Excess Weight Gain Accelerates 1-Methyl-1-Nitrosourea– Induced Mammary Carcinogenesis in a Rat Model of Premenopausal Breast Cancer

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Abstract

In contrast to the null effects generally reported, high-risk premenopausal women (Gail score $\geq$1.66) enrolled in the Breast Cancer Prevention P-1 Trial were recently reported to be at increased risk for breast cancer when overweight (HR = 1.59) or obese (HR = 1.70). To investigate this clinical observation in a preclinical setting, ovary-intact female rats were intraperitoneally injected with 50 mg/kg 1-methyl-1-nitrosourea at 21 days of age to simulate premenopausal women with increased risk. Two commercially available strains of Sprague–Dawley rat (Taconic Farms) were used, which are dietary resistant (DR) or dietary susceptible (DS) to excess weight gain when fed a purified diet containing 32% kcal from fat, similar to levels consumed by the typical American woman. DS rats were approximately 15.5% heavier than DR rats at study termination and plasma leptin indicated a marked difference in adiposity. DS rats had higher incidence (26% increase), multiplicity (2.5-fold increase), and burden (5.4-fold increase) of mammary carcinomas with a concomitant reduction in cancer latency (16% earlier detection) compared with DR rats (P < 0.001 for all analyses), and displayed a higher proportion of hormone receptor negative tumors compared with DR rats [OR = 1.78; 95% confidence interval (CI), 0.83–3.81]. Circulating levels of several breast cancer–risk factors, including leptin, adiponectin:leptin ratio, insulin, insulin-like growth factor (IGF)-1, IGF-1:IGF-1 binding protein-3 ratio, and calculated insulin resistance (HOMA-IR) were negatively impacted in DS rats (P < 0.05 for all analyses). These findings support further investigation of the effects of excess weight in high-risk premenopausal women and demonstrate a useful preclinical model for rapid evaluation of mechanistic hypotheses. *Cancer Prev Res; 7(3); 310–8. ©2014 AACR.*

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in U.S. women (1). Current estimates indicate that 17% of breast cancer cases in the United States are preventable by maintaining a healthy weight (2). This relationship is of considerable public health importance given the ongoing obesity epidemic, in which 2 of 3 women are reportedly overweight or obese as defined by body mass index (BMI) $\geq$25 kg/m^2 (3, 4). Obese women with breast cancer typically have larger tumors, higher rates of metastasis, higher rates of recurrence, and increased all-cause and breast cancer–related mortality at any age compared with normal weight women with breast cancer (5, 6).

The nature of the association between obesity and breast cancer has been reported to hinge on menopausal status. In postmenopausal women, a positive association between excess body weight and breast cancer risk has been consistently reported (2). Conversely, most studies report a null or inverse association of excess weight with breast cancer risk in premenopausal women, although this topic remains controversial (2, 7–9). Recently, in a study based on the National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention (P-1) Trial, Cecchini and colleagues provided evidence contrary to this mainstream of thinking. In the Cecchini study, a large cohort of premenopausal women ages $\geq$35 years at high risk for breast cancer (Gail score $\geq$1.66) was evaluated at 3 tiers of BMI: <25.0 (normal weight), 25.0 to 29.9 (overweight), and $\geq$30.0 kg/m^2 (obese). Risk of invasive breast cancer was increased in overweight and obese women (HR = 1.59 and 1.70, respectively) compared with women of BMI <25 (10), suggesting that in women with multiple breast cancer–risk factors, which comprise a subpopulation of premenopausal women, excess weight gain may further increase risk.

Despite the abundance of preclinical studies evaluating the relationship between dietary fat, adiposity, and breast cancer risk, several gaps remain in our understanding of the effect of overweight and obesity on breast carcinogenesis as

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well as in available preclinical tools to fill these gaps. Although most strains are resistant to diet-induced obesity (DIO), MacLean and colleagues have characterized subpopulations of Wistar rats with sensitivity or resistance to diet-induced excess weight gain; however, these studies have only been performed in a postmenopausal model for breast cancer (11–14). Studies in mice, although mechanistically informative and likewise trending toward identification of obesity-resistant and sensitive subpopulations in reports from Cleary and colleagues (15–17) are limited by 2 factors. First, in most mouse models for breast cancer, predominantly estrogen and progesterone receptor–negative tumors are induced, a molecular subtype of the disease, which is less common in premenopausal women (reviewed in refs. 18 and 19). Second, preclinical models of diet-induced obesity in susceptible mouse strains generally utilize high-fat diets containing 45% to 60% kcal from fat, levels of fat above relevance to human populations. Moreover, nonobese control mice are frequently fed a low-fat diet thus adding differences in dietary composition as potentially confounding variables in those investigations.

