Mechanisms Mediating the Effects of Prepubertal (n-3) Polyunsaturated Fatty Acid Diet on Breast Cancer Risk in Rats1,2

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ABSTRACT Dietary exposures during childhood may influence later breast cancer risk. We tested in an animal model the hypothesis that prepubertal intake of (n-3) PUFAs, present mainly in fish, reduces susceptibility to breast cancer. Between postnatal days 5 to 25, rat pups were fed (n-3) PUFA-containing diets at a 2:1 ratio of (n-6):(n-3) PUFAs (typical of prehistoric societies) or a control (n-6) PUFA diet at a 17:1 ratio of (n-6):(n-3) PUFAs (comparable with current Western societies). These fatty acids were given in a low- or high-fat context (16 or 39% energy from fat). The low-(n-3) PUFA diet reduced while the high-(n-3) PUFA diet increased carcinogen-induced mammary tumorigenesis. The low-(n-3) PUFA diet reduced mammary cell proliferation and increased apoptosis, particularly in the terminal end buds (the mammary source of malignant breast tumors). The high-(n-3) PUFA diet had opposite effects on these 2 key biomarkers and increased phospho-Akt levels, a survival factor. Microarray analyses identified genes that were permanently upregulated in the low-(n-3) PUFA–exposed glands and function in oxidative damage repair. Serum levels of 8-hydroxy-2′ deoxyguanosine, a marker of DNA damage, were significantly reduced in these low-(n-3) PUFA–fed rats, and increased in the high-(n-3) PUFA–exposed group. The latter group exhibited reduced expression of BRCA1, a DNA repair gene. Our results indicate that the opposing susceptibilities to mammary tumorigenesis between the low- versus high-fat (n-3) PUFA–exposed groups were associated with altered DNA damage repair and gene expression linked to proliferation, survival, and differentiation.


KEY WORDS: • (n-3) polyunsaturated fatty acids • prepubertal diet • mammary tumorigenesis

A puzzle that remains to be solved is the apparent role of diet in breast cancer but the absence of a clear-cut association between any specific dietary factor and the risk of developing this disease. An illustrative example is dietary fat: 15 to 20 y ago, data generated in humans suggested that a high fat intake would increase breast cancer risk (1,2), but cohort studies performed from the mid-1990s onward failed to show such an association (2–7). Because dietary fat is composed of different types of fats—mono- and polyunsaturated fatty acids, saturated fatty acids, and trans fatty acids—studies have also assessed whether these different fats have similar or different effects on the breast.

There are two types of PUFAs: (n-6) PUFAs, which are obtained primarily from vegetable oils, such as corn oil, and (n-3) PUFAs, which are derived from cold-water fish and some plants, such as canola and flax. Data from animal studies show that (n-6) PUFAs promote tumorigenesis (8–10), but most human studies do not support a role for (n-6) PUFAs in increasing breast cancer risk (2,5). (n-3) PUFAs have been linked to reduced breast cancer risk (11,12). However, this view was recently challenged by the failure of large cohort studies to indicate any association between (n-3) PUFAs and breast cancer (13,14). Animal studies have also generated conflicting data: some studies show that dietary intake of (n-3) PUFAs inhibits tumorigenesis (15), whereas others show that it does not (16,17).

Novel concept: timing of dietary exposures determines their effect on breast cancer risk

One explanation for the controversy regarding dietary fat exposures and breast cancer is that the age when PUFAs are consumed and the types and levels of PUFAs consumed may determine how they affect breast cancer risk. An example of the sensitivity of age to the time of dietary exposures is body weight and breast cancer risk. High and low birth weights increase (18–21) whereas obesity at puberty reduces breast cancer risk (22–27). Obesity during adult life reduces premenopausal breast cancer risk (28–31) but increases the risk of postmenopausal breast cancer (28,31). Excessive weight gain

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during pregnancy also increases breast cancer risk (32). Other examples include different effects among in utero (33), prepubertal (34,35), premenopausal, and postmenopausal exposure (36–40) to genistein (a phytoestrogen present in soybeans) or soy on breast cancer risk. Common to the different dietary factors, each of which affects breast cancer risk uniquely depending on the age when consumed, is that they modify estrogen levels or exhibit estrogenic activities. Substantial evidence indicates that the timing of estrogenic exposures critically determines their effect on future breast cancer risk (41).

