Dietary Modulation of Pregnancy Estrogen Levels and Breast Cancer Risk among Female Rat Offspring

Leena Hilakivi-Clarke, Eliasabeth Cho, Ana Cabanes, Sonia DeAssis, Susan Olivo, William Helferich, Marc E. Lippman, and Robert Clarke

Department of Oncology, Lombardi Cancer Center, Washington, DC 20007 [L. H.-C., E. C., A. C., S. D., S. O., R. C.]; Department of Nutrition, University of Illinois, Urbana, Illinois 61801 [W. H.]; and Department of Medicine, University of Michigan, Ann Arbor, Michigan 48109 [M. E. L.]

ABSTRACT

Purpose: Against the hypothesis that high estrogen levels in utero increase the risk of developing breast cancer in later life are data showing that pregnancy estrogen levels are significantly higher in Asian women who have low breast cancer risk than in Caucasian women. We investigated whether maternal dietary intake of genistein or n-3 polyunsaturated fatty acids (PUFAs), which are typical to Asian but not Caucasian diet, affect pregnancy estrogen levels and susceptibility to mammary tumorigenesis among offspring.

Experimental Design: For that purpose, pregnant female Sprague Dawley rats were fed isocaloric AIN-93-based diets containing either at 15 mg (low), 150 mg (medium), or 300 mg (high)/kg genistein/diet or low- or high-fat (16 versus 39% energy from fat) diet composed either of n-3 PUFA menhaden oil or n-6 PUFA corn oil. All diets were switched to regular AIN-93 diet when pups were born.

Results: Maternal intake of n-3 PUFA diets significantly increased pregnancy 17β-estradiol (E2) levels (48% increase when compared with high n-6 PUFA diet; \( P < 0.0045 \)). High genistein exposure also increased pregnancy estrogen levels, but the increase did not reach statistical significance (\( P < 0.14 \)). The offspring of high-fat n-3 PUFA-consuming dams were significantly less likely to develop 7,12-dimethylbenz(a)anthracene-induced mammary tumors (38% of these rats developed tumors during week 17 versus 64% of high n-6 PUFA offspring; \( P < 0.003 \)). Maternal genistein intake did not affect offspring’s tumor incidence. The mammary glands of high fat n-3 PUFA offspring contained more lobules (\( P < 0.07 \)) and were thus more differentiated, whereas the glands of high genistein offspring contained more terminal end buds (\( P < 0.0015 \)), which are the sites of malignant transformation.

Conclusions: Our findings indicate that the elevated estrogen levels in the n-3 PUFA mothers were linked to reduced rather than increased breast cancer risk among their offspring, suggesting that other effects of n-3 PUFA may counteract the effects of high fetal estrogenicity on the mammary gland. High maternal genistein intake did not reduce offspring’s breast cancer risk, and therefore high maternal soy intake in Asian women may not be associated with daughters’ low breast cancer risk.

INTRODUCTION

Some breast cancers may be preinitiated already in utero by an exposure to high levels of fetal estrogens (1). For example, high birth weight or being a twin, both of which are indicators of elevated in utero estrogen exposure (2, 3), are associated with an increased risk of developing breast cancer (4–8). At a particularly high risk are twins whose birth weight was high (9). Consistent with these data, low maternal estrogen levels may reduce breast cancer risk in daughters. Women whose mothers suffered from pre-eclampsia during pregnancy, which is associated with low pregnancy estrogen levels, are at a reduced risk to develop breast cancer (10). Animal studies also support the hypothesis. Maternal exposure to either E2 or the synthetic estrogen diethylstilbestrol increases an offspring’s breast cancer risk (11, 12). Furthermore, maternal estrogen levels can be modified by diet to affect an offspring’s mammary tumor incidence. For example, exposure to a high-fat n-6 PUFA diet elevates pregnancy estrogen levels (11, 13) and increases both spontaneous mammary tumorigenesis in mice (14) and carcinogen-induced mammary tumorigenesis in rats (11).

The hypothesis that high in utero estrogen levels increase breast cancer risk has recently been challenged by the observation that Asian women, whose breast cancer risk is low, have significantly higher estrogen levels during pregnancy than Caucasian women (15, 16). It is not known why their pregnancy estrogen levels are high; among nonpregnant women, circulating estrogens are 40% lower in Asian than Caucasian women (17–19). One distinct difference between these women is their diet. Asians consume high levels of soy protein (20) and n-3

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2 To whom requests for reprints should be addressed, at Research Building, Lombardi Cancer Center, Room W-405, Georgetown University, 3970 Reservoir Road, Northwest, Washington, DC 20007-2197. Phone: (202) 687-7237; Fax: (202) 687-7505; E-mail: Clarkel@gunet.georgetown.edu.

