Identification of a Molecular Signature Underlying Inhibition of Mammary Carcinoma Growth by Dietary N-3 Fatty Acids

Weiqin Jiang1, Zongjian Zhu1, John N. McGinley1, Karam El Bayoumy2, Andrea Manni2, and Henry J. Thompson1

Abstract
An increased ratio of dietary n-3 relative to n-6 fatty acids has been shown to inhibit the development of mammary cancer in animal models. However, the molecular mechanisms by which n-3 fatty acids affect tumor growth remain unknown. Here, we investigated the effects of varying dietary ratios of n-3:n-6 fatty acids on cell signaling in a rat model of chemically induced mammary carcinoma. Cell proliferation was reduced by 60% in carcinomas from the high n-3:n-6 treatment group compared with the low n-3:n-6 treatment group. These changes were associated with decreased cyclin-D1 and phospho-retinoblastoma protein expression and increased levels of cyclin-dependent kinase inhibitors, CIP1 (p21) and KIP1 (p27). In addition, the apoptotic index was increased in carcinomas from the high n-3:n-6 group and was associated with elevated apoptotic protease-activating factor 1 and a higher ratio of Bax/Bcl-2. Interestingly, changes in protein expression were consistent with reduced inflammation and suppressed mTOR activity, and the molecular signature associated with high n-3:n-6 treatment revealed changes in PPARγ activation and suppression of lipid synthesis. Together, our findings indicate that the molecular effects of high dietary n-3 to n-6 ratios are heterogeneous in nature but point to consistent changes in lipid metabolism pathways, which may serve as potential therapeutic targets for cancer prevention and control. This study identifies the pathways modulated by dietary fatty acid ratios in a rat model of breast cancer, with implications for cancer prevention. Cancer Res; 72(15); 3795–806. ©2012 AACR.
anti-pRB/RB, anti-Apf1, anti-pFOXO1&3/FOXO1&3, anti-NFkB-p65, anti-SRT-1, anti-FASN, anti-PISKp110, anti-pAMPK/AMPK, anti-pACC/ACC, anti-pAkt/Akt, anti-pmTOR/mTOR, anti-p70S6/P70S6, anti-pE-BP1/E-BP1, anti-p-Raptor/Raptor, anti-p-PRAS40/PRAS40, anti-rabbit immunoglobulin-horse-radish peroxidase (HRP)-conjugated secondary antibody, and LumiGLO reagent with peroxide were purchased from Cell Signaling Technology; anti-IGF1R-1, anti-p21\(^{\text{G}_{1}}\), anti-PPAR\(\gamma\), \(\beta\), \(\gamma\), anti-HMGCR, anti-SREBP-1, and anti-mouse immunoglobulin-HRP-conjugated secondary antibody were from Santa Cruz; and mouse anti-\(\beta\)-actin primary antibody was obtained from Sigma-Aldrich. Biotinylated donkey anti-rabbit, donkey anti-goat secondary antibodies, and normal donkey serum were obtained from Jackson ImmunoResearch; HRP-conjugated streptavidin was obtained from Dako; and stable 3,3'-diaminobenzidine was obtained from Invitrogen.

**Animals and experimental design**

The tissue evaluated in this study was from a previously reported experiment. Briefly, in that study, 21-day-old rats were injected with 50 mg MNU/kg body weight (intrapерitoneally) as previously described (11). Seven days following carcinogen injection, all rats were randomized into treatment groups, 30 rats per group, and were fed their respective experimental diets. The actual fatty acid content of each diet groups, 30 rats per group, and were fed their respective experimental diets. The actual fatty acid content of each diet was determined by gas chromatography-mass spectroscopy (GC-MS) as previously described (1), and the fatty acid data are available in Supplementary Table S1. The rats were fed diets in which the ratio of n-3:n-6 was either 0.7 (low n-3, control) or 14.6 (high n-3). Diet and water were provided ad libitum. Rats were weighed weekly and palpated for detection of mammary tumors twice per week. Animal rooms were maintained at 22°C ± 1°C with 50% relative humidity and a 12-hour light/12-hour dark cycle. At necropsy, detectable mammary pathologies were excised and weighed. A section of each lesion was fixed in neutral-buffered formalin and prepared for histologic classification; the remainder of each lesion was snap-frozen in liquid nitrogen for molecular determination; only con

**Determination of rates of cell proliferation and apoptosis**

KI-67 immunohistochemical staining was used as an index of tumor growth fraction and was determined as previously described (12). Ten representative images of each KI-67-stained section were chosen at random and acquired using a Zeiss Axioskop II (Carl Zeiss, Inc.) at a magnification of \(\times 400\) and analyzed using Image Pro Plus 4.5 (Media Cybernetics, Inc.). Apoptosis was quantified using the criteria developed by Kerr for its detection (13, 14): 10 images of corresponding hematoxylin and eosin-stained serial sections were acquired at \(\times 400\). Apoptotic and normal cells were marked and counted using the count tool in Adobe Photoshop CS4 (Adobe Systems, Inc.). The apoptotic index was computed as the number of apoptotic cells divided by the total number of cells counted and was expressed as a percentage.