Why is the age when specific dietary factors are consumed so critical? The breast undergoes extensive changes during a woman’s lifetime, mainly in relation to dramatic hormonal alterations associated with puberty, pregnancy, and menopause. The effect of a specific dietary factor is likely to depend on the developmental stage of the mammary fat pad: at its inception, when a primitive epithelial tree first appears; at puberty, when the epithelial tree begins to grow in response to ovarian estrogen production; or during adulthood, when the gland is fully mature. The mammary gland may also be particularly sensitive to dietary exposures during pregnancy when it undergoes extensive proliferation and differentiation in preparation for lactation. Findings in animals support these sensitive periods, indicating, for example, that an exposure to estrogens during fetal life alters fetal mammary gland development and that these changes can persist after birth and sometimes throughout life (42). An exposure to estrogens before puberty onset (43) or during pregnancy (44) also causes long-lasting changes in mammary gland morphology.

A structure in the mammary gland that leads the growth of the epithelial ductal tree, allowing it to invade the mammary fat pad, is the terminal end bud (TEB; in humans, called a terminal ductal lobular unit). TEBs contain the highest level of proliferating cells, and also the sites where malignant transformation occurs (45). In normal development, TEBs regress to terminal ducts or differentiate into alveolar buds that further differentiate into lobules; when the epithelial tree has filled the fat pad at about 9 to 10 wk of age, no TEBs can be detected. Pregnancy induces further differentiation of the mammary epithelial tree, thus making the breast highly resistant to malignant transformation (46).

Our work investigating the effects of early life dietary exposures on mammary gland morphology, gene expression, and breast cancer risk indicate that in utero estrogenic exposures increase mammary cancer risk (42). The mammary glands of animals exposed in utero to 17β estradiol contain more target cells for malignant transformation, high numbers of proliferating cells, and a low rate of apoptosis, and they overexpress oncogenes (Shajahan AN, unpublished data). In contrast, prepubertal estrogenic exposures reduce mammary cancer risk, induce differentiation of the mammary epithelial tree, decrease cell proliferation, increase apoptosis, and up-regulate the expression of antioxidant and DNA repair genes [(43,47), Shajahan AN, unpublished data]. Furthermore, the size of the epithelial tree (mammographic density in humans) is reduced (43). Because high mammographic density is a strong predictor of breast cancer risk in women (48–50), prepubertal estrogenic exposures may reduce breast cancer risk by reducing the size of the epithelial tree (causing a low mammographic density). Human studies support this view: women who had high BMIs during childhood (i.e., they had higher levels of circulating estrogens originating from adipose tissue before ovarian estrogen production) have persistently lower mammographic densities than women who had normal BMIs during childhood (51).

We have recently investigated caveolin-1, a scaffolding protein that may function as a tumor suppressor in the mammary gland (Shajahan AN, unpublished data), as a potential mediator of the protective effects of prepubertal dietary exposures on the breast. Caveolin-1 forms cholesterol- and sphingolipid-rich omega-shaped vesicles called caveolae at the plasma membrane, and it is involved in various functions including cholesterol trafficking, vesicle transport, and signal transduction (52). Because caveolin-1 sequesters and regulates the function of various resident membrane proteins that include G-proteins, Src-like kinases, endothelial nitric oxide synthase, H-Ras, and epidermal growth factor receptor (53,54), their interactions may lead to the ability to prevent the initiation of breast cancer. A finding that downregulation of the gene encoding caveolin-1 (CAV1) causes constitutive activation of membrane estrogen receptor-α (55) suggests that caveolin-1, whether directly or indirectly, interacts with the estrogen receptor in affecting breast cancer risk. We (Shajahan AN, unpublished data) found that high caveolin-1 expression in rats exposed to 17β estradiol during prepuberty correlates with reduced cell proliferation and cyclin D1 and phospho-Akt protein levels and increased apoptosis. In support of the role of caveolin-1 as a negative regulator of a variety of progrowth signaling proteins, we (Shajahan AN, unpublished data) detected decreased levels of Src and ErbB2 in animals exposed to 17β estradiol during prepuberty. Our data thus suggest that prepubertal estrogenic exposures affect the expression and function of a putative tumor suppressor caveolin-1, in a manner that is consistent with observed changes in decreased susceptibility to mammary tumorigenesis.