3 The abbreviations used are: E2, estradiol; PUFA, polyunsaturated fatty acid; TEB, terminal end bud; DMBA, 7,12-dimethylbenz[a]anthracene; COX-2, cyclooxygenase 2; PPAR, peroxisome proliferator-activated receptor.
fatty acids, which are present in fish and other marine products (21), whereas Caucasian diets are generally low in these dietary components. Both higher soy (or more specifically the phytoestrogen genistein present in soy) and n-3 PUFA intakes have been linked to reduced breast cancer risk, although the evidence is inconsistent (22, 23). Furthermore, these dietary components may reduce circulating estrogen levels in nonpregnant women (24–27). We investigated whether maternal exposure to either genistein in soy or n-3 PUFAs influences pregnancy estrogen levels and offspring’s breast cancer risk.

To understand how maternal diet and/or in utero estrogenicity mediate their effects on offspring’s breast cancer risk, we also monitored changes in mammary gland morphology. In rats, carcinogens interact with TEB structures to produce malignant breast tumors (28). Corresponding structures in the human breast (terminal ductal lobular units) are the sites in which most breast tumors are initiated (29). Earlier studies in rats have shown that in utero estrogenic exposures increase mammary epithelial density and the number of TEBs (11, 30). Human studies indicate that women who had high birth weight have increased breast epithelial density (31), which in turn is associated with increased breast cancer risk (32). Therefore, we investigated whether maternal genistein or n-3 PUFA diet intake alters mammary gland development in female offspring.

**MATERIALS AND METHODS**

**Animals.** Ten-week-old Sprague Dawley rats were obtained from Charles River. Upon arrival, they were fed American Institute of Nutrition (AIN-93)-based diets. All rats were housed in groups of 5/cage, males and females separately, in standard rat plexiglas cages at a constant temperature and humidity under a 12-h light-dark cycle (lights on 06:00 h). Studies were performed in accordance with the appropriate institutional and federal regulations.

**Maternal Dietary Exposures**

- **n-3 PUFA.** Animal feeds were prepared commercially by Bioserv (Frenchtown, NJ) to our specifications. Upon arrival, 43 female rats were assigned to four groups (\( n = 10–13/\text{group} \)) consuming AIN-93-based diets containing either high (39% energy from fat) or low (16% energy from fat) levels of n-3 PUFA or n-6 PUFA. Menhaden oil was the source of n-3 PUFA, and corn oil was the source of n-6 PUFA. These diets are described in Table 1. The low-fat n-6 PUFA diet is similar to the standard AIN-93 diet, except that the source is soybean oil that contains more n-3 PUFAs than corn oil. Because menhaden oil does not contain enough n-6 PUFAs for adequate fetal growth, all four diets were composed of both menhaden and corn oil in appropriate ratios. The diets were isoenergetic. After the females had been on the special diets for 7 days, they were mated by housing 1–2 female rats with one male. Male rats were removed 19 days later and females were housed singly. On the day of labor, all animals were switched to a low-fat n-6 PUFA control diet (AIN 93 diet).

**Genistein.** Dietary modifications were slightly different from described above. Upon arrival, all animals were fed control AIN-93 diet for 7 days. Sixty female rats were then switched to one of three AIN-93-based formulations containing either low, medium, or high levels of genistein in the same amount of soy isolate (\( n = 17–23/\text{group} \)) and mated a few days later. Each soy isolate was added at 20% of the diet as the sole source of protein. The soy-based diet provided 2% protein, and 2.2 g of methionine were added to each kilogram of soy isolate diet so that all of the essential amino acids requirements were met. The soy protein isolate (Protein Technologies International, St. Louis, MO) provided three different levels of genistein: 0.075; 0.75; and 1.5 mg of genistein (aglycone equivalents)/g of product. The final concentrations of genistein in the three soy isolate diets were 15, 150, and 300 mg genistein/kg diet, respectively. Previous studies indicate that serum genistein levels in animals consuming medium or high genistein containing soy isolate diet are at same range than those seen in Asians consuming high levels of soy (33, 34). Males were removed 19 days later and females were housed singly. When female rats gave birth, their diets were switched back to the control AIN-93 diet.