**Western blotting**

Twenty-two mammary carcinomas, each from a different rat (11 per group) were homogenized in lysis buffer [40 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 0.25 mol/L sucrose, 3 mmol/L EGTA, 3 mmol/L EDTA, 50 mmol/L \(\beta\)-mercaptoethanol, 1 mmol/L phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Calbiochem)]. The lysates were centrifuged at 7,500 \(\times g\) for 10 minutes at 4°C and supernatant fractions were collected and stored at −80°C. Supernatant protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad). Western blotting was conducted as described previously (15). Briefly, 40 \(\mu\)g of protein lysate per sample was subjected to 8% to 16% SDS-PAGE after being denatured by boiling with SDS sample buffer [63 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L dithiothreitol (DTT), and 0.01% bromophenol blue] for 5 minutes. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The levels of IGF1R, pIRS, IRS, PISKp110, cyclin-D1, p27\(^{\text{G}_{1}}\), p21\(^{\text{G}_{1}}\), pRB, Bax, Bcl-2, Apaf-1, c-cPARPs (PARP89 and PARP116), pAMPK, AMPK, pACC, ACC, HMGCR, SREBP-1 (precursor, not mature form), FASN, pAkt/Akt, mTOR, mTOR, p70S6, p70S6, p-E-BP1/E-BP1, p-Raptor, Raptor, pPRAS40, PRAS40, PISKp, \(\beta\), \(\gamma\), GFR120, NFKBp65, pFOXO1&3a, FOXO1&3a, HIF-1\(\alpha\), SIRT-1, GADD153, and \(\beta\)-actin were determined using specific primary antibodies, followed by treatment with the appropriate peroxidase-conjugated secondary antibodies and visualized by LumiGLO reagent Western Blotting Detection System. The chemiluminescence signal was captured using a ChemiDoc densitometer (Bio-Rad) that was equipped with a CCD camera having a resolution of 1,300 \(\times\) 1,030. Quantity One software (Bio-Rad) was used in the analysis. All of the Western blotting signals were within a range where the signal was linearly related to the mass of protein. The Quantity One software has a warning algorithm that notifies the user if pixel density is approaching saturation so that all signals used for analysis are in the linear range. The actin-normalized scanning density data were used for analysis.

**Statistical analyses**

Differences among groups in KI-67 staining, apoptotic index, and the actin-normalized Western blot data were analyzed by the Kruskal–Wallis rank test as implemented in Systat version 13 (Systat Software, Inc.; ref. 16). All \(P\) values are 2-sided and statistical significance was set a priori at \(P < 0.05\).

**Soft independent modeling of class analogue analysis**

Principal components analysis (PCA) is an unsupervised cluster analysis method for summarizing a set of correlated variables by transforming them, by means of an eigen decomposition, into a new set of uncorrelated variables, typically reducing the dimensionality of the original data set. The procedure is carried out with no prior knowledge of class membership. The first principal component (PC) is the linear combination of the features (actin-normalized scanning data from Western blotting) that passes through the centroid of the
full data set while minimizing the square of the perpendicular distance of each point to that line; each subsequent PC is constructed in a similar manner, subject to the constraint of being mutually orthogonal (17). The PCA model can be written as

\[ X = Xbar + TP' + E \]  

where \( X \) is the matrix of Western blotting values, \( Xbar \) is a vector of means (all 0 when the data are centered), \( T \) is a matrix of scores that summarize the \( X \) variables, \( P' \) is a matrix of loadings, and \( E \) is a matrix of residuals.