**Prepubertal diet and later breast cancer risk**

**Human studies.** Based on a mathematical model that is an extension of the Pike model of breast cancer, Colditz and Frazier (56) proposed that the years before birth of the first child are the most important in determining breast cancer risk. The difficulty in addressing the possible importance of dietary exposures during childhood for later breast cancer risk is the virtual lack of reliable information on childhood dietary exposures in women who develop breast cancer. Proxy measures, such as famine or severe eating disorders during adolescence, are complicated by several confounding factors. Perhaps reflecting these confounding factors, studies report that childhood famine may either increase or reduce later breast cancer risk (57,58). Women who suffered from anorexia nervosa in their youth are possibly at a reduced risk (59). Thus, caloric restriction during childhood may protect against later development of breast cancer. However, high childhood BMI also is linked to reduced breast cancer risk (24,25,32,60,61).

Despite the apparent difficulties in addressing childhood dietary exposures retrospectively, the effects of diet during childhood on breast cancer risk have been studied by a few groups. The results are variable and do not provide any strong associations implicating one particular dietary factor, such as dietary fat, with breast cancer (62–64). These studies suggest that high intake of fruits and vegetables, eggs, vegetable fat, or milk reduces breast cancer risk, whereas high chicken or butter consumption increases the risk (62,65,66). However, findings pointing to associations between a single dietary factor during childhood and breast cancer relate to individual studies rather
than representing consistency across all studies. An exception is adolescent soy intake: two studies that investigated its relation to future breast cancer risk both observed a protective effect (38,39).

**Animal studies.** Similar to human studies, results are consistent in showing that prepubertal or pubertal exposure to genistein, a phytoestrogen present at high levels in soy that is administered either via injections or feed, reduces the incidence and/or multiplicity of subsequent carcinogen-induced mammary tumors in animals (34,35,67). The mechanisms mediating the protective effects are still being explored but are likely to include a persistent alteration in rate of cell proliferation, apoptosis, or both (34); a reduced number of TEBs and increased epithelial differentiation (34,35); and upregulation of breast cancer gene 1 (BRCA1) (43).

Except for flaxseed and vitamin A, we are not aware of pubertal dietary exposures that have been studied in the context of their effect on later breast cancer risk. Rats fed a flaxseed diet from conception until weaning exhibit changes in mammary gland morphology that are predictive of a reduced risk for developing mammary tumors (68,69). Specifically, early life exposure to flaxseed reduces the number of TEBs and promotes their differentiation to lobuloalveolar structures. A recent study by the same group of investigators indicated that prepubertal exposure to a 10% flaxseed diet reduces the risk of developing 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors (70). However, in our hands, prepubertal exposure to a 10% flaxseed diet increased later breast cancer risk (Hilakivi-Clarke L, Khan G, unpublished data, 2005). Findings concerning vitamin A also are difficult to interpret. Metz and Schedin (71) found that pubertal exposure to supraoptimal levels of vitamin A and, conversely, vitamin A deficiency increase mammary tumorigenesis in rats.

Here we describe the results of our study investigating the effects of prepubertal exposure to (n-3) PUFAs on later carcinogen-induced mammary tumorigenesis in rats. Some of the findings have been published (47) and some have been submitted for publication (Olivo S, Zhu Y, Galam K, Wang J, Zwartz A, Lee R, Clarke R, Hilakivi-Clarke L, unpublished data, 2005).