To circumvent these issues, this study was conceived as a means to rapidly evaluate Cecchini and colleagues's findings in a reverse translational setting, and to our knowledge has not been previously attempted. We describe the integration of a novel model for the study of diet-induced obesity introduced in 1997 by BE Levin of Veterans Affairs Medical Center in East Orange, NJ, with a well-characterized approach to cancer induction that was developed by our laboratory in 1995. The model for polygenic obesity developed by Levin utilizes 2 strains of Sprague–Dawley rats selectively bred for >20 generations for resistance (DR) or susceptibility (DIO or DS, as used herein) to diet-induced excess weight gain when fed diet containing ∼32% kcal as fat. This model has been extensively characterized by Levin's group (e.g., refs. 20–22). When fed the 32% fat diet, DS rats rapidly gain excess weight and have expanded peripheral and visceral fat depots by 3 months of age (21), display hyperlipidemia (total cholesterol and triglycerides) by 2 months, hyperleptinemia by 3 months, and pronounced fat infiltration of the liver by 6 months of age (21). DS rats display prediabetic measures of glucose homeostasis including hyperinsulinemia by 2 months, insulin resistance by 3 months, worsened oral glucose tolerance by 2 months, and eventual reduced pancreatic insulin secretion by 9 months of age, although rats do not fully progress to diabetes up to 2 years of age (21). Our model for breast cancer induction involves injecting weanling Sprague–Dawley rats, which are highly sensitive to mammary cancer induction, with a nontoxic dose of a chemical carcinogen (23). Tumors begin to emerge after sexual maturity and incidence, multiplicity, and latency of mammary tumors are dependent on carcinogen dose (23–25). The histogenesis, morphological stages of disease development, and the types of carcinomas induced recapitulate the process of breast carcinogenesis in women (18, 26). In ovary-intact animals, the disease process provides a biologically relevant model for premenopausal breast cancer. Using this integrated preclinical model, we evaluated Cecchini and colleagues's hypothesis that excess weight gain resulting in overweight or obesity increases incident cancer in premenopausal women with increased risk for breast cancer.

Materials and Methods

Animal breeding and husbandry

Breeder pairs (approximately 30 pairs each Levin DR and DS) were obtained from Taconic at 5 to 7 weeks of age. These outbred strains of Sprague–Dawley rats were originally obtained by Taconic from BE Levin after 20 generations of selective breeding for rapid weight gain on sucrose and moderate fat (32%; SUMO32) diet and were subsequently outbred using a rotational breeding scheme for an additional 30+ generations, and are commercially available from the Taconic repository [strain: TacLevin:CD(SD)DIO, stock #DS; TacLevin:CD(SD)DR, stock #DR]. In-house breeding was conducted using a Poiley rotational breeding scheme, in which breeder pairs are systematically rotated in each breeding cycle (27).

Pups were weaned at 3 weeks of age and were immediately switched to SUMO32 diets. Post-weaning, rats were housed 3 per cage, maintained on 12-hour light:dark cycle at $24 \pm 2°C$ with 30% relative humidity, and given *ad libitum* access to SUMO32 diet and distilled water. Animals were weighed weekly. To initiate mammary carcinogenesis according to the rapid emergence model first developed by our laboratory (23), female ovary-intact DR ($n = 103$) and DS ($n = 101$) rats were injected intraperitoneally (50 mg/kg) with 1-methyl-1-nitrosourea (MNU; Ash Stevens—prepared fresh in acidified saline) at 21 days of age as previously described (24). Biweekly palpations for detection of mammary tumors began 24 days after carcinogen and continued until study termination. All animal studies were performed in accordance with the Colorado State University Institutional Animal Care and Use Committee.

Diet formulation and composition

The sucrose and moderate 32% fat (SUMO32) diet is a purified formulation described in Table 1. Anhydrous milk fat and corn oil together contribute 32.1% of kcal in the SUMO32 diet, composed of 29.2% saturated fatty acids (9.4% of total dietary kcal), 28.4% monounsaturated fatty acids (9.1% of total dietary kcal), and 42.4% polyunsaturated fatty acids (13.6% of total dietary kcal; ref. 28, manufacturer's label). The SUMO32 diet provided 16.7% protein and 51.2% carbohydrate by kcal, comparable to the macronutrient composition of the average American woman's diet as reported in Table 1 (29). The SUMO32 rodent diet provided 4.35 kcal/g (18.2 kJ/g) and was mixed onsite at our laboratory's diet mixing facility and stored at $−20°C$ until used.