**Table 1** Nutritional composition of maternal n-3 and n-6 PUFA diets

<table>
<thead>
<tr>
<th>Ingredients (g)</th>
<th>16% fat: Low fat</th>
<th>39% fat: High fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn oil</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>Menhaden oil</td>
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<td>5</td>
</tr>
<tr>
<td>Protein</td>
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<td>240</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>530</td>
<td>420</td>
</tr>
<tr>
<td>Fiber</td>
<td>52.5</td>
<td>102.5</td>
</tr>
<tr>
<td>Alphacel</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>AIN mineral mix</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AIN vitamin mix</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.014</td>
<td>0.014</td>
</tr>
<tr>
<td>TBHQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total grams</td>
<td>1,000</td>
<td>1,000</td>
</tr>
<tr>
<td>Kcal density/g</td>
<td>3.8</td>
<td>4.1</td>
</tr>
<tr>
<td>% kcal from fat</td>
<td>16</td>
<td>39</td>
</tr>
<tr>
<td>% kcal from protein</td>
<td>26.4</td>
<td>22</td>
</tr>
<tr>
<td>% kcal from carbohydrates</td>
<td>54</td>
<td>39</td>
</tr>
</tbody>
</table>

**Postnatal Care**

Pups were cross-fostered on postnatal day 2 by removing all male pups and housing female pups of 3–4 different litters with one nursing dam. This was done to randomize the genetic background or its interaction with maternal exposure. Pups housed with a surrogate dam were all born to dams that were fed the same diet than the surrogate mother was during pregnancy. Pups were weaned on postnatal day 22.

**Serum E2 Levels in Pregnant Rats and Their Offspring**

A separate set of 36 female Sprague Dawley rats arrived in our laboratory on gestation day 7. Upon arrival, they were divided into 6 groups, which were fed either a low or high menhaden oil diet or a high corn oil diet (maternal dietary fat exposures), or diets containing low, medium, or high amount of genistein in soy protein isolate (maternal genistein exposures).
Diets were those described above. On gestation day 18 (PUFAs) or 19 (genistein), the pregnant female rats were anesthetized using methoxyflurane inhalant to obtain their blood by cardiac puncture and killed immediately afterward by cervical dislocation.

In the offspring (n = 5–7/group), blood was obtained at the ages of 3 (genistein exposure only) and 8 weeks. On the third postnatal week, none of the female rats had vaginal openings, indicating that they had not undergone puberty and had not yet started their estrus cycling. On the eighth postnatal week, uterine morphology was used to determine estrus stage for each rat. For the final analysis, E2 levels obtained from blood samples of rats that were in proestrus were excluded because E2 levels are known to peak at this stage but be relatively similar in the other estrus stages. Proestrus was characterized by thick uterine horns that were filled with fluid. The smears obtained in proestrus consisted mainly of nucleated cells, with some markedly swollen that were filled with fluid. The smears obtained in proestrus were excluded because E2 levels are known to peak at this stage but be relatively similar in the other estrus stages. Proestrus was characterized by thick uterine horns that were filled with fluid. The smears obtained in proestrus consisted mainly of nucleated cells, with some markedly swollen.

Serum was separated, frozen, and kept in −80°C until assayed. Total serum 17β-E2 concentrations were determined using a specific double antibody kit from ICN Biomedicals, Inc. (Irvine, CA), according to the manufacturer’s instructions. Serum samples were analyzed in four batches containing blood obtained from pregnant rats exposed genistein or PUFAs or from their offspring. The intra-assay coefficient for the duplicates for each sample was 6.7–7.0%.

**Mammary Wholemounts**

Wholemounts of the fourth abdominal glands of 3- and 8-week-old female offspring/group were prepared. None of these animals were exposed to a carcinogen. The removed glands were stained with carmine aluminum after a procedure developed by Dr. Banerjee, referenced by Haslam et al. (35). Analysis of mammary epithelial structures in the wholemounts was based on visual evaluation under an Olympus dissecting scope, using a visual scale we have developed (30). The following characteristics of the coded mammary glands were evaluated using a 6-point scale (from 0 = absent, 5 = numerous): (a) density of epithelial ducts; (b) density of alveolar buds; and (c) density of lobules. The total number of TEBs were also counted. These various epithelial structures are well-characterized by Russo and Russo (28). Mammary gland morphology was evaluated blindly by two investigators who were reviewing the glands together.

**Inducing Mammary Tumorigenesis**

Mammary tumors were induced by administration of 10 mg of (~50 mg/kg body weight) DMBA (Sigma, St. Louis, MO) to 47-day-old female rats (n = 23–27/group). This is a suboptimal dose that induces tumors in approximately two-thirds of the control group, thus enabling assessments of both reductions and increases in the endpoints of tumorigenicity. More than 75% of the tumors induced by 10 mg of DMBA are adenocarcinomas (36); in our previous experiments, the proportion of adenocarcinomas in the control group was 80–100% (37, 38). The carcinogen was dissolved in peanut oil and administered by oral gavage in a volume of 1 ml.