**Dietary exposures.** To determine whether prepubertal exposure to (n-3) PUFAs modifies later susceptibility to malignant transformation, we fed rat pups diets containing (n-3) PUFAs at a 2:1 ratio of (n-6):(n-3) PUFAs (typical of early societies) and a control (n-6) PUFA diet (AIN93) at a 17:1 (n-6):(n-3) PUFA ratio (comparable with current Western societies) between postnatal d 5 and 25. The fat sources were menhaden oil, which is high in (n-3) PUFAs, and corn oil, which contains high levels of (n-6) PUFAs. To modify the absolute (n-3) PUFA content without affecting the (n-6): (n-3) PUFA ratios, these fatty acids were given in a low-fat [16% energy from fat; low-fat (n-3) and low-fat (n-6) groups] or high-fat [39% energy from fat; high-fat (n-3) and high-fat (n-6) groups] context.

**Mammary tumorigenesis.** Mammary tumors were induced using 7,12-dimethylbenz[a]anthracene (DMBA) following the protocol used in our laboratory. This breast cancer animal (rat) model was invented by Huggins et al. (72) and further characterized by Russo and Russo (46,73). The Russos’ work has been groundbreaking, guiding all investigators who use this carcinogen. Mammary epithelial cells in TEBs metabolize DMBA to polar metabolites (epoxides) that cause DNA dam-

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- Supplementary Information

**Mechanisms mediating the effects of prepubertal (n-3) PUFA diets on mammary tumorigenesis**

To understand how prepubertal dietary exposure to (n-3) PUFAs can both reduce and increase breast cancer risk de-
pending on whether a diet contains high or moderate levels of these fatty acids, we investigated changes in mammary gland morphology and endpoints linked to the mechanism of action of (n-3) PUFAs [increased lipid peroxidation and a consequent increase in apoptosis, reduced cell proliferation and cyclooxygenase (COX)-2 levels, and increased peroxisome proliferator-activated receptor γ (PPARY) activity] (77). Because PPARY inhibits cyclin D1 (78), we also studied changes in the gene coding for cyclin D1. In addition, we performed gene microarrays to further characterize differences between the 2 groups exposed prepubertally to (n-3) PUFAs that exhibited opposite changes in mammary tumorigenesis.

**Mammary gland morphology, cell proliferation, and apoptosis.** Mammary gland morphology was studied in whole mounts to determine whether prepubertal (n-3) PUFA exposures alter TEBs or their differentiation to alveolar buds and further lobuloalveolar units (LAUs) (45). The mammary glands of low- and high-fat (n-3) PUFA groups contained fewer TEBs than did those of the low-fat (n-6) PUFA group (reference diet) or the high-fat (n-6) PUFA group. In addition, the glands of these rats contained a higher number of LAUs than did those of the low-fat (n-6) PUFA group. Thus, the mammary glands of (n-3) PUFA groups were more differentiated than those of (n-6) PUFA groups. The findings in the low-fat (n-3) PUFA group were consistent with increased epithelial differentiation being associated with reduced risk. However, similar changes noted in the mammary glands of the high-fat (n-3) PUFA group were unexpected in the context of their increased susceptibility to mammary tumorigenesis.

**Lipid peroxidation levels in PUFA-fed animals.** One of the changes induced by (n-3) PUFAs is an increase in lipid peroxidation, which can result in an increase in apoptosis (79,80). Lipid peroxidation was examined by measuring lipid hydroperoxide levels. As expected, the high-fat (n-3) PUFA group had the highest levels of lipid hydroperoxides, whereas the low-fat (n-6) PUFA group had the lowest levels. It is of interest that the increased levels persisted for adulthood. The low-fat (n-3) PUFA group also had modestly increased lipid peroxidation (45).

**Apoptosis.** The (n-3) PUFA groups had increased apoptosis in various tissues (81,82), perhaps reflecting lipid peroxidation induced by (n-3) PUFAs. We found that the level of apoptosis, assessed using the TUNEL assay, was significantly higher in the low-fat (n-3) PUFA group than in the low-fat (n-6) PUFA group. In contrast, the high-fat (n-3) PUFA group had a significantly lower level of apoptosis in both the lobuloalveolar structures and the TEBs than did the low-fat (n-6) PUFA group. This surprise finding may be related to reduced levels of phospho-Akt we noted in the mammary glands of the high-fat (n-3) PUFA group (45). Akt is a survival factor that inhibits apoptosis (83).

