Published OnlineFirst July 23, 2013; DOI: 10.1158/1940-6207.CAPR-13-0152

Research Article

Cancer Prevention Research

# Proteomic Changes Induced by Effective Chemopreventive Ratios of n-3:n-6 Fatty Acids and Tamoxifen against MNU-Induced Mammary Cancer in the Rat

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## Abstract

We used a proteomic approach to gain insights into the mechanisms of protection at the protein level by a high n-3:n-6 ratio in the absence and presence of Tamoxifen. Four groups were treated with 1-methyl-1-nitrosourea (MNU) and fed the following diets with varied n-3:n-6 ratios; group 1 = 1:1; groups 2 and 3 = 10:1 and 25:1, respectively; group 4: (25:1) plus Tamoxifen (1 mg/kg diet). The plasma from six rats/group was pooled and analyzed with the isobaric tags for relative and absolute quantitation method; 148 proteins were identified with 95% confidence by ProteinPilot 4.0. In plasma of rats fed 10:1, 25:1 n-3:n-6, and 25:1 plus Tamoxifen, the number of proteins that met our criteria ($P \leq 0.05$, error factor $\leq 2$) were 10, 14, and 19 proteins, respectively. Selected proteins were further validated by Western blotting. Compared to 1:1, both 10:1 and 25:1 diets upregulated vitamin D binding protein, gelsolin, and 14-3-3 sigma, reported to have tumor suppressive effects, whereas alpha-1B-glycoprotein, which has been reported to be elevated in the serum of breast cancer patients was decreased. Compared to 25:1, the 25:1 plus Tamoxifen diet down-regulated apolipoprotein E, haptoglobin, and inter-α-inhibitor H4 heavy chain. Ingenuity pathway analysis determined that the trends of specific proteins were related to lipid metabolism in the 25:1 n-3:n-6 group, whereas the 25:1 n-3:n-6 plus Tamoxifen group included proteins involved in cancer and inflammation. Our results show that several proteins were altered in a manner consistent with chemoprevention. Such proteins may serve as biomarkers to monitor efficacy of n-3 and Tamoxifen in future clinical chemoprevention trials. *Cancer Prev Res; 6(9); 979–88. ©2013 AACR.*

## Introduction

Breast cancer is the second leading cause of cancer death in women (1). Although effective local and synthetic therapies are available, prevention represents the best approach to reduce breast cancer morbidity and mortality. The antiestrogens, Tamoxifen and Raloxifene, have been shown to be effective chemopreventive agents reducing the risk of breast cancer in high-risk women by about 50% and 38%, respectively (2). However, only a very small portion of high-risk women take these drugs for chemoprevention primarily because of fear of side effects, particularly thromboembolic events (2). Furthermore, antiestrogens are only able to inhibit the development of estrogen receptor (ER) positive tumors, whereas they are totally ineffective at inhibiting ER negative ones. Clearly there is an urgent need to develop mechanistically based naturally occurring chemopreventive agents which can be used individually and in combination with antiestrogens. The combined approach may allow the use of lower doses of antiestrogens to minimize their side effects.

It has been postulated that the risk of breast cancer can be influenced by certain dietary components such as the amount and type of dietary fats ingested (3–7). Among the fatty acids, omega-3 (n-3) and omega-6 (n-6) fatty acids have been suggested to decrease and increase breast cancer risk, respectively. Despite the prevailing hypothesis that the ratio of n-3:n-6 may be important for chemoprevention, the optimum ratio has not been rigorously tested in many previously published reports (7). Furthermore, several chemoprevention studies in animal models used diets that were high in the percent of dietary calories provided from fat which are not consistent with the U.S. Dietary Guidelines (8). Because both issues (% calories from fat and n-3:n-6 ratio) are problematic from the translational perspectives, this stimulated our team (4) to formulate a series of experiments testing the chemopreventive efficacy of purified diets modified to reflect levels of dietary fat which are currently

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**Note:** Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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**doi:** 10.1158/1940-6207.CAPR-13-0152

Published OnlineFirst July 23, 2013; DOI: 10.1158/1940-6207.CAPR-13-0152



recommended in the U.S. Dietary Guidelines (8). In addition, within the polyunsaturated fatty acids the ratio of n-3:n-6 fatty acids was varied from 25:1 to 1:25 to provide a robust evaluation of the role of this ratio in affecting the postinitiation phase of 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis in the rat (4). Our laboratories were the first to show that the combination of n-3 fatty acids with Tamoxifen was a more effective chemopreventive regimen than the individual interventions (4, 9). Specifically, we observed that high n-3:n-6 ratios (10:1 and 25:1) were able to suppress carcinogenesis in Tamoxifen-treated animals, thus suggesting that they are able to inhibit the development of hormone-resistant tumors.