Necropsy

The study was terminated 63 days after carcinogen when rats were 84 days of age, whereupon fasted rats were euthanized within a 4-hour window via CO_2 inhalation and cervical dislocation. Blood was collected into EDTA

Table 1. Sucrose and moderate fat (32% kcal) (SUMO32) diet formulations

Ingredient	Source	g/100 g	%kcal/ 100 g	SUMO32 diet (% kcal)	U.S. woman's diet (% kcal)
Anhydrous milk fat	Catalog #1075	4.2	8.7		
	Hunter Walton & Co. Inc.			Total fat: 32.1	Total fat: 33.5
				Saturated fat: 9.4	Saturated fat: 11.1
Corn oil	Mazola brand, local retailers	11.3	23.4		
Sucrose (granulated sugar)	Kroger or Great Value brand, local retailers	27.8	25.6		
Dextrose monohydrate	Catalog #12205	7.2	6.6		
	Batory Foods			Carbohydrate: 51.2	Carbohydrate: 50.5
Corn starch	Catalog #15400	20.6	19.0		
	Batory Foods				
Lactic casein ($\geq$85% protein)	Catalog #3G202	18.2	16.7	Protein: 16.7	Protein: 15.5
	Batory Foods				
Solka-Floc (Cellulose)	Catalog #3F5741	2.9	—	—	—
	Batory Foods				
DL-methionine	Catalog #402950	0.3	—	—	—
	Dyets Inc.				
Choline bitartrate	Catalog #400750	0.2	—	—	—
	Dyets Inc.				
Calcium carbonate	Catalog #400400	0.5	—	—	—
	Dyets Inc.				
Sodium bicarbonate	Catalog #404090	0.5	—	—	—
	Dyets Inc.				
Potassium citrate monohydrate	Catalog #403600	1.3	—	—	—
	Dyets Inc.				
Mineral mix	Catalog #210025	3.8	—	—	—
	Dyets Inc.				
Vitamin mix	Catalog #310025	1.1	—	—	—
	Dyets Inc.				
Total		100.0	100.0		

VacuTainers (Becton-Dickinson) and centrifuged to separate plasma. After separation, plasma was kept on ice before freezing at −20°C until use. Rats were skinned and mammary gland chains were examined under translucent light; grossly visible tumors were excised, weighed, and processed for histopathologic analysis and classification by hematoxylin and eosin staining as previously described (30).

Plasma analyses

Body weights of both DR and DS strains are normally distributed; therefore, to maximize statistical power, animals chosen for plasma analysis were purposely sampled from nonoverlapping areas of the distributions, for example lean DR and heavy DS. All systemic analytes were determined using commercial ELISA kits performed according to manufacturer's specifications. Specifically, glucose was determined using a kit obtained from Thermo Fisher. Insulin, leptin, interleukin (IL)-6, IL-1β, and TNF-α were determined using a Multiplex Kit, whereas adiponectin and IGF-1 were separately quantified using commercial single-plex kits from Millipore. C-reactive protein was determined using a commercial rat ELISA from Helica Biosystems Inc.

Estradiol, progesterone, and sex hormone binding globulin were determined using commercial ELISAs from GenWay Biotech. IGF-1 binding protein (IGFBP)-3 Kit was from BioVendor. Homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated as previously described (31).

Immunohistochemical assessment of hormone receptor status

The estrogen and progesterone receptor status of mammary carcinomas was determined using our previously published procedure (32). Specificity of the staining is illustrated in Supplementary Fig. S1.

Statistical analyses

Statistical analyses were performed on data from palpable mammary adenocarcinomas and 95% CIs were constructed for each outcome. Differences were assessed for statistical significance as follows: final body weight at study termination was assessed by an unpaired t test with Welch–Satterthwaite correction; cancer incidence (%) and proportion of estrogen and progesterone positive and negative tumors

were assessed by a χ^2 test of equal proportions; cancer multiplicity (# carcinomas/rat) was assessed using Poisson regression; carcinoma burden (g/rat) was assessed using a 2-stage model combining χ^2 (incidence) and log-linear (burden) P values; and cancer latency was assessed using a log-rank (Mantel–Cox) test in Kaplan–Meier survival analysis, where animals without palpated tumors were right-hand censored 60 days after carcinogen, the last day rats were palpated for detectable tumors before study termination (censored: DR, $n = 39$; DS, $n = 9$). Between-group differences in plasma analytes were assessed by an unpaired t test with Welch–Satterthwaite correction and $\log_{10}$ data transformation as needed to satisfy statistical assumptions.

