mice. **Ba**, **Bb** and **Bc**, AL (n=26), CCR (n=23), ICR-Refed (n=15–16) and ICR-Restricted (n=15) mice. **Ba**. Terminal Leptin serum levels. ANOVA $P < 0.05$. **Bb**. Terminal Adiponectin serum levels. ANOVA $P > 0.05$. **Bc**. Terminal Adiponectin:Leptin

Ratio. ANOVA $P < 0.01$. **Ca**. Terminal Leptin serum levels for mice with and without mammary tumors. t-test $P > 0.05$. **Cb**. Terminal Adiponectin serum levels for mice with and without mammary tumors. t-test $P > 0.05$. **Cc**. Terminal Adiponectin:Leptin Ratio for mice with and without mammary tumors. t-test $P = 0.03$. **Ca**, **Cb**, **Cc** AL without mammary tumors (n=13), AL with mammary tumors (n=13). CCR without mammary tumors (n=7), CCR with mammary tumors (n=16). ICR-Refed without mammary tumors (n=13), ICR-Refed with mammary tumors (n=2). ICR-Rest without mammary tumors (n=12), ICR-Rest with mammary tumors (n=3). In **A** (**a**, **b**, **c**), **B** (**a**, **b**, **c**) and **C** (**a**, **b**, **c**) ICR-Refed mice were euthanized during a refeeding period and ICR-Restricted mice were euthanized during a restriction period. *, †, ‡ Columns with different superscripts are significantly different from each other.

Figure 3.

Protein Expression of Adiponectin, AdipoR1, AdipoR2, Leptin, ObR, ObRb in Mammary Tissue and Adiponectin, Leptin in Mammary Fat Pad of MMTV-TGF- α Female Mice. **A.** Adiponectin, AdipoR1, AdipoR2, Leptin, ObR and ObRb protein expression in Mammary Tissue. **B.** Adiponectin, AdipoR1, AdipoR2, Leptin, ObR and ObRb protein expression in Mammary Tumor. **C.** Adiponectin and Leptin protein expression in Mammary Fat Pad of mice without mammary tumor. **D.** Adiponectin and Leptin protein expression in Mammary Fat Pad of mice with mammary tumor. **A, B, C, D.** Adiponectin, AdipoR1, AdipoR2, Leptin, ObR and ObRb protein expression levels were detected by Western blot analysis. β -actin protein was probed as a loading control, ICR-Refed were euthanized during a refeeding period, ICR-Restricted mice were euthanized during a restriction period. The arrows at the bottom of the blots represent division between dietary groups.

Figure 4.

Serum Adiponectin, Leptin Levels and Ratio of Adiponectin:Leptin Levels for Ad libitum-Fed (AL), Chronic Calorie Restricted (CCR) and Intermittent Restricted (ICR) MMTV-TGF- α Mice with and without Mammary Tumors over the Course of the Study. A, Serum Adiponectin Levels for AL, CCR and ICR mice during cycles 1, 3, 5, 8, and 11 for mice that never developed mammary tumors compared to those that did. Bars represent means of Adiponectin concentrations. t-test $P > 0.05$. **Aa**, AL without mammary tumors ($n=10$), AL with mammary tumors ($n=9$). **Ab**, CCR without mammary tumors ($n=12$), CCR with mammary tumors ($n=11-12$). **Ac**, ICR-Restricted. **Ad**, ICR-ReFed. **Ac** and **Ad** ($n=16$ for mice without mammary tumors and $n=4$ for mice with mammary tumor).

B, Serum Leptin Levels for AL, CCR and ICR mice during cycles 1, 3, 5, 8, and 11 for mice that never developed mammary tumors compared to those that did. Bars represent means of Adiponectin concentrations. t-test $P>0.05$. **Ba**, AL without mammary tumors (n=10), AL with mammary tumors (n=9). **Bb**, CCR without mammary tumors (n=12), CCR with mammary tumors (n=11–12). **Bc**, ICR-Restricted. **Bd**, ICR-Refed. **Bc** and **Bd** (n=15–16 for mice without mammary tumors and n=4 for mice with mammary tumor).

C, Serum Adiponectin:Leptin Ratio for AL, CCR and ICR mice during cycles 1, 3, 5, 8, and 11 for mice that never developed mammary tumors compared to those that did. Bars represent means of Adiponectin concentrations. t-test $P>0.05$. **Ca**, AL without mammary tumors (n=10), AL with mammary tumors (n=9). **Cb**, CCR without mammary tumors (n=12), CCR with mammary tumors (n=11–12). **Cc**, ICR-Restricted. **Cd**, ICR-Refed. **Cc** and **Cd** (n=15–16 for mice without mammary tumors and n=4 for mice with mammary tumor).

In **A** (**a, b, c, d**), **B** (**a, b, c, d**) and **C** (**a, b, c, d**) ICR-Refed mice were euthanized during a refeeding period and ICR-Restricted mice were euthanized during a restriction period. ^{*}, [†], [‡] Columns with different superscripts are significantly different from each other.