In this study, a proteomics approach using the isobaric tags for relative and absolute quantitation (iTRAQ) method was employed to provide insights into the mechanism at the protein level responsible for the chemopreventive action of the high n-3:n-6 fatty acid ratios in the absence and presence of Tamoxifen in the MNU mammary tumor model. Furthermore, this study presents our initial attempt to identify biomarkers which could be used to monitor the efficacy of n-3 fatty acids and Tamoxifen in future clinical chemoprevention studies.

## Materials and Methods

### Reagents and chemicals

Albumin/immunoglobulin G (IgG) removal kits were purchased from EMD Millipore. iTRAQ reagents were obtained from Applied Biosystems Inc. DC protein assay reagents for protein quantification were obtained from Bio-Rad Laboratories. Reagents for Western blots were obtained from Bio-Rad Laboratories and primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Abcam, and Cell Signaling. Antibodies against vitamin D binding protein (VDBP; Santa Cruz Biotechnolog; SC-32899), 14-3-3 sigma (Abcam, ab14123), alpha-1B-glycoprotein (A1BG; Santa Cruz Biotechnology; sc-132613), gelsolin (Abcam, ab134183), apolipoprotein E (Abcam, ab20874), haptoglobin (Abcam, ab117316), and inter-α inhibitor H4 heavy chain (ITIH4; Abcam, ab118283) were used. Chemilumnescent immunodetection reagents and autoradiography film were obtained from GE Healthcare.

### Experimental set-up

The plasma evaluated in this study was obtained from rats used in a recently published study (4), testing the chemopreventive effects of different n-3:n-6 ratios individually and in combination with Tamoxifen. Briefly, female Sprague Dawley rats were injected intraperitoneally at 21 days of age with 50 mg of MNU/kg body weight as previously described (4). Seven days postadministration of the carcinogen, all rats were randomized into treatment groups and were fed their respective experimental diets (30 rats/group): *ad libitum* consisting of 1:25, 1:10, 1:5, 1:1, 5:1, 10:1, 25:1 n-3:n-6 fatty acids with or without Tamoxifen (1.0 mg Tamoxifen citrate/kg diet). The ratio of polyunsaturated:saturated:monounsaturated fatty acids was 10%:10%:10%, according to FDA recommendation (8). The rats were weighed and palpated for detection of mammary tumors twice a week. At the end of 8 weeks the rats were sacrificed by cervical dislocation. Blood was collected through the retro-orbital-sinus and distributed into heparinized capillary tubes and EDTA-coated tubes. All samples were aliquoted and kept at −80°C.

### Plasma samples

The experimental procedures to collect plasma samples were previously described (4). In this study, we selected 6 plasma samples per group at random from rats fed a ratio of n-3:n-6 of 1:1 (control; Group 1), 10:1 (Group 2), 25:1 (Group 3), and 25:1 with Tamoxifen (1 mg Tamoxifen citrate/kg diet; Group 4).

### Protein depletion and quantitation

The abundant blood proteins, IgG and serum albumin, were mostly depleted from all plasma samples using an Albumin/IgG removal column (CalBiochem; Cat. No. 122642). To ensure proper removal of albumin, an aliquot from each depleted sample was separated on a 10% SDS-PAGE and stained with Coommasie Blue (Bio-Rad 161-0786). The samples were then precipitated according to manufacturer's procedure for the CalBiochem ProteoExtract Protein Precipitation Kit for volumes larger than 200 μL. After the precipitation 150 μL of MilliQ water, 33 μL of 6M urea, and 10 μL of 2% SDS solution was added to each precipitated pellet for solubilization. Following depletion, precipitation, and solubilization, aliquots of all samples were submitted to a DC Protein Assay to determine total protein concentration (Bio-Rad Laboratories).