Results

SUMO32 diet induces rapid excess weight gain in DS rats

All rats were fed SUMO32 diet (Table 1) *ad libitum* throughout the 9-week duration of the study. Rats sensitive (DS) to diet-induced excess weight gain ($n = 101$) were 3 g heavier than rats resistant (DR) to diet-induced excess weight gain ($n = 103$) at the outset of the study (body weights for DS and DR: 56.7 ± 8.7 g vs. 53.6 ± 6.3 g, respectively, $P = 0.004$). As depicted in Fig. 1A, body weights of DS and DR rats began to diverge immediately with SUMO32 feeding, and DS rats continued to gain weight at an accelerated rate for the duration of the study ($P_{Trend} < 0.001$). At study termination 63 days after carcinogen, DS rats were 15.5% heavier than DR rats (217.0 ± 24.5 vs. 187.9 ± 14.6, respectively, $P < 0.001$). Plasma leptin, which is related to fat mass (33, 34), was substantially higher in DS versus DR rats at study termination (5.6 ± 1.4 vs. 0.9 ± 0.8 for DS vs. DR, respectively; $P < 0.001$).

Chemically induced mammary carcinogenesis is markedly accelerated in DS rats

To determine the effect of excess weight gain on the development of chemically induced mammary carcinogenesis, female ovary-intact rats (DR, $n = 103$; DS, $n = 101$) were injected intraperitoneally with 50 mg/kg 1-methyl-1-nitrosurea (MNU) at 21 days of age as delineated in the rapid emergence model of mammary carcinogenesis (23). Latency to the first palpated mammary carcinoma per rat was evaluated by to time-to-event analysis with tumor-free rats right-hand censored at 60 days after carcinogen, the last day rats were palpated before study termination (censored: DS, $n = 9$; DR, $n = 39$). Cancer outcomes are reported in Table 2 and are based on data for palpable histopathologically confirmed mammary adenocarcinomas. As shown in Fig. 1B, cancer-free survivor functions for DR and DS rats were significantly different ($P < 0.001$) and 50% incidence of mammary adenocarcinoma in the DS group was achieved by 39 days after carcinogen, compared 49 days after carcinogen in the DR group. Final cancer incidence was 91% in DS rats versus 65% in DR rats. Cancer multiplicity and cancer burden were also markedly higher in the DS versus DR group (Fig. 1C and Table 2; $P < 0.001$ for all analyses). The carcinomas used to construct Fig. 1B were
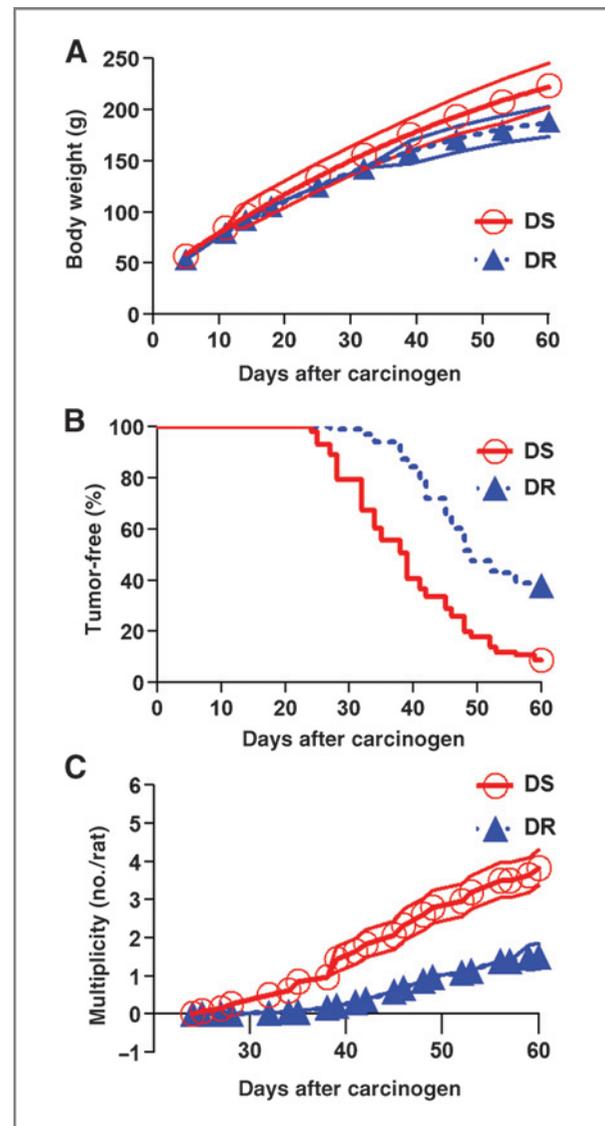