The animals were examined for mammary tumors by palpation once per week. The end points for data analysis were: (a) latency to tumor appearance; (b) the number of animals with tumors (tumor incidence); (c) the number of tumors per animal (tumor multiplicity); and (d) the number of animals surviving until the end of the follow-up period. During the follow-up, those animals in which tumor burden approximated 10% of total body weight were sacrificed, as required by our institution. All surviving animals, including those that did not appear to develop mammary tumors, were sacrificed 17 weeks after carcinogen administration.

**Statistical Analyses**

Results for the data obtained on (a) pregnancy and other reproductive function-related parameters (uterine wet weight, serum E2 levels) and body weight, (b) mammary gland morphology (total number of TEBs and density of epithelial tree, alveolar buds, and lobules in the whole mounts), and (c) some mammary tumor end points (latency and multiplicity) were analyzed using one- or two-way ANOVA. Where appropriate, between-group comparisons were done using Fisher’s Least Significant Difference test. Differences in the tumor incidence during week 17 and the number of surviving animals were determined using a χ² test. The time to tumor presentation was measured as the number of weeks from DMBA exposure to the time first tumor per animal could be palpated. Estimations of tumor presentation were calculated by the methods developed by Kaplan and Meier (39). Differences among the treatment arms were tested using an extension of the log rank test and both Gehan and Peto’s generalized Wilcoxon tests as implemented in STATISTICA (40). The differences were considered significant if the P < 0.05. All probabilities were two-tailed.

**RESULTS**

**Effect on Pregnancy**

The effect of different diets on maternal weight gain, percentage of successful pregnancies, gestation length, offspring’s birth weight, and litter sizes were monitored.

**PUFA Diets.** Gestation was longer in the animals exposed to low or high-fat n-3 PUFA diets or high-fat n-6 PUFA diet (which contained 3.5, 7, or 1.5 g/100 g food of menhaden oil, respectively) than in the animals exposed to low-fat n-6 PUFA diet (contained 0.5 g/100 g food of menhaden oil). However, these differences did not reach statistical significance. No significant differences were noted in the other pregnancy-related parameters either among the four groups that were fed either high or low fat diets composed of mixtures of corn and menhaden oil (Table 2a). There was a tendency for more female offspring to be born to dams kept on the low-fat n-6 PUFA diets than to the other dams, but the difference did not reach statistical significance.

**Genistein.** Maternal exposure to diets containing three different levels of genistein did not significantly affect pregnancy, as shown in Table 2b. Rats fed the highest level of genistein tended to have the lowest number of successful pregnancies, but the difference was not statistically significant. Pregnancy weight gain, length of pregnancy, or number of pups per litter were all similar in the three groups.
Effects on Offspring’s Body Weight Gain

Neither maternal exposure to a high or low PUFA diet composed of menhaden and corn oil or maternal exposure to genistein affected postnatal body weights (Table 2, a and b).

Pregnancy E2 Levels

PUFA Diets. Pregnant rat dams exposed to menhaden oil diets, either at low or high total energy level, had significantly higher E2 levels on day 18 of gestation than those dams exposed to high-fat corn oil diet [F (2, 15) = 8.0, P < 0.0045; Fig. 1a].

Genistein. When determined on gestation day 19, genistein feeding during pregnancy dose dependently increased serum E2 levels, although the difference among the three groups in one-way ANOVA test did not reach statistical significance [F (2, 15) = 2.26, P < 0.14; Fig. 1b].

E2 Levels in Offspring

PUFA Diets. Serum E2 levels in the offspring were determined only during week 8. The results indicated that the levels were significantly lower in the offspring of mothers kept on the high-fat n-6 PUFA diet during pregnancy than in the offspring of mothers kept on the other three diets [F (1, 13) = 6.91, P < 0.02; Fig. 2a].

Genistein. Serum E2 levels were determined before puberty onset during weeks 3 and 8. During week 3, no differences in circulating E2 levels were seen in pups whose mothers were exposed to different amounts of genistein during pregnancy. However, in adult rats, serum E2 levels were significantly lower in the offspring of high genistein fed mothers when compared with offspring of low genistein fed mothers [F (2, 10) = 4.33, P < 0.045; Fig. 2b]. It is to be noted that we only assessed total E2 levels and, therefore, cannot determine whether the levels of free estrogens were affected.

Effects on Mammary Gland Morphology

PUFA Diets. Mammary gland morphology was examined during postnatal weeks 3 and 8. During week 3, the glands obtained from offspring of dams fed a high-fat n-6 PUFA diet contained significantly more TEBs than the glands of the three other dietary groups [F (3, 11) = 4.77, P < 0.02; Fig. 3a]. Density of the epithelial tree was also different among the groups [F (3, 13) = 4.29, P < 0.03]. Glands of the high-fat n-6 PUFA offspring were the most dense; glands of high-fat n-3 PUFA offspring were the least dense (Fig. 3b).