The plasma samples from each of the 4 groups (1:1 n-3:n-6, 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus Tamoxifen) were assigned an isobaric tag for a 4-plex experiment as described in Fig. 1. A total of 6 samples of Sprague Dawley rat plasma from each group was pooled (500 μg protein) together. From this pool 115 μg of protein was denatured, reduced, alkylated, and digested with 2% SDS, 5 mmol/L tris-(2-carboxyethyl) phosphine, 84 mmol/L iodoacetamide (Sigma-Aldrich), and trypsin (5:1; Promega), respectively. The samples were dried under vacuum for 2 hours. To the contents of each tube an appropriate amount of 0.5M TEAB with ethanol was added until the pH of all samples was between 7.0 and 8.0. Each iTRAQ Tag (Applied Biosciences) was added to its designated pool of protein for a specific group and incubated in the dark, followed by drying by vacuum. Once samples were tagged all samples from each group were mixed and submitted to the Pennsylvania State University College of Medicine Proteomic/MS Core Facility. The samples were separated by 2D-LC and analyzed by MALDI-TOF/TOF mass spectroscopy as described previously (10). Figure 1 summarizes the steps mentioned earlier.

### Database search and statistical analysis

Following the submission of the samples to 2D-LC and MALDI-TOF/TOF, the samples were analyzed by the Paragon Algorithm in ProteinPilot 4.0 software. Using this

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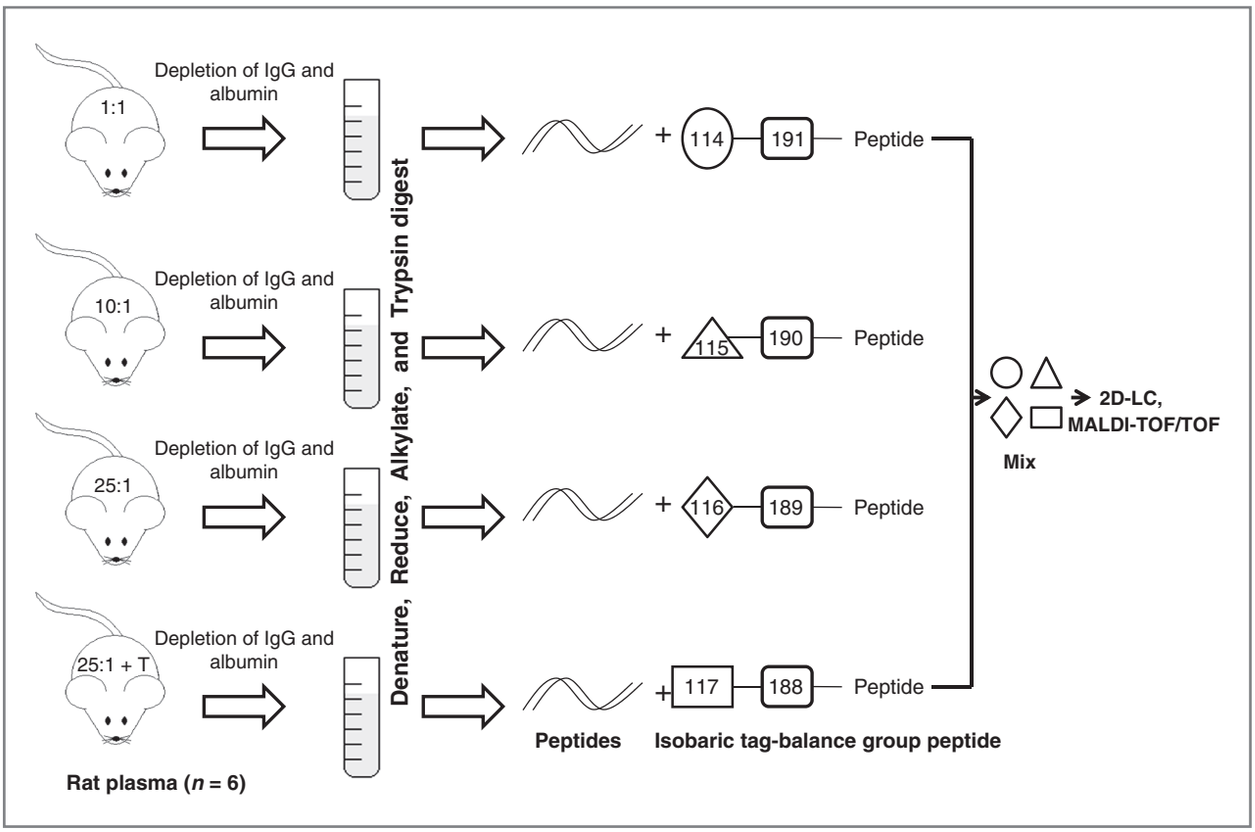