Figure 1. Excess weight gain accelerates mammary carcinogenesis. Diet-induced excess weight gain susceptible (DS; $n = 101$) and resistant (DR; $n = 103$) rats were switched from chow to sucrose and moderate fat (32% dietary fat by kcal; SUMO32) diet *ad libitum* at weaning. Mammary carcinogenesis was initiated by injecting rats intraperitoneally with 50 mg/kg MNU at 21 days of age. Study was terminated 63 days (9 weeks) after carcinogen; only palpable confirmed mammary adenocarcinomas were included for analysis. A, rats were weighed weekly while on study; data, mean ± SD. Weight gain trend was analyzed with nonlinear regression. DS display significantly more weight gain compared with DR rats, $P_{Trend} < 0.001$. B, rats were palpated twice weekly for the detection of mammary tumors. Data were subjected to Kaplan–Meier survival analysis with the log-rank (Mantel–Cox) test, and cancer-free survival (survivor function) for DS rats was markedly reduced compared with DR rats (censored: DR, $n = 39$; DS, $n = 9$). Mammary adenocarcinoma incidence was increased in DS compared with DR rats, $P < 0.001$. C, cancer multiplicity (#/rat) was analyzed by Poisson regression; data, mean ± 95% CI. Multiplicity was significantly elevated in DS compared with DR rats, $P < 0.001$.

assessed for estrogen and progesterone receptor status. The percent of carcinomas that were estrogen/progesterone receptor negative was increased in the DS group (34.9%)

Table 2. Excess weight accelerates mammary carcinogenesis in rats resistant (DR) or susceptible (DS) to diet-induced weight gain

Group	N	Body weight[a] (g)	Incidence[b] (%)	Relative risk[c]	Multiplicity[d] (#/rat)	Burden[e] (g/rat)	Latency[f] (days)
DS	101	217.0 (215.9, 225.2)	91.1 (83.8, 94.8)	1.40 (1.20, 1.63)	4.3 (3.8, 5.0)	7.15 (5.99, 8.31)	39 (35, 41)
DR	103	187.9 (185.1, 190.8)	65.1 (55.0, 74.2)	—	1.7 (1.4, 2.1)	1.33 (0.91, 1.75)	49 (48, 56)

NOTE: Data based on palpable adenocarcinomas.
[a]Values are means (95% CI) at study termination. Groups differ ($P < 0.001$) by an unpaired t test with Welch–Satterthwaite correction.
[b]Values are percentages (95% CI). Groups differ ($P < 0.001$) by a χ^2 test of equal proportions.
[c]Values are point estimates of incidence risk in DS compared with DR (95% CI), from the χ^2 test of equal proportions.
[d]Values are means (95% CI). Groups differ ($P < 0.001$) by Poisson regression.
[e]Values are medians (95% CI). Groups differ ($P < 0.001$) by 2-stage model combining χ^2 (incidence) and log-linear (burden) P values.
[f]Values are point estimates at 50% quartile (95% CI of linear-transformed survivor functions). Survivor functions differ ($P < 0.001$) by the log-rank (Mantel–Cox) test. Censored: DR, $n = 39$; DS, $n = 9$.

versus DR group (22.8%), although this difference did not achieve statistical significance [$P = 0.134$; odds ratio = 1.78; 95% confidence interval (CI), 0.83–3.81].

DS rats display widespread alterations in circulating analytes

Given previous reports describing metabolic alterations of DS rats, we evaluated circulating levels of a broad panel of analytes in plasma from DR ($n = 10$) and DS ($n = 10$) rats, with values reported in Table 3. DS rats displayed statistically significant elevations in fasted plasma insulin, IGF-1, IGF-1:IGFBP-3 ratio, leptin, and adiponectin, with a concomitant reduction in adiponectin:leptin ratio versus DR rats. Calculated insulin resistance, estimated through HOMA-IR, was elevated in DS versus DR rats. A trend toward elevated TNF-α and estradiol in DS rats was observed but differences between these and other evaluated analytes did not reach statistical significance.