By week 8, the glands of the offspring whose mothers were fed the high-fat n-3 PUFA diet contained significantly fewer TEBs than the glands of the other three groups [F (3, 19) = 11.4, P < 0.002]. Density of lobules was also altered. Offspring of n-3 PUFA fed mothers had more lobules than the offspring of

Table 2a Pregnancy-related parameters in rat dams fed different types of PUFA diets during pregnancy and developmental parameters in their pups. The values are means ± SE. No significant differences were observed between the groups.

<table>
<thead>
<tr>
<th>Maternal diets</th>
<th>Low n-3</th>
<th>High n-3</th>
<th>Low n-6</th>
<th>High n-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mated female rats</td>
<td>10</td>
<td>13</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>No. of litters</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>% successful pregnancies</td>
<td>100%</td>
<td>70%</td>
<td>70%</td>
<td>80%</td>
</tr>
<tr>
<td>Weight gain during pregnancy (g)</td>
<td>155.6 ± 3.0</td>
<td>161.3 ± 4.6</td>
<td>159.3 ± 5.3</td>
<td>168.8 ± 1.9</td>
</tr>
<tr>
<td>Length of pregnancy (days)</td>
<td>24.4 ± 0.5</td>
<td>25.1 ± 0.5</td>
<td>23.7 ± 0.5</td>
<td>25.3 ± 0.3</td>
</tr>
<tr>
<td>Number of pups/litter</td>
<td>13.6 ± 0.8</td>
<td>13.3 ± 0.8</td>
<td>13.6 ± 0.5</td>
<td>13.2 ± 1.0</td>
</tr>
<tr>
<td>Pup weight (g)</td>
<td>8.8 ± 0.5</td>
<td>8.5 ± 0.4</td>
<td>8.8 ± 0.2</td>
<td>8.7 ± 0.6</td>
</tr>
</tbody>
</table>
| on day 2
| 33.2 ± 0.5 | 34.3 ± 0.4 | 33.0 ± 0.6 | 34.6 ± 0.7 |
| on day 14
| 47.4 ± 3.9 | 42.8 ± 4.11 | 60.0 ± 3.3 | 46.3 ± 5.7 |

% females per litter

Table 2b Pregnancy-related parameters in rat dams fed low, medium, or high genistein diet during pregnancy and developmental parameters in their pups. The values are means ± SE. No significant differences were observed between the groups.

<table>
<thead>
<tr>
<th>Maternal treatments</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mated female rats</td>
<td>23</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>No. of litters</td>
<td>16</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>% successful pregnancies</td>
<td>70%</td>
<td>71%</td>
<td>55%</td>
</tr>
<tr>
<td>Weight gain during pregnancy (g)</td>
<td>98.3 ± 5.5</td>
<td>95.3 ± 8.2</td>
<td>106.3 ± 5.0</td>
</tr>
<tr>
<td>Length of pregnancy (days)</td>
<td>23.7 ± 0.4</td>
<td>23.7 ± 0.4</td>
<td>23.4 ± 0.5</td>
</tr>
<tr>
<td>Number of pups/litter</td>
<td>11.4 ± 0.5</td>
<td>11.5 ± 0.6</td>
<td>12.9 ± 0.8</td>
</tr>
<tr>
<td>Pup weight (g)</td>
<td>7.7 ± 0.1</td>
<td>7.1 ± 0.2</td>
<td>7.0 ± 0.1</td>
</tr>
</tbody>
</table>
| on day 2
| 9.3 ± 0.6 | 9.3 ± 0.4 | 8.8 ± 0.3 |
| on day 14
| 32.9 ± 1.3 | 32.9 ± 1.6 | 32.5 ± 0.8 |
| on day 21
| 46.7 ± 1.2 | 47.3 ± 1.8 | 47.3 ± 1.8 |
| % females per litter | 56.0 ± 10.5 | 52.7 ± 6.2 | 46.6 ± 4.8 |
n-6 PUFA fed mothers, but the difference was of borderline statistical significance [F (3, 19) = 2.83, P = 0.07].

Genistein. Maternal exposure to the high genistein diet during pregnancy affected mammary gland development in the offspring but only in the 8-week-old animals. At that age, the high genistein offspring had more TEBs than the other two groups [F (2, 14) = 10.8, P = 0.0015; Fig. 3b]. The glands of the medium genistein group also contained more TEBs than the glands of the low genistein group, but the difference did not reach statistical significance. The density of lobules was significantly lower in the offspring of dams fed high genistein diet during pregnancy than in the offspring of low or medium genistein fed dams [F (2, 15) = 6.6, P < 0.009]. Changes in the density of the epithelial tree were also detected. However, the difference occurred only between the medium and high genistein offspring, and no differences were noted when either one of these groups were compared with the low genistein offspring.