Figure 1. Experimental set-up of 4-plex iTRAQ analysis. Plasma from 6 rats representing each group was depleted of albumin and IgG and combined. The plasma was submitted to denaturation, reduction, alkylation, and trypsin digestion, followed by isobaric tagging to prepare for 2D-LC and MALDI-TOF/TOF, used in iTRAQ analysis.

algorithm a list of high-confidence protein identifications was developed, requiring a ProteinPilot Unused Score of greater than 1.3 (greater than 95% confidence interval). The stringent local false discovery rate (FDR) estimation, using the PSPEP algorithm (11) based on simultaneously searching a Decoy database composed of the exact reverses of each protein sequence in the normal forward database, were used to independently estimate the FDR. All proteins considered confidently identified had a local FDR estimation no higher than 5%.

The log ratios of the proteins identified with high confidence in groups given 10:1 n-3:n-6, 25:1 n-3:n-6, 25:1 n-3:n-6 plus Tamoxifen were compared to the log ratios of the 1:1 n-3:n-6 group (control-Tag 114) using a Student two-tailed *t* test in Microsoft Excel; a comparison between 25:1 n-3:n-6 and 25:1 n-3:n-6 plus Tamoxifen was also made. Only proteins that had a log ratio with a *P*-value of less than or equal to 0.05 and an error factor (EF) less than or equal to 2 in the groups 10:1 n-3:n-6, and 25:1 n-3:n-6, and 25:1 n-3:n-6 fatty acids plus Tamoxifen, when compared with the control group, were further investigated.

### Western blot analysis

Proteins from diluted/undiluted plasma were submitted to acrylamide gels that ranged from 10% to 20% acrylamide (Bio-Rad Laboratories) using 14 to 35 μg protein per well. They were transferred to PVDF (Bio-Rad Laboratories), blocked with 5% milk, and probed overnight at 4°C with antibodies to validate the differences in protein levels discovered in the iTRAQ analysis. Proteins that were validated include VDBP, 14-3-3 sigma, A1BG, gelsolin, apolipoprotein E, haptoglobin, and ITIH4. The images were captured with Bio-Rad's GS800 Calibrated Densiometer and quantified with the Quantity One v4.5.0 1D Analysis Software (Bio-Rad Laboratories). The equal loading technique was used based on determining protein concentration using the Bio-Rad DC Protein Assay. Staining of the PVDF membrane with Ponceau S confirmed that the proteins were loaded equally. A Student *t* test was used to determine significance of results which was considered a *P*-value of $\leq 0.05$.

## Results

As compared to control rats treated with MNU and fed a diet containing n-3:n-6 ratio of 1:1, tumor incidence was significantly decreased by 12%, 21%, and 72% in rats treated with MNU, but fed 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus Tamoxifen, respectively (4, 12). Tumor multiplicity was also decreased by 26%, 30%, and 80% in the rats fed 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus Tamoxifen, respectively.

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Figure 2 illustrates a representative MS and peptide MS/MS spectrum of the corresponding amino acid sequence, AGKEPGLQIWR, used in the identification and quantification of one of the proteins identified in this study, gelsolin. The 4-plex iTRAQ analysis of the plasma samples from rats treated with set ratios of n-3 and n-6 fatty acids identified 148 proteins with high confidence (unused protein score with 95% confidence interval or greater). From this list 10, 14, and 19 proteins were modulated significantly ($P \leq 0.05$) and with an EF less than or equal to 2 (SD $\leq$ 20%) in the groups given 10:1 n-3:n-6, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus Tamoxifen, respectively, when compared to the control group given 1:1 n-3:n-6 ratio (Supplementary Table S1).

### Proteins are modulated in rats administered a diet of n-3:n-6 fatty acids in a ratio of 10:1, 25:1, and 25:1 plus Tamoxifen

Out of 148 proteins identified, 10 proteins were significantly upregulated in plasma of the Sprague Dawley rats ($P \leq 0.05$, EF $\leq 2$) given a dietary ratio of 10:1 n-3:n-6 fatty acids compared to the control group given the 1:1 ratio (Supplementary Table S2).