Discussion

This study characterizes a preclinical rat model designed to interrogate the effects of excess weight and fat mass accumulation on breast cancer risk. This preclinical model was developed in response to a recent report by Cecchini and colleagues in which high-risk women ages ≥35 years enrolled in the Breast Cancer Prevention P-1 Trial with BMI ≥25 had substantially elevated risk of invasive breast cancer compared with women with BMI <25. Cecchini and colleagues concluded that overweight and obesity are not protective among premenopausal women in a high-risk population, and that the relationship between BMI and breast cancer may not be the same for all women (10). The Cecchini study challenges currently held conventions in the field of premenopausal breast cancer research (reviewed in ref. 9), and has paradigm-shifting potential as it provokes a shift in thinking away from considering individuals with elevated BMI as a homogeneous population, and toward viewing this population as highly diverse with wide variance in diet,

lifestyle, genetics, and biology, and the accompanying risk tiers that these factors, in addition to BMI, confer.

In preclinical studies, use of a carcinogen to induce cancer simulates a population of individuals at increased risk in that whereas carcinogen treatment establishes risk, the biology of each animal determines the response. Not all animals treated with a chemical carcinogen develop cancer; rather the process is stochastic in nature. Using the selectively outbred Levin strains of diet-induced excess weight gain susceptible (DS) or resistant (DR) rats, we evaluated the effects of excess weight gain on development of mammary cancer. DS rats gained excess weight despite both strains consuming identical SUMO32 diet; thus, these strains allow for investigation of excess weight gain independent of dietary macronutrient composition, unlike models of diet-induced obesity that use high-fat formulations for the obese group and low-fat formulations for the referent control.

In this study, *ad libitum* feeding of SUMO32 diet resulted in a 15.5% difference in body weights between DS and DR rats, a relevant comparison to the clinical population as stratified by BMI tiers for normal weight (BMI <25 kg/m^2), overweight (25.0–29.9 kg/m^2), or obese (≥30.0 kg/m^2) women. For example, a 5 feet 4 inch adult woman weighing 140 lbs with BMI 24.0 kg/m^2 is considered normal weight, whereas a 5 feet 4 inch woman weighing 161.7 lbs (15.5% increase) with BMI 27.8 kg/m^2 is considered overweight and, if preexisting risk factors are present, may be at increased risk of breast cancer compared with the normal weight woman (HR = 1.59), based on Cecchini's findings (10). This elevated risk conferred by excess weight was recapitulated in this study, in which moderate excess weight gain in DS rats (15.5%) was accompanied by elevated risk of mammary adenocarcinoma (relative risk, 1.40), a level of risk strikingly similar to the value reported in the clinical study. DS rats displayed marked acceleration of mammary carcinogenesis compared with DR rats, with statistically significant increases in cancer incidence (26% increase), multiplicity (2.5-fold increase), and tumor

Table 3. Rats susceptible to diet-induced excess weight gain (DS) display alterations in circulating factors compared with dietary-resistant (DR) rats