Effects on Mammary Tumorigenesis

PUFA Diets. Mammary tumors began to appear 7 weeks after carcinogen administration, consistent with other reports (28). The animals were followed for 17 weeks and then sacrificed. By week 17, 48% of the rats in the low-fat n-6 PUFA group, 52% of the rats in the low-fat n-3 PUFA group, 64% of the animals in the high-fat n-6 PUFA group, and 38% of the rats in the high n-3 PUFA group had developed mammary tumors (χ² = 13.9, df = 3, P < 0.003). Thus, tumor incidence was lowest in the offspring of mothers consuming a high-fat n-3 PUFA diet and highest in the offspring of mothers consuming a high-fat n-6 PUFA diet during pregnancy. Estimations of the time to tumor presentation indicated that the difference in tumor incidence during the 17-week follow-up between the high n-3 PUFA and high n-6 PUFA groups almost reached statistical significance (P = 0.06; Fig. 4a). The difference between the low and high n-6 PUFA groups was not statistically significant (P = 0.10). Menhaden oil in n-6 PUFA diets might have reduced n-6 PUFA’s tumor preinitiating properties seen in an earlier study in which pregnant rats were fed fats composed only of corn oil (11).

The likelihood of survival, as determined by the proportion of rats per group that had to be sacrificed before the end of the 17-week follow-up period, was different among the offspring. Highest survival rates were seen in rats whose mothers consumed a low-fat n-3 PUFA diet during pregnancy (χ² = 18.9, df = 3, P < 0.0003). Survival rates were similar in the offspring of low-fat n-6 or high-fat n-3 PUFA mothers and lowest in the high n-6 PUFA offspring. These results indicate that although
The medium and high genistein groups (Table 3) did not alter tumor incidence, but tumors grew slower than in the other three groups. No differences were noted in the latency of tumor appearance or multiplicity of tumors among the groups (Table 3a).

**Genistein.** Maternal exposure during pregnancy to genistein in soy isolate did not significantly affect the time to tumor presentation among the offspring (Fig. 4b). However, tumor incidence during week 17 was significantly higher in the high genistein group (82% of animals had developed tumors) when compared with the incidence in the low and medium genistein groups (67%; $\chi^2 = 7.44, df = 2, P < 0.025$). The proportion of animals that survived to week 17 was significantly reduced in the medium and high genistein groups ($\chi^2 = 9.92, df = 2, P < 0.007$), indicating that tumors grew faster in these animals. Both latency to tumor appearance and tumor multiplicity were similar in the three groups (Table 3b).

**DISCUSSION**

Results of the present and previous animal and human studies (11, 41) indicate that different dietary components can clearly affect pregnancy estrogen levels. However, high in utero estrogen levels do not consistently increase offspring’s breast cancer risk. We found that maternal dietary intake of n-3 PUFA increased pregnancy estrogens but reduced the risk of developing mammary tumors among the offspring. In contrast, maternal intake of a high genistein diet nonsignificantly increased pregnancy E2 levels and some aspects of an offspring’s mammary tumorigenesis. The findings regarding pregnancy estrogen levels parallel to data obtained in Asian women who consume high levels of fish or soy and have low breast cancer risk. Pregnant Asian women have significantly higher estrogen levels than pregnant Caucasian women (15, 16), whereas nonpregnant Asian women have ~40% lower serum estrogen levels than nonpregnant Caucasian women (17–19). It is to be noted that because we did not investigate whether simultaneous dietary exposure to both n-3 PUFA and genistein affect pregnancy estrogen levels and/or tumorigenesis, our study does not allow evaluation of a possible interaction between the two dietary components.

Although total fat intake may not be linked to circulating estrogens, those women who reduce fat intake exhibit a significant reduction in serum E2 levels (26). In contrast, high dietary n-3 PUFA consumption is inversely linked to low serum E2 levels in postmenopausal women (27). Data showing that high maternal n-3 PUFA intake may increase birth weight (42), and high birth weight is linked to high maternal estrogen levels (2), suggesting that n-3 PUFA consumption may increase pregnancy estrogen levels. In animal studies, a high-fat n-6 PUFA diet increases serum estrogen levels in pregnant rodents when compared with an isocaloric low-fat n-6 PUFA diet (13). The findings obtained in this study suggest that isocaloric diets containing 1:1 (low fat) or 1:2 (high fat) n-3:n-6 PUFA ratio significantly increase pregnancy estrogen levels, compared with a high-fat corn oil diet with 1:12 n-3:n-6 PUFA ratio. It is not immediately apparent why these maternal n-3 PUFA dietary exposures increased pregnancy E2 levels in rats. The mechanisms could include increased placental or maternal/fetal production of estrogens, perhaps via increased aromatase activity, or inhibition of estrogen metabolism, which would lead to accumulation of E2.