**Cellular proliferation.** (n-3) PUFAs generally inhibit cell growth in vivo and in vitro studies (84). We studied cell proliferation by measuring proliferating cell nuclear antigen protein expression by immunohistochemistry. Consistent with the in vitro data, the low-fat (n-3) PUFA group exhibited lower cell proliferation in the ducts, lobuloalveolar structures, and TEBs than did the low-fat (n-6) group (47). However, we observed significantly higher levels of proliferating cells in the LAUs and TEBs in the high-fat (n-3) PUFA group than in the low-fat (n-6) group.

To begin to understand what was driving the increased proliferation in the high-fat (n-3) PUFA group, we measured the expression of genes that induce cell differentiation while inhibiting cell proliferation and are regulated by (n-3) PUFAs [i.e., PPARg, which encodes PPARγ (85), and BRCA1 (86)]. These genes may function as mammary tumor suppressors.

**PPARγ and cyclin D1.** PPARγ is a nuclear receptor that plays an important role in adipocyte differentiation (87) and is also associated with epithelial cell differentiation (88). PPARγ is activated by (n-3) PUFAs (87) and interacts with cyclin D1, inhibiting its activation (78). It has been suggested that an important physiological function of cyclin D1 is to regulate cellular metabolism, in particular, fat metabolism, through PPARγ. We determined the protein levels of these genes in the mammary glands of 8-wk-old animals, assessed separately in the TEBs, lobular structures, and ducts, using immunohistochemistry (Khan G, Olivo S, Hilakivi-Clarke L, unpublished data, 2005). Our results showed that the levels of PPARγ significantly increased in the mammary glands of the low-fat (n-3) PUFA group and were lowest in the high-fat (n-3) PUFA group, compared with both the low- and high-fat (n-6) PUFA groups. Cyclin D1 levels did not change in the low-fat (n-3) PUFA group but significantly increased in the high-fat (n-3) PUFA group. These findings are consistent with the data generated from in vitro systems, suggesting an inverse relation between PPARγ and cyclin D1 (12).

**BRCA1.** BRCA1 is a tumor suppressor gene that affects DNA damage repair and is strongly implicated in a high proportion of inherited breast cancers (89,90). A recent study done in a 3-dimensional culture model indicates that inhibition of BRCA1 expression prevents cell differentiation and induces proliferation (91), suggesting that this gene has a critical role in controlling the cell cycle. This is further evidenced by an apparent association between BRCA1 and cyclin D1 signaling (92). We found that the high-fat (n-3) PUFA group had a significant decrease in BRCA1 expression compared with the high-fat (n-6) PUFA group, whereas the low-fat (n-3) PUFA group had a nonsignificant increase in BRCA1 expression (Olivo S, Zhu Y, Galam K, Wang J, Zwartz A, Lee R, Clarke R, Hilakivi-Clarke L, unpublished data 2005).

**Caveolin-1.** As mentioned above, CAV1, which encodes the scaffolding protein caveolin-1 and is a potential tumor suppressor gene, is expressed in differentiated cells (52). Previous studies conducted in vitro and in vivo suggest that (n-3) PUFAs and PPARγ upregulate CAV1 expression (93,94). Consistent with these observations, the (n-3) PUFA groups exhibited higher caveolin-1 expression in the mammary glands (Shajahan A, Goel S, de Assis S, Yu B, Clarke R, Hilakivi-Clarke L, unpublished data, 2005). Thus, although upregulation of CAV1 in the low-fat (n-3) PUFA group could be linked to reduced mammary tumorigenesis, it was not sufficient to prevent the development of high levels of mammary tumors in the high-fat (n-3) PUFA group.