Analyte/analyte ratio	DS	DR	Fold change[a]	t statistic[b]	P
Insulin signaling					
Insulin (pg/mL)	1402.4 ± 851.9	398.9 ± 577.5	3.5	4.12	**<0.001**
Glucose (mg/dL)	136.4 ± 78.7	119.1 ± 63.5	1.1	0.64	0.53
HOMA-IR	12.0 ± 6.6	4.6 ± 7.9	2.6	3.64	**0.003**
IGF-1 (ng/mL)	285.7 ± 28.1	235.3 ± 39.5	1.2	3.24	**0.005**
IGFBP-3 (ng/mL)	82.7 ± 14.0	80.6 ± 12.9	1.0	0.36	0.73
IGF-1:IGFBP-3[c]	14.6 ± 2.0	12.3 ± 2.3	1.2	2.36	0.03
Adipokines					
Leptin (ng/mL)	5.6 ± 1.4	0.9 ± 0.8	5.9	7.15	**<0.001**
Adiponectin (μg/mL)	31.8 ± 13.3	17.1 ± 4.7	1.9	3.63	**0.002**
Adiponectin:leptin[c]	0.5 ± 0.3	3.3 ± 2.8	0.2	4.21	**0.001**
Inflammatory cytokines					
CRP (μg/mL)	695.3 ± 315.0	617.1 ± 200.5	1.1	0.65	0.52
IL-1β (pg/mL)	17.3 ± 25.7	7.8 ± 9.6	2.2	1.01	0.33
IL-6 (pg/mL)	46.3 ± 43.44	111.2 ± 235.6	0.4	0.31	0.76
TNF-α (pg/mL)	3.3 ± 1.0	2.6 ± 0.9	1.3	1.65	0.12
Sex steroid hormones					
Estradiol (pg/mL)	46.1 ± 11.7	39.8 ± 8.6	1.2	1.37	0.19
Progesterone (ng/mL)	6.3 ± 4.1	5.2 ± 2.4	1.2	0.48	0.64
SHBG (nmol/L)	12.8 ± 4.7	14.5 ± 7.7	0.9	0.25	0.81
SHBG:estradiol[c]	84.2 ± 47.8	99.3 ± 49.9	1.1	0.86	0.40
Progesterone:estradiol[c]	126.7 ± 91.5	113.1 ± 41.2	1.1	0.13	0.90
Progesterone:estradiol:SHBG[c]	10.8 ± 8.9	9.5 ± 5.3	1.1	0.06	0.95

NOTE: Circulating analytes in plasma from DS ($n = 10$) and DR ($n = 10$) rats, presented as means $\pm$ SD. Analytes were log-transformed before analysis and the Welch–Satterthwaite method was used as necessary to satisfy statistical assumptions.

Abbreviations: CRP, C-reactive protein; HOMA-IR, homeostasis model assessment-estimated insulin resistance; IGF-1, insulin-like growth factor-1; IGFBP-3, IGF binding protein-3; IGF-1:IGFBP-3, ratio of IGF-1 to IGFBP-3; SHBG, sex hormone binding globulin; estradiol:SHBG, ratio of estradiol to SHBG; estradiol:progesterone, ratio of estradiol to progesterone; estradiol:progesterone:SHBG, ratio of estradiol to progesterone to sex hormone binding globulin.

[a]Fold change is mean DS value in relation to mean DR value.

[b]t statistics are absolute values.

[c]Analyte concentrations were converted to molarity before ratio determination.

burden (5.4-fold increase) with a concomitant reduction (16% earlier detection) in cancer latency in DS rats. In addition, a trend toward an increased proportion of estrogen/progesterone receptor negative mammary carcinomas was observed in the DS group (11.7% increase compared with DR rats), a finding that parallels the 14% increase in estrogen/progesterone receptor negative breast cancer reported in the clinical study (10). Also of interest was the dramatic increase in tumor burden in DS compared with DR rats, as multiple reports describe a trend toward larger breast tumors in obese women compared with normal weight women (35, 36). As the carcinogen responsiveness of the Levin rat strains was unknown, these studies utilized very large sample sizes; however, future studies seeking to declare differences in tumor incidence between DR and DS groups are estimated to be well powered (>80.8% power) with $n = 40$ rats per group. Studies in which multiplicity is the primary endpoint are estimated to

be well powered (>95% power) with $n = 20$ rats per group, due to the magnitude of the difference in tumor multiplicity between groups.

Although this study identified alterations in a broad panel of circulating analytes in DS compared with DR rats (Table 3), a great deal of further investigation is required to establish the mechanisms driving this phenomenon. However, we would be remiss if we failed to briefly examine results of the initial assessment of plasma biomarkers in the context of multiple mechanisms that have been proposed to explain the relationship between obesity and breast cancer, including altered adipokine and growth factor signaling, chronic inflammation, and increased production of sex hormones (reviewed in ref. 37–40).

Statistically significant elevations in circulating insulin, IGF-1, and IGF-1:IGFBP-3 ratio were observed in plasma from DS compared with DR rats. Moreover, as an indirect assessment of insulin resistance, HOMA-IR was elevated

in DS rats, suggesting that insulin and insulin-related signaling may be deregulated in DS rats. Several studies report that insulin resistance is an independent risk factor for breast cancer in clinical populations and is positively correlated with tumor angiogenesis and metastasis (reviewed in ref. 40). Furthermore, Calori and colleagues reported that obese insulin-resistant individuals had elevated risk of cancer mortality (HR = 1.52) compared with obese insulin-sensitive individuals (HR = 1.04; ref. 41), again suggesting that a population of individuals with elevated BMI is heterogeneous with an array of associated risk. In the Cecchini study, a greater percentage of obese (5.9%) and overweight (2.3%) women were diabetic than lean women (1.4%); however, full multivariable adjustment for several variables, including history of diabetes, only minimally reduced HRs from the final multivariable assessment of 1.59 and 1.70 for overweight and obesity to 1.55 and 1.66, respectively (10), suggesting that other factors or interaction of factors are influencing these risk ratios.