The plasma from rats administered a diet of 25:1 n-3:n-6 fatty acids had 12 known proteins upregulated and 2 proteins downregulated according to our criteria ($P \leq 0.05$, EF $\leq 2$) as illustrated in Supplementary Table S3. Proteins such as VDBP and A1BG were differentially altered in a manner consistent with chemoprevention (13–16). VDBP was found to be upregulated, whereas A1BG was downregulated in response to increasing levels of n-3 fatty acids.

Supplementary Table S4 illustrates 19 proteins were regulated by the dietary intervention of 25:1 n-3:n-6 fatty acids with Tamoxifen according to our criteria ($P \leq 0.05$, EF $\leq 2$); 11 proteins were upregulated and 8 proteins were downregulated. The statistically significant changes in observed iTRAQ ratios showed that gelsolin was upregulated, whereas A1BG was downregulated further.

### Verification by Western blot analysis of proteins identified by iTRAQ

Next, we conducted Western blot analysis to verify the alterations in protein levels identified by iTRAQ. Several proteins were selected based on their known contributions to carcinogenesis and the availability of their antibodies. Gelsolin was found to be upregulated in the 10:1 n-3:n-6, but significantly higher yet in the 25:1 n-3:n-6 group (Fig. 3A). A similar trend was seen with VDBP (Fig. 3B). However, the protein A1BG was found to be downregulated in the 10:1 n-3:n-6, and was significantly lower in the 25:1 n-3:n-6 group (Fig. 3C). The protein 14-3-3 sigma, not identified by iTRAQ analysis but still of interest due to its well-known tumor suppressor activity in breast cancer (17–19), was observed to be increased significantly in the 10:1 and 25:1 n-3:n-6 groups (Fig. 3D).

Proteins from depleted rat plasma representing the 25:1 and 25:1 n-3:n-6 plus Tamoxifen groups were also analyzed by Western blot analysis. The proteins apolipoprotein E, haptoglobin, and ITIH4 were shown to decrease in the plasma obtained from rats given a combination of 25:1 n-3:n-6 plus Tamoxifen, in comparison to the 25:1 n-3:n-6 fatty acids alone, as shown in Fig. 4A–C, respectively.

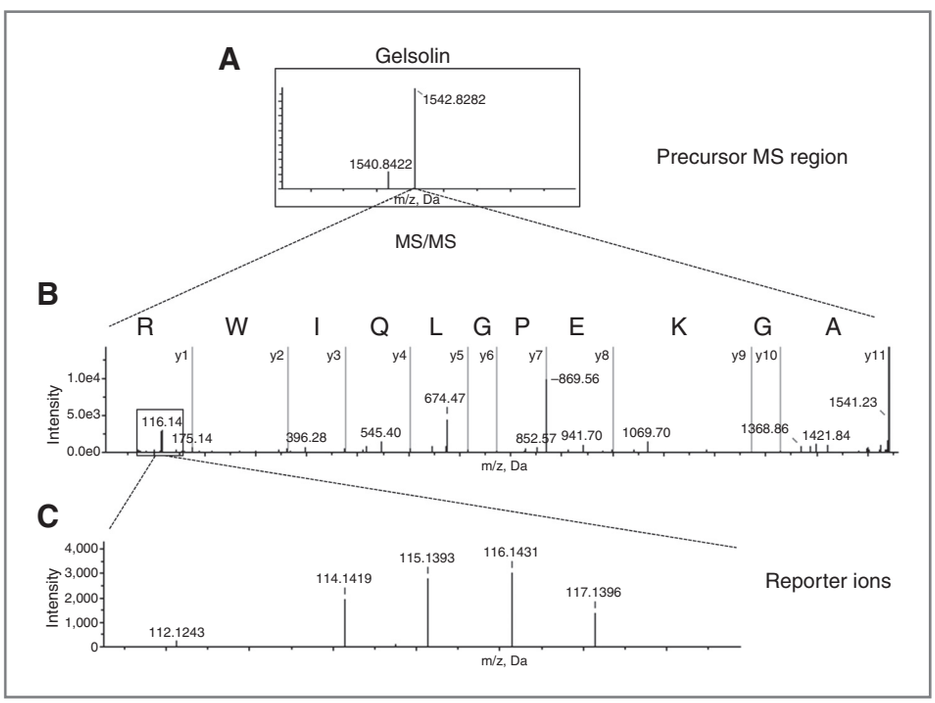


Figure 2. Identification of gelsolin. MALDI-TOF MS (A) for gelsolin and its tandem MS (B) showing the unique peptide AGKEPGLQIWR for the identification of gelsolin. The protein was quantitated by observing the intensities of the reporter ions (C) for groups 114, 115, 116, and 117.