**Gene microarrays.** To further identify signaling pathways that might mediate the contrasting effects of prepubertal exposure to low- and high-fat (n-3) PUFA diets on mammary gland morphology and breast cancer risk, we performed gene microarray analyses using GF300DS rat filters. A novel profile selection algorithm was applied, and comparisons were made between the low- and high-fat (n-3) PUFA array filters and, further, to (n-6) PUFA array filters. These comparisons were anticipated to identify genes that may characterize low and high breast cancer risk, rather than exposure to a diet either low or high in (n-3) PUFAs. In the mammary glands of 8-wk-old rats, 50 genes were differentially expressed between the high- and low-fat (n-3) PUFA groups. Seventy-five genes were differentially expressed in the high-fat (n-3) PUFA group and 29 in the low-fat (n-3) PUFA group, compared with both (n-6) PUFA groups. Some genes that were differentially expressed between the high- and low-fat (n-3) PUFA groups
were also differentially expressed between the high-fat (n-3) and (n-6) PUFA groups (11%) and between the low-fat (n-3) and (n-6) PUFA groups (31%). These overlapping genes (a total of 17 genes) may be most strongly linked to altered breast cancer risk, and they included antioxidant genes and genes that regulate cyclin Ds, such as Akap95 [upregulated by prepubertal exposure to a low-fat (n-3) PUFA diet] (95) or genes that activate the Src and Ras oncogenic pathways, such as cyclophilin and syndecan 2 [upregulated by a high-fat (n-3) PUFA diet] (96).

**DNA damage.** Several changes seen in DNA damage suggested that prepubertal exposure to a low-fat (n-3) PUFA diet decreased DNA damage, whereas exposure to a high-fat (n-3) PUFA diet increased DNA damage. To determine whether that was the case, we measured the levels of 8-hydroxy-2′-deoxyguanosine (8-OhdG), an oxidative DNA product induced by oxygen radicals (97,98). The low-fat (n-3) PUFA group exhibited reduced DNA damage (reduced 8-OhdG levels), whereas the high-fat (n-3) PUFA group exhibited increased DNA damage.

**COX-2 expression.** Nonsteroidal anti-inflammatory drugs inhibit COX activity and have been shown to inhibit tumorogenesis (99). Conversely, cox-2 overexpression in mice is sufficient to induce mammary tumors (100). Earlier findings in rats fed (n-3) PUFAs indicated increased incorporation of (n-3) PUFAs into the membrane phospholipids and subsequent inhibition of cox-2 expression (101,102). In agreement with these observations, we found that both the low- and high-fat (n-3) PUFA groups had decreased cox-2 expression. Because the prepubertal exposure to the high-fat (n-3) PUFA diet increased whereas the low-fat (n-3) PUFA diet decreased mammary tumorigenesis, downregulation of cox-2 expression was not sufficient to prevent increased tumorigenesis in the high-fat (n-3) PUFA group. Whether the reduced expression contributed to the reduced tumorigenesis in the low-fat (n-3) PUFA group remains to be established.

**Final remarks**

The biological changes that could explain the opposing effects of prepubertal dietary exposures to a low- or high-fat (n-3) PUFA diet on later susceptibility to mammary tumorigenesis are summarized in Figures 1 and 2. Our study indicated that the reduced mammary tumorigenesis in the rats prepubertally fed a low-fat (n-3) PUFA diet was associated with reduced cell proliferation and increased cell death, and these changes were accompanied by altered expression of genes that regulate these two events. Gene microarray analysis identified upregulation of several antioxidant genes and reduced 8-OhdG levels, which indicate reduced accumulation of DNA damage. Increased risk of developing mammary tumors in the rats fed the high-fat (n-3) PUFA diet was linked to increased cell proliferation and reduced apoptosis that might be caused by increased expression of cyclin D1 and reduced expression of BRCA1. Because these animals also exhibited high levels of lipid peroxidation and reduced expression of antioxidant genes, these cellular events could have led to increased levels of DNA damage.

If these data generated in an animal model are relevant to susceptibility to breast cancer in women, it is advisable to consume some dietary (n-3) PUFAs during childhood. However, there is likely to be an upper limit for (n-3) PUFA consumption; levels higher than such a limiting level may have adverse effects on the mammary gland and increase susceptibility to breast cancer.

**LITERATURE CITED**

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