Another key finding of this study was the nearly 6-fold elevation of leptin in DS compared with DR rats. Leptin is strongly correlated with adiposity (33, 34). Although we did not measure adiposity *per se*, Levin has previously reported elevations in circulating leptin in 12-week-old male DS animals of a similar magnitude as found in our 12-week-old females (male rats: DS, 5.1 ng/mL; DR: 1.8 ng/mL), which was associated with a 2- to 4-fold increase in individual fat pad mass and a cumulative doubling of total adiposity compared with DR rats (21). Breast tumor expression of leptin receptor in conjunction with elevated circulating leptin has been associated with poor prognosis and tumor metastasis (42, 43); conversely, studies examining the relationship between circulating adiponectin and breast cancer risk in both premenopausal and postmenopausal women report mixed outcomes (44–46). In this study, we observed increased levels of circulating adiponectin with a concomitant reduction in the ratio of adiponectin:leptin in DS compared with DR rats. Although the elevated adiponectin in DS rats was unexpected, previous studies utilizing obese (fa/fa) Zucker rats have reported elevated levels of circulating adiponectin with reduced tissue expression of the adiponectin receptor 1 in obese versus lean animals, whereby adiponectin signaling may be impaired despite elevated circulating levels (47). Furthermore, it has recently been suggested that the adiponectin:leptin ratio, rather individual adipokine levels, may be more informative for evaluating breast cancer risk profile (37, 48).

Both chronic low-grade inflammation and increased conversion of androgen to estrone by the cytochrome P450 aromatase in peripheral fat tissues have been implicated in mammary carcinogenesis, the latter particularly in the development of hormone-responsive tumors (49, 50); interestingly, Cecchini and colleagues report that BMI was significantly associated with elevated risk of estrogen receptor-positive tumors in premenopausal women. Although inflammatory cytokines and sex hormones were not significantly altered at the systemic level in the current study, effects on the carcinogenic process were observed at an early stage of body fat accumulation. The published work of Levin indicates that DS rats continue to accumulate both central and peripheral adipose tissue as well as in tissues such as the liver (21). Trends in both preclinical and population data suggest that inflammation and sex hormones may play a role in tumor promotion and progression. An advantage of our carcinogenesis model is the relative ease with which carcinogen dose is modified and hence rate of tumor development, and consequently study duration and fat accumulation, is regulated, enabling future investigation of the effects of early, intermediate, and late-phase adiposity-associated metabolic sequelae on the carcinogenic process.

In conclusion, this study describes a model with potential value to a wide range of researchers, from basic scientists to clinicians, in which to study the impact of excess weight and adiposity on mammary carcinogenesis. Furthermore, the findings highlight the potential importance of identifying and characterizing at-risk groups, which stratify a population with elevated BMI. The Levin strains of selectively outbred DR and DS rats in conjunction with the rapid emergence model of mammary carcinogenesis provide a platform on which to conduct preclinical research into the effects of excess weight gain on a population at high risk of malignancies of the breast as recently reported by Cecchini, and represent rapid and cost-effective preclinical tools with high relevance to clinical populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.B. Matthews, Z. Zhu, W. Jiang, H.J. Thompson
Development of methodology: S.B. Matthews, Z. Zhu, W. Jiang, H.J. Thompson
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.B. Matthews, Z. Zhu, W. Jiang, J.N. McGinley, E.S. Neil, H.J. Thompson
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.B. Matthews, Z. Zhu, W. Jiang, J.N. McGinley, H.J. Thompson
Writing, review, and/or revision of the manuscript: S.B. Matthews, Z. Zhu, W. Jiang, J.N. McGinley, E.S. Neil, H.J. Thompson
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.B. Matthews, Z. Zhu, J.N. McGinley, E.S. Neil
Study supervision: Z. Zhu, H.J. Thompson

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Cancer Prevention Research

Excess Weight Gain Accelerates 1-Methyl-1-Nitrosourea–Induced Mammary Carcinogenesis in a Rat Model of Premenopausal Breast Cancer

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