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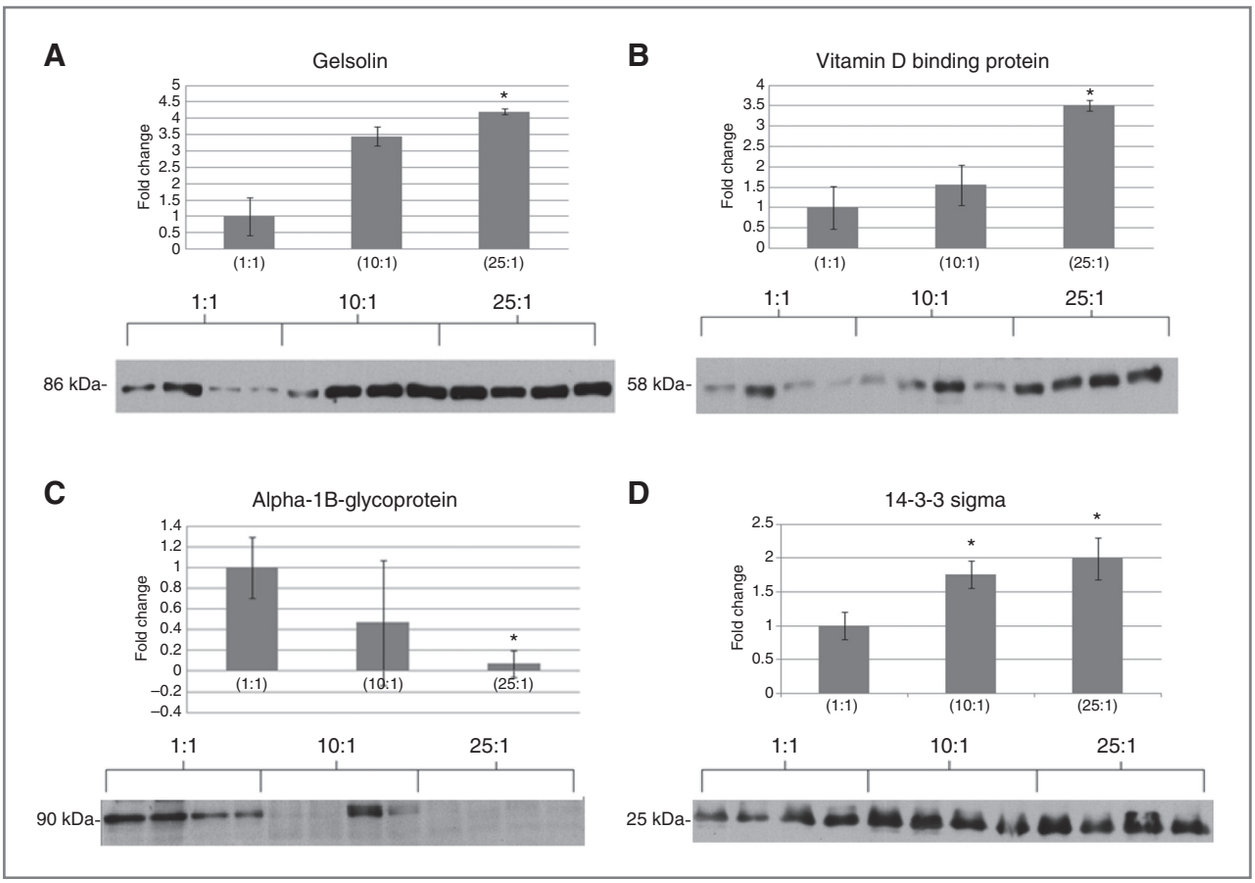


Figure 3. Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 1:1, 10:1, and 25:1 n-3:n-6). A, gelsolin expression. B, vitamin D binding protein expression. C, alpha-1B-glycoprotein expression. D, 14-3-3 sigma expression