The role of dietary fat intake in affecting breast cancer risk is controversial, with results obtained in most cohort studies arguing against any major involvement (43). Nevertheless, some evidence suggests that n-3 PUFA might be protective toward breast cancer in women (44–47), but this is not supported by all studies. In animal models, dietary n-3 PUFA exposure reduces both the growth and metastasis of human breast cancer cells in nude mice (47). High n-3 PUFA intake has also been reported to inhibit the growth of spontaneous or carcinogen-induced mammary tumors in mice and rats (48–51). Our results suggest that maternal exposure to a high-fat n-3 PUFA diet reduces offspring’s mammary tumorigenesis.

In addition to perhaps affecting estrogen levels, n-3 PUFA has many other biological effects (23), some of which might be responsible for reducing breast cancer risk after an in utero exposure. For example, PUFA-induced changes in COX-2 activity and/or PPARγ levels might be important. Overexpression of COX-2 in transgenic mice induces mammary tumorigenesis (51), and it is known that n-6 PUFAs increase and n-3 PUFAs reduce COX-2 activity (52). PUFAs can also directly regulate gene expression by binding to PPARγ, a nuclear transcription factor.
factor and member of the steroid receptor superfamily. n-3 PUFAs have been shown to inhibit transactivation of PPARγ, whereas n-6 PUFAs activate it in MCF-7 and MDA-MB-231 human breast cancer cells (53). However, because PPARγ ligands inhibit estrogen biosynthesis in human breast adipose tissue (54), PPARγ may have therapeutic properties in the treatment of breast cancer. Future studies will determine whether in utero exposures to n-3 or n-6 PUFAs affect mammary tumorigenesis by inducing changes in COX-2 or PPARγ.

Mammary gland morphology is clearly associated with breast cancer risk. For example, high epithelial density increases breast cancer risk by 6–8-fold (32), making it one of the strongest predictors of this disease. High in utero estrogenicity (birth weight) is also associated with increased breast density in women (31). Animal studies show that high in utero estrogenicity may lead to increased mammary epithelial density but also to an increased number of TEBs and/or reduced differentiation to lobular structures (11). We found that the mammary glands of offspring whose mothers were exposed to a high-fat n-3 PUFA diet during pregnancy were more differentiated than those of the high-fat n-6 PUFA offspring. Differentiated mammary glands are not susceptible for chemical carcinogenesis (28), and this could explain the reduced mammary tumor incidence among the high-fat n-3 PUFA offspring.

At the physiological concentrations achieved by dietary consumption of soy, genistein acts as an estrogen, activates the estrogen receptor, and induces proliferation of normal and malignant mammary epithelial cells (22). However, soy intake has also been suggested to reduce serum estrogen levels (17–19), although these findings have not been supported by all studies (55). Furthermore, the reduction may not be caused by genistein because isoflavone/genistein-free diets have a similar effect on serum estrogens than isoflavone-containing diets (56). The impact of soy intake on pregnancy hormone levels in women is not known.

Soy is believed to reduce breast cancer risk, although our recent meta-analysis shows that soy intake has no protective effect in postmenopausal women, providing a modest protective effect (30% reduction in risk) only in premenopausal women (57). Earlier studies (58) have shown that in utero exposure via s.c. maternal injections to genistein increases carcinogen-induced mammary tumorigenesis in the offspring. Neonatal genistein exposure increases the incidence of malignant uterine tumors (59), mimicking the effects of neonatal diethylstilbestrol exposure. In this study, a maternal dietary exposure to high genistein levels in soy protein isolate did not significantly affect mammary tumor development (weekly tumor incidence, tumor latency, or multiplicity). However, this exposure increased the final mammary tumor incidence and shortened offspring survival.

If genistein is an estrogenic compound and in utero exposure increases later risk of developing mammary tumors (58), why do Asian women who consume considerable levels of soy during pregnancy have a reduced breast cancer risk? One explanation is that the genistein in soy has different effects on the breast than genistein alone. Other components in soy may oppose the actions of genistein. This explanation is partially supported by our study showing that only an exposure to high but not medium genistein levels increases some aspects of offspring’s breast cancer risk. Thus, at high genistein concentrations, the protective components in soy may not be able to reverse genistein’s estrogenic properties. Alternatively, Asian women are exposed to soy throughout their life, at least in Asian countries, and exposure during childhood may counteract the effects of in utero exposure. Human and animal studies indicate that prepubertal estrogenic exposures, including genistein, reduce later breast cancer risk (37, 60). Animal studies additionally show that genistein exposure from conception until adulthood reduces mammary tumor multiplicity (61). Thus, prepubertal genistein exposure can reverse the tumor-inducing effects of in utero genistein exposure.