Table 1

Correlation between Terminal BW, Total Fat Pad Weight, Mammary Fat Pad Weight and Leptin, Adiponectin or Adiponectin:Leptin Ratio.

	Leptin	Adiponectin	Adiponectin:Leptin Ratio
Body Weight	positive correlation $r=0.7687$; $P<0.0001$	no correlation $r=-0.0267$; $P>0.05$	negative correlation $r=-0.2625$; $P<0.0187$
Total Fat Pad Weight	positive correlation $r=0.7886$; $P<0.0001$	no correlation $r=0.01260$; $P>0.05$	negative correlation $r=-0.3440$; $P<0.0018$
Mammary Fat Pad Weight	positive correlation $r=0.8005$; $P<0.0001$	no correlation $r=0.0043$; $P>0.05$	negative correlation $r=-0.3220$; $P<0.0036$

Number of XY pairs for each correlation is eighty. AL (n=27), CCR (n=24), ICR-Refed (n=15) and ICR-Restricted (n=14) mice. ICR-Refed mice were euthanized during a refeeding period and ICR-Restricted mice were euthanized during a restriction period.

Table 2

Protein expression of Adiponectin, AdipoR1, AdipoR2, Leptin, ObR, ObRb in mammary tissue and Adiponectin and Leptin in Mammary Fat Pad of MMTV-TGF- α mice with and without mammary tumor; densitometry units (optical density of protein/optical density of β -actin)

Mammary tissue				
	AL n=5	CCR n=5	ICR-Restricted n=5	ICR-Refed n=5
Adiponectin	1.00 $\pm$ 0.03	0.99 $\pm$ 0.07	0.88 $\pm$ 0.13	0.95 $\pm$ 0.01
AdipoR1	0.44 $\pm$ 0.04 [†]	0.47 $\pm$ 0.05 ^{*,†}	0.61 $\pm$ 0.05 [*]	0.56 $\pm$ 0.01 ^{*,†}
AdipoR2	0.46 $\pm$ 0.03	0.56 $\pm$ 0.05	0.53 $\pm$ 0.03	0.47 $\pm$ 0.00
Leptin	0.90 $\pm$ 0.05 [*]	0.60 $\pm$ 0.03 [†]	0.63 $\pm$ 0.08 [†]	0.73 $\pm$ 0.03 ^{*,†}
ObR	0.63 $\pm$ 0.06 [*]	0.58 $\pm$ 0.13 ^{*,†}	0.42 $\pm$ 0.08 ^{*,†}	0.26 $\pm$ 0.04 [†]
ObRb	0.79 $\pm$ 0.10 [*]	0.68 $\pm$ 0.04 ^{*,†}	0.53 $\pm$ 0.03 [†]	0.61 $\pm$ 0.06 ^{*,†}
Mammary tumor				
	AL n=5	CCR n=5	ICR-Restricted n=3	ICR-Refed n=3
Adiponectin	0.75 $\pm$ 0.12	0.53 $\pm$ 0.06	0.55 $\pm$ 0.15	0.63 $\pm$ 0.01
AdipoR1	0.40 $\pm$ 0.05	0.35 $\pm$ 0.03	0.52 $\pm$ 0.10	0.42 $\pm$ 0.02
AdipoR2	0.53 $\pm$ 0.07 [†]	0.49 $\pm$ 0.05 [†]	0.76 $\pm$ 0.03 [*]	0.49 $\pm$ 0.04 [†]
Leptin	0.77 $\pm$ 0.03	0.79 $\pm$ 0.02	0.75 $\pm$ 0.06	0.76 $\pm$ 0.02
ObR	0.62 $\pm$ 0.01	nd	0.42	nd
ObRb	0.55 $\pm$ 0.05	nd	0.63	nd
Mammary Fat Pad (tumor-free mice)				
	AL n=5	CCR n=5	ICR-Restricted n=5	ICR-Refed n=5
Adiponectin	0.73 $\pm$ 0.08 [†]	0.70 $\pm$ 0.06 [†]	1.00 $\pm$ 0.03 [*]	0.95 $\pm$ 0.05 ^{*,†}
Leptin	1.25 $\pm$ 0.05	1.22 $\pm$ 0.14	1.26 $\pm$ 0.05	1.32 $\pm$ 0.14
Mammary Fat Pad (tumor-bearing mice)				
	AL n=5	CCR n=5	ICR-Restricted n=3	ICR-Refed n=3
Adiponectin	nd	nd	nd	1.12
Leptin	1.42 $\pm$ 0.23	1.48 $\pm$ 0.20	1.49 $\pm$ 0.03	1.59 $\pm$ 0.06

NOTE: Values are mean $\pm$ SE, n – number of probed samples

^{*}, [†] Values within a row with different superscript symbol are significantly different by ANOVA

nd – not detected