Orthogonal projections to latent structures for discriminant analysis (OPLS-DA) is a supervised, class-based method where class membership is assigned and used to elicit maximum data separation (18–21). The OPLS-DA model can be written as

\[ X = TpPp + TpP0 + E \]  

The interpretation of (B) is similar to that for the PCA model; however, an additional rotation has been applied using the class information to partition \( TP^+ \) into a predictive, \( TpPp \), and an orthogonal, \( TpP0 \), component. The number of predictive and orthogonal components in the models was determined by cross-validation. Three key statistics describe the fit of each model. First, \( R2X(cum) \) is the total amount of variation (predictive + orthogonal) explained in \( X \); \( R2Y(cum) \) is the total amount of the variation explained in \( Y \); and third, \( Q2Y(cum) \) is the total amount of predicted variability in \( Y \) (the goodness of prediction), estimated by \( 7 \)-fold cross-validation. The contribution of each component partitioned into between (predictive) and within (orthogonal) class is also estimated and summarized as \( R2Xp \) and \( R2Xo \), respectively. \( R2Xp \) and \( R2Xo \) sum to \( R2X(cum) \). The ability of the model to classify the observations into the defined classes is reflected in misclassification rates for each model, where the target proteins of mammary carcinomas determined by Western blotting were classified on the basis of the modeled probability of a single observation belonging to a particular class.

All analyses were conducted using soft independent modeling of class analogue (SIMCA) analysis, SIMCA-P+ v.12.0.1 (Umetrics).

**Results**

**Carcinogenic response**

In the high versus low n-3:n-6 treatment groups, cancer incidence was reduced by 21%, cancer multiplicity by 30%, cancer mass by 79.9%, and cancer latency was prolonged by 15.8%. These effects were statistically significant as shown in Supplementary Table S2. The focus of the analyses reported herein was to investigate the mechanisms that accounted for the 79.9% reduction in tumor burden, as the reduction in tumor burden was clearly greater than could be accounted for by the effects of the high n-3 ratio in delaying cancer latency, which would decrease the length of time over which tumors grew. To that end, 11 carcinomas were analyzed from each group of the different animals. The carcinomas selected were non-necrotic, sufficiently large for an extended number of analyses, and representative of the differences in carcinoma mass and latency in the treatment groups from which the carcinomas were obtained (Supplementary Table S2). The values for the selected carcinomas evaluated herein were carcinoma mass, 2.56 ± 1.68 g versus 0.19 ± 0.13 g (mean ± SD; \( P = 0.001 \)), time to detection, 37.5 [95% confidence interval (CI), 34.5–40.5] days and 44.3 (95% CI, 41.5–47.0; \( P = 0.004 \); Mantel test), respectively, for the low versus high n-3 treatment groups. Estimated tumor growth rates (mg/d) were 181 ± 70 and 48 ± 48 (mean ± SD, \( P < 0.005 \)), respectively, for the low and high n-3:n-6 treatment groups.

**Cell proliferation and death**

To ascertain what cellular process(es) accounted for the differences observed in carcinoma mass per rat and estimated tumor growth rate, both the Ki-67 and apoptosis labeling indices were computed. As shown in Table 1, Ki-67 was reduced by 60% (\( P < 0.0001 \)) and the apoptotic index was 129% higher (\( P < 0.0001 \)) in the high versus low n-3 treatment groups (representative photomicrographs in Supplementary Fig. S1). As the next step in analysis, levels of key proteins that regulate the G1–S transition in the cell cycle and the induction of apoptosis were assessed. The levels of cyclin-D1 and phospho-Rb were reduced and levels of 2 cyclin-dependent kinase inhibitors, p21 and p27, were elevated in the high versus low n-3:n-6 treatment group suggestive of a block in cell-cycle transit at the G1–S transition (Table 1; representative Western blot analyses in Supplementary Fig. S2A). Relative to apoptosis and consistent with the elevated apoptotic index observed in the high n-3:n-6 group, the level of cleaved PARP (PARP89/116 ratio) was elevated as were levels of Bax and Apaf-1, whereas the level of Bcl-2 was not significantly affected. These changes are indicative of the induction of apoptosis via the intrinsic pathway (Table 1; representative Western blot analyses in Supplementary Fig. S2B).

The processes of cell proliferation and apoptosis are generally linked and consequently changes in regulatory mechanisms can be highly correlated. Moreover, the standard manner of reporting data can mask the ability to visualize differences among the responses of animals within a treatment group as well as the occurrence of overlapping responses between treatment groups. To better understand the proliferative and apoptotic response in this regard, the data were subjected to unsupervised cluster analysis via PCA and to supervised cluster analysis via OPLS-DA, as described in Materials and Methods. As shown in Fig. 1A and B, 100% of the carcinomas were correctly identified with low and high n-3:n-6 group assignment, and the degree of heterogeneity among responses within both groups is shown in the dendrogram that is Fig. 1C. More heterogeneity in response is apparent in the low versus high n-3:n-6 group. Figure 1D shows the 95% CIs for covariance of the 7 proteins evaluated in the first PC sorted in ascending order, which relegates target proteins with elevated expression in the low n-3:n-6 group compared with the high n-3:n-6 group to the distal end of the x-axis. These jack-knifed CIs (JKCI) identified proteins with high reliability (green bars) versus low reliability (red bars) as shown in Fig. 1E. Proteins with low reliability, based on error bars crossing 0 provide no useful information relative to identifying the treatment group from...
which a carcinoma was obtained. Figure 1F shows the ranked importance of proteins and clearly indicated that the proteins contributing most to the classification of carcinomas were associated with the cell-cycle regulation.

Transcription factors

The n-3:n-6 ratio has been reported to affect gene expression by mechanisms dependent on and independent of binding to fatty acid–binding proteins. Given that a considerable body of evidence indicates that n-3 fatty acids mediate effects on cell signaling via binding to PPARα, β/δ, and γ, an initial set of Western blot analyses was conducted to determine levels of each receptor in carcinomas. As shown in Table 2, PPARγ content was increased by 31% in the high versus low n-3 treatment group (P < 0.001), whereas the level of β/δ was 14.6% lower (P = 0.015) and PPARα was unaffected (representative Western blot analyses are shown in Supplementary Fig. S3). Another family of cell surface receptors that can activate gene expression following fatty acid binding are G-protein–coupled receptors. One such protein, GRP120, is associated with macrophages, which was of interest because the n-3:n-6 ratio has also been reported to affect transcriptional activity related to inflammation, intermediary metabolism, and cell fate, effects on transcriptional factors were assessed, that is, the content of phospho-NF-κB p65Ser536, phospho-FOXO-1Thr24, phospho-FOXO-3α Thr32, Hif-1α, and SIRT-1 were assessed. As shown in Supplementary Fig. S3), consistent with the FOXO data, a downstream product associated with FOXO-mediated transcription, GADD153, was 46.5% higher in the high versus low n-3:n-6 treatment group.

To determine the extent to which these changes in protein concentration could correctly classify tumors according to treatment group, data were subjected to PCA and OPLS-DA. As shown in Supplementary Fig. S4, PCA correctly classified 88% of the tumors in the high and low n-3:n-6 treatment groups, whereas OPLS-DA correctly classified 100% of the tumors in the high and low n-3:n-6 treatment groups.

Table 1. Effect of dietary n-3:n-6 ratio on cellular processes regulating cell proliferation and apoptosis

<table>
<thead>
<tr>
<th>Dietary n-3:n-6 ratio</th>
<th>Low 0.6</th>
<th>High 14.6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell proliferation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67 index (%)</td>
<td>34.9 ± 1.6</td>
<td>14.0 ± 0.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RbSer780 ratio</td>
<td>0.41 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cyclin-D1</td>
<td>1.302 ± 29</td>
<td>967 ± 29</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p21</td>
<td>465 ± 31</td>
<td>664 ± 40</td>
<td>0.001</td>
</tr>
<tr>
<td>p27</td>
<td>326 ± 11</td>
<td>393 ± 10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptotic index (%)</td>
<td>1.71 ± 0.05</td>
<td>3.92 ± 0.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bax</td>
<td>188 ± 8</td>
<td>242 ± 9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>589 ± 28</td>
<td>527 ± 26</td>
<td>0.117</td>
</tr>
<tr>
<td>Bax/Bcl-2</td>
<td>0.32 ± 0.01</td>
<td>0.47 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>392 ± 9</td>
<td>457 ± 17</td>
<td>0.005</td>
</tr>
<tr>
<td>PARP89</td>
<td>795 ± 26</td>
<td>577 ± 53</td>
<td>0.002</td>
</tr>
<tr>
<td>PARP116</td>
<td>547 ± 15</td>
<td>292 ± 28</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PARP89/116 ratio</td>
<td>1.45 ± 0.02</td>
<td>1.99 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: Values are means ± SEM (n = 11). The methods for determining the Ki-67 and apoptotic index are described under Materials and Methods. The resulting count data were evaluated by the Kruskal-Wallis rank test. Actin-normalized Western blotting data, which are semiquantitative estimates of protein expression, were analyzed by Kruskal-Wallis rank test. Ratio is the ratio of phospho-protein (arbitrary units of optical density) to non-phospho-protein (arbitrary units of optical density).

Abbreviations: Bax, Bcl-associated X; Bcl, B-cell leukemia oncogene.

Growth factor–related signaling

The evaluation of insulin-related signaling involved a large number of proteins in the Akt-mTOR-AMPK–regulated network. As shown in Table 2 (representative Western blot analyses in Supplementary Fig. S5), signaling that is associated...
Multivariate discriminant analysis of the actin-normalized intensities for each protein in cell cycle and apoptosis that was Western blotted was used to examine the correlation structure among proteins and distinguish carcinomas based on the treatment group from which they were obtained (low n-3:n-6 or high n-3:n-6). Each point represents an individual carcinoma. A, to visualize inherent clustering patterns, the score scatter plot shows the first 2 score vectors of the PCA model that accounts for 82.9% of the variability in protein expression. No observations lie outside the 95% confidence ellipse. B, OPLS-DA was used to fit a 2-class supervised model and partition the sources of variation. The scatter plot shows the first predictive and first orthogonal components. Complete separation of the carcinomas from each treatment group was observed, and the wide scatter of carcinomas within treatment group along the y-axis indicates high within-class variation. C, to visualize the misclassification rate, the dendrogram depicts hierarchical clustering patterns among carcinomas within a treatment group. Node height of cluster 6.27 from 0 confirms the elevated diversity seen within high n-3:n-6 treatment group. To determine the proteins responsible for distinctness of carcinomas in the 2 treatment groups, the OPLS-DA model was reparameterized to compare all low n-3:n-6 carcinomas with all high n-3:n-6 carcinomas. D, the S-plot shows the relationship between the modeled correlation (vertical axis) and the modeled covariance (horizontal axis) from the 2-class OPLS-DA model. Top right and bottom left regions of S-plots contain candidate biomarkers with both high reliability and high magnitude. E, to determine the statistical reliability of the proteins identified as influential in D, JKCI were estimated for the first predictive component for the proteins evaluated and were sorted in ascending order on the basis of expression in the low n-3:n-6 (control) group; target proteins with JKCI including 0 were indicated by red bars, because they are unreliable predictors of treatment group assignment. F, rank of importance in target proteins.
effect of dietary n-3:n-6 ratio on cellular processes regulating cell transcription factors and insulin signaling

<table>
<thead>
<tr>
<th>Dietary n-3</th>
<th>Low</th>
<th>High</th>
<th>P</th>
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<tbody>
<tr>
<td>PPARα</td>
<td>84 ± 12</td>
<td>83 ± 14</td>
<td>0.980</td>
</tr>
<tr>
<td>PPARβ</td>
<td>446 ± 19</td>
<td>381 ± 15</td>
<td>0.015</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1,212 ± 42</td>
<td>1,589 ± 62</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>GPR120</td>
<td>282 ± 4</td>
<td>333 ± 5</td>
<td>0.001</td>
</tr>
<tr>
<td>NF-κB p65Ser536 ratio</td>
<td>5.2 ± 0.4</td>
<td>3.2 ± 0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>FOXO1Thr24 ratio</td>
<td>0.71 ± 0.07</td>
<td>0.38 ± 0.01</td>
<td>0.001</td>
</tr>
<tr>
<td>FOXO3 Thr32 ratio</td>
<td>0.52 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hif-1α</td>
<td>799 ± 60</td>
<td>541 ± 88</td>
<td>0.026</td>
</tr>
<tr>
<td>SIRT-1</td>
<td>55 ± 7</td>
<td>43 ± 9</td>
<td>0.324</td>
</tr>
<tr>
<td>GADD153</td>
<td>71 ± 4</td>
<td>104 ± 5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 2. Effect of dietary n-3:n-6 ratio on cellular processes regulating cell transcription factors and insulin signaling

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**Table 2.** Effect of dietary n-3:n-6 ratio on cellular processes regulating cell transcription factors and insulin signaling

with cell proliferation and cell survival was downregulated in high versus low n-3:n-6 treatment group, that is, insulin-like growth factor-1 receptor (IGF-1R; 21.2%; P = 0.039), phospho-IRα Ser376/381 (20.3%; P < 0.0001), PI3Kp110 (19.9%; P = 0.005), AKT (22.0%; P < 0.0001), mTOR (16.2%; P = 0.001), PRAS40 (44.9%; P < 0.0001), P70S6K (22.1%; P < 0.0001), and 4E-BP1 (26.2%; P < 0.0001). To determine the extent to which these changes in protein concentration could correctly classify tumors according to treatment group, data were subjected to PCA and OPLS-DA. As shown in Supplementary Fig. S5, 19 of 22 carcinomas were correctly classified, there were no carcinomas that were outliers, but there was considerable within-group variability in the response pattern. The OPLS-DA analysis correctly classified 100% of the carcinomas according to treatment group. The variable with the greatest effect in correct classification was phosphorylated P70S6K, whereas IGF-1Rα had the greatest influence in identifying carcinomas from the low n-3:n-6 treatment group (positively associated with carcinoma growth), and phosphorylated AMPK had the greatest influence in identifying carcinomas from the high n-3:n-6 treatment group (negatively associated with carcinoma growth).

**Lipid metabolism**

In view of these findings, key regulatory points in lipid metabolism were assessed. As shown in Table 3, evidence of significantly decreased lipid biosynthesis [fatty acid synthase (FASN), 20.0% lower, P < 0.0001; HMGCR 13.2% lower, P < 0.0001; SREBP-1 (precursor form) 27.5% lower, P < 0.0001] and significantly increased phospho-ACC (1.3-fold, P < 0.0001) were observed in the high versus low n-3:n-6 treatment group (representative Western blot analyses shown in Supplementary Fig. S5). As shown in Supplementary Fig. S8, all carcinomas were correctly classified by unsupervised PCA and supervised OPLS-DA cluster analysis. The variable with the greatest effect in correct classification was FASN, which had the greatest influence, and overexpression was positively associated with carcinoma growth, whereas phospho-ACC had the greatest influence in identifying carcinomas from the high n-3:n-6 treatment group (negatively associated with carcinoma growth).

To take advantage of the analytic power of OPLS-DA, one additional analysis was conducted. As shown in Fig. 2, all
signaling proteins were included in the OPLS-DA model statement. Twenty-one of 22 carcinomas were correctly classified by PCA (Fig. 2A). The OPLS-DA analysis correctly classified 100% of the carcinomas according to treatment group (Fig. 2B). The degree of heterogeneity among responses within both groups is shown in the dendrogram that is Fig. 2C. Elevated PPARγ expression was most important to identifying carcinomas in the high n-3:n-6 treatment group (negatively associated with carcinoma growth; Fig. 2D and E), whereas overexpression of FASN was most important to identifying carcinomas in the low n-3:n-6 treatment group (positively associated with carcinoma growth). The variable with the greatest effect in correct classification was FASN (Fig. 2F).

Discussion

Recent evaluations of the literature on the effects of n-3 fatty acids in the prevention and control of breast cancer have provided a mixed view with evidence indicating inhibition, no effect, and enhancement of carcinogenesis (7, 22, 23). In our review (22), a series of recommendations were made concerning the experimental approaches that would serve to guide the design of experiments with the potential of resolving the fish oil–breast cancer conundrum. In pursuit of that objective, our laboratories recently reported a series of experiments in which the dietary ratio of n-3 to n-6 fatty acids varied across a wide range to identify the critical chemistry underlying protection against breast cancer and to define mechanisms associated with inhibition of various aspects of the carcinogenic process without limiting the experimental design by levels of intake currently achievable by the dietary consumption of fish or fish oil supplements (1). It was observed that experimentally verified dietary n-3 to n-6 ratios of 4.9 and 1.4 rendered protection against chemically induced rat mammary cancer in the presence or absence of treatment with tamoxifen. Dietary ratios of n-3 to n-6 less than 4.9 had no effect. Analysis of plasma from treated animals indicated that host systemic factors associated with adipocyte function (leptin and adiponectin) and with growth factor availability homeostasis (IGF-1) were affected by dietary n-3 to n-6 ratios in excess of 4.9. These systemic factors have been implicated in various aspects of breast carcinogenesis, suggesting a potential role in mediating protection (1, 24–26).

Host systemic factors clearly exert effects on the heterogeneous collection of diseases referred to as breast cancer (27). During the process of cellular transformation and the progression of transformed cells to invasive and metastatic stages of cancer, the regulation of cell signaling pathways that is normally subject to interactions with neighboring cells in the tissue and to blood- and lymph-borne systemic factors becomes increasingly independent of those factors, that is, regulation becomes autonomous (28). Thus, the signaling pathways deregulated within carcinomas provide clues about etiology as well as valuable information about potential targets for cancer prevention and control.

The expression of the Ki-67 protein is strictly associated with cell proliferation being present during all active phases of the cell cycle, G1, S, G2, and mitosis but absent from resting cells in G0. This makes Ki-67 an excellent marker for determining growth fraction, that is, the proportion of cells not in G0, for a given cell population (29). As shown in Table 1, the growth fraction was significantly reduced in the high n-3:n-6 group, and it appeared that this was accompanied by a partial block in cell-cycle progression at the G1–S transition. As reviewed in the work of Peters and colleagues (7) and Foti and colleagues (10), n-3 fatty acids have been reported to mediate effects on cell proliferation by repressing cyclin-D1 and inducing p21. Strikingly, as shown in Fig. 1, the nonbiased assessment of effects on cyclin-D1 and p21 in the high n-3:n-6 group, the analyses shown in Fig. 1 did not identify factors in the apoptotic pathway as exerting a strong effect in identifying the treatment group from which the carcinomas were obtained, a finding suggestive of a dominant effect of n-3 fatty acids on the proliferative machinery of the cell (30).

A large body of literature indicates that n-3 fatty acids exert biologic effects, in part, via the activation of PPAR receptors, which regulate transcription of genes involved in cell proliferation and survival and cellular metabolism (7). The PPAR family of genes codes for α, β, δ, and γ isoforms of receptors. As shown in Table 2, PPARγ was the dominant form of the protein in mammary carcinomas and was increased in carcinomas of

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Table 3. Effect of dietary n-3:n-6 ratio on proteins regulating lipid metabolism

<table>
<thead>
<tr>
<th>Dietary n-3</th>
<th>Low</th>
<th>High</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCser79</td>
<td>2.35 ± 0.13</td>
<td>3.12 ± 0.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FASN</td>
<td>1,714 ± 39</td>
<td>1,372 ± 34</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HMGCR</td>
<td>859 ± 25</td>
<td>746 ± 16</td>
<td>0.001</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>385 ± 12</td>
<td>279 ± 5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: Values are means ± SEM (n = 11). Actin-normalized Western blot analysis data, which are semiquantitative estimates of protein expression, and the ratio data were analyzed by Kruskal–Wallis rank test. Ratio is the ratio of phospho-protein (arbitrary units of optical density) to non-phospho-protein (arbitrary units of optical density).

Abbreviations: ACC, acetyl-CoA carboxylase; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; SREBP-1, sterol regulatory element-binding protein 1.
Multivariate discriminant analysis of the actin-normalized intensities for each protein in cellular signaling network that was Western blotted was used to examine the correlation structure among proteins and distinguish carcinomas based on the treatment group from which they were obtained (low n-3:n-6 or high n-3:n-6). Each point represents an individual carcinoma. A, to visualize inherent clustering patterns, the score scatter plot shows the first 2 score vectors of the PCA model that accounts for 64.8% of the variability in protein expression. No observations lie outside the 95% confidence ellipse. B, OPLS-DA was used to fit a 2-class supervised model and partition the sources of variation. The scatter plot shows the first predictive and first orthogonal components. Complete separation of the carcinomas from each treatment group was observed, and the wide scatter of carcinomas within treatment group along the y-axis indicates high within-class variation. C, to visualize the misclassification rate, the dendrogram depicts hierarchical clustering patterns among carcinomas within a treatment group. Node height of cluster 6.89 from 0 confirms the elevated diversity seen within high n-3:n-6 treatment group. To determine the proteins responsible for distinctness of carcinomas in the 2 treatment groups, the OPLS-DA model was reparameterized to compare all
the high n-3:n-6 treatment group. This finding is consistent with evidence that the γ-receptor plays a primary role (31) in breast carcinogenesis, as its activation has been a target of drug development for use in cancer prevention and control. While level of protein does not indicate its activation state, canonical patterns of pathway activity associated with PPAR activation were observed in the high n-3:n-6 group, including NF-κB phosphorylation with reduced inflammation (32), reduced phosphorylation of FOXO-1, and at sites consistent with its transcriptional activation, and the elevated level of GADD153, associated with the transcriptional activity of FOXO, observed in the carcinomas from the high n-3:n-6 treatment group (33). Hypoxia-inducible factor (Hif)-1α has also been reported to be co-regulated when PPAR is activated, and the level of this protein was reduced in the carcinomas from the high n-3:n-6 group (34, 35). Thus, in aggregate, the data shown in Table 2 provide a strong case for future investigations that focus on transcriptional activation as a mechanism of interest in elucidating how the n-3:n-6 ratio inhibits tumor growth.

PPARs are 1 of the 4 components of the intracellular energy sensing apparatus, and these receptor proteins monitor fatty acid concentrations and respond to extracellular changes by regulating cellular metabolism, particularly, lipid synthesis and degradation (6, 36). There are many facets to lipid regulation, several of which integrate with other extracellular signals such as insulin and IGF-1 as well as the intracellular energy charge that is monitored by AMPK (37). Given that an inverse association has been reported between plasma IGF-1 and the potential role of the n-3 to n-6 ratio in limiting intratumoral lipid biosynthesis merits scrutiny. Nonetheless, both measures of enzyme activity of proteins involved in lipid synthesis and GC- or liquid chromatography-time-of-flight-MS of tissue lipid profiles are required to confirm effects on the biosynthetic process.

Heterogeneity of response

A novel aspect of the data analyses used herein was the application of supervised and unsupervised clustering techniques in an effort to minimize interpretation bias and to characterize the heterogeneity of responses observed both within and between treatment groups. The use of the unsupervised PCA method, shown in Figs. 1A and 2A and Supplementary Figs. S4, S6, and S8, shows the extent of overlap in response between treatment groups for each category into which we divided the proteins that were evaluated, recognizing that some proteins could be listed in more than one category. Such overlap between groups, while embedded in mean and SE statistics, can easily be overlooked in data interpretation, which the graphic analysis helps keep in perspective. The supervised clustering approach that we also applied to the data, remaining panels in those figures with test statistics summarized in Supplementary Table S3, has the advantage that it divides the variance in response to the component associated with treatment group, the variance within a treatment group not associated with the treatment, and a final component that is unexplained variance and is frequently viewed as the biologic noise in the system. From the OPLS-DA

![Graph](www.aacajournals.org)
analyses reported herein was on synthesis, it is critical that these studies be extended to lipid catabolism. The work reported is also limited in that eicosanoid metabolism was not considered. This is a highly complex topic and will be the subject of a distinct line of investigation that is beyond the scope of the work reported herein.

**Summary of potential impact**

The evidence presented indicates considerable heterogeneity in the nature of carcinoma responsiveness to n-3 fatty acids indicating a need for assessing how various molecular subtypes of breast cancer respond to an abundance of n-3 relative to n-6 fatty acids in the diet. This information is required to better understand factors that affect cancer risk but even more importantly to understand whether n-3 fatty acids could play a role in the management of various molecular subtypes of the disease. The fact that such a high ratio of dietary n-3:n-6 fatty acids was required to achieve the profound effects on tumor growth reported herein not only indicates that these biologic activities are not likely to be achieved by dietary consumption of fish but also that there are specific metabolites of n-3 fatty acids that account for these effects and that are likely to be endogenously synthesized metabolites, the production of which is normally rate-limited. The rate-limited synthesis of specific n-3 fatty acid metabolites may account, at least in part, for the current conundrum in the n-3 fatty acid, breast cancer literature. The identification of the penultimate metabolites that account for anticancer activity is made feasible by the exaggeration of the dietary ratios of n-3: n-6 fatty acids as reported herein and is the focus of our ongoing investigations.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Jiang, Z. Zhu, J.N. McGinley, H.J. Thompson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Jiang, Z. Zhu, J.N. McGinley, H.J. Thompson

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**Acknowledgments**

The authors thank John Richie and Bogden Prokopczyk who conducted the lipid analyses referenced in this manuscript.

**Grant Support**

This work was supported by the Susan G. Komen Foundation (K081632). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 19, 2012; revised April 24, 2012; accepted May 10, 2012; published OnlineFirst May 31, 2012.

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**Figures**

**Figure 3.** Cellular processes regulating transcription factors, insulin signaling, and lipid synthesis that are likely to account for the effects on cell proliferation and apoptosis in mammary carcinomas of rats fed high versus low (control) dietary ratio of n-3:n-6 fatty acids. Diameter of red (decreased expression) and green arrows (increased expression) indicates magnitude of effect and font size of stated proteins indicates relative importance as determined by OPLS-DA. PPARγ and to a lesser extent, G-protein-coupled protein receptor 120 (GPR120) attenuate inflammation via direct or indirect effects on NF-κB and HIF-1α. PPARγ affects multiple targets in lipid metabolism including FASN. In addition, high dietary n-3:n-6 is accompanied by reduced activity of the mTOR as reflected in the reduced phosphorylation of its downstream targets including 70-kDa ribosomal protein S6 kinase (RPS6KB) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which in turn, exert effects on cell proliferation and cell survival. Mechanisms by which mTOR activity is downregulated include (i) downregulation of IGF-1R, phosphorylated insulin receptor substrate 1 (pIRS1), phosphoinositide 3-kinase 3-kinesin (P3K), phosphorylated Akt, phosphorylated Forkhead box O, and (ii) upregulation of pAMPK by increased adiponectin and decreased leptin, phosphorylated acetyl-CoA carboxylase (ACC), and phosphorylated regulatory-associated protein of mTOR (Raptor). Decreased phosphorylated mTOR and increased pAMPK further attenuate fatty acids synthesis via reduction of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) and of sterol regulatory-element-binding protein-1 (SREBP1) that results in decrease of FASN. The overall consequence of these changes in cell signaling is a decrease in cell proliferation and an increase in cell death by apoptosis.

**Limitations**

Lipid metabolism has 2 important facets, synthesis and degradation, and they are interrelated. While the focus of the
References


Identification of a Molecular Signature Underlying Inhibition of Mammary Carcinoma Growth by Dietary N-3 Fatty Acids