Consumption of soy is known to affect the breast epithelium. Both data obtained in animal models and humans indicate that dietary soy exposure induces epithelial proliferation in normal breast tissue (22). Perhaps not surprisingly, high soy intake has been associated with increased breast density in women (62). Our data indicate that the offspring of mothers consuming a high genistein diet during pregnancy
had significantly more TEBs and less lobules than those consuming a low genistein diet, consistent with earlier results in mice perinatally exposed to genistein (63). Because carcinogens initiate malignant transformation by interacting with the cells in the TEBs (28), the glands of the high genistein offspring would be expected to be more susceptible to carcinogenesis.

In summary, a maternal diet during pregnancy containing high levels of n-3 PUFAs reduced the susceptibility to mammary carcinogenesis among female offspring. In contrast, exposure to genistein only in utero might be harmful because it could increase later breast and ovarian cancer risk (58, 59). An additional concern is findings showing that in utero genistein exposure could potentially increase the risk of infant acute myelogenous leukemia, perhaps by causing site-specific DNA breaks (64, 65). Finally, our results suggest that dietary-induced changes in pregnancy estrogen levels are likely to be only one of the mechanisms that might alter breast cancer risk among female offspring.

REFERENCES

Table 3a Effect of maternal dietary genistein intake on mammary tumor growth among female offspring

Data represent the mean ± SE of latency to tumor appearance, tumor multiplicity, and the proportion of animals that did not survive until week 17 (sacrificed because of tumor size exceeding 10% of total body weight).

<table>
<thead>
<tr>
<th>Genistein Intake</th>
<th>No. animals with tumors (%)</th>
<th>Tumor latency (wk)</th>
<th>Multiplicity (tumors per rat)</th>
<th>% Animals sacrificed prior wk 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low n-6 PUFA (n = 25)</td>
<td>12 (48%)</td>
<td>11.3 ± 0.6</td>
<td>1.2 ± 0.1</td>
<td>28%</td>
</tr>
<tr>
<td>High n-6 PUFA (n = 25)</td>
<td>16 (64%)</td>
<td>10.0 ± 0.6</td>
<td>1.1 ± 0.1</td>
<td>40%</td>
</tr>
<tr>
<td>Low n-3 PUFA (n = 23)</td>
<td>12 (52%)</td>
<td>11.2 ± 1.0</td>
<td>1.2 ± 0.1</td>
<td>13%</td>
</tr>
<tr>
<td>High n-3 PUFA (n = 24)</td>
<td>9 (38%)</td>
<td>11.9 ± 1.1</td>
<td>1.3 ± 0.2</td>
<td>25%</td>
</tr>
</tbody>
</table>

*χ² = 13.9, df = 3, P < 0.003; bχ² = 18.9, df = 3, P < 0.0003.

Table 3b Effect of maternal dietary genistein intake on mammary tumor growth among female offspring

Data represent the mean ± SE of latency to tumor appearance, tumor multiplicity, and the proportion of animals that did not survive until week 17 (sacrificed because of tumor size exceeding 10% of total body weight).

<table>
<thead>
<tr>
<th>Genistein Intake</th>
<th>No. animals with tumors (%)</th>
<th>Tumor latency (wk)</th>
<th>Multiplicity (tumors per rat)</th>
<th>% Animals sacrificed prior wk 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low genistein (n = 27)</td>
<td>18 (67%)</td>
<td>10.6 ± 0.6</td>
<td>2.1 ± 0.4</td>
<td>37%</td>
</tr>
<tr>
<td>Medium genistein (n = 27)</td>
<td>18 (67%)</td>
<td>10.4 ± 0.6</td>
<td>2.0 ± 0.2</td>
<td>51%</td>
</tr>
<tr>
<td>High genistein (n = 27)</td>
<td>22 (82%)</td>
<td>11.1 ± 0.6</td>
<td>2.0 ± 0.2</td>
<td>59%</td>
</tr>
</tbody>
</table>

*χ² = 7.44, df = 2, p < 0.02; bχ² = 9.92, df = 2, P < 0.007.


Dietary Modulation of Pregnancy Estrogen Levels and Breast Cancer Risk among Female Rat Offspring

Leena Hilakivi-Clarke, Elisabeth Cho, Ana Cabanes, et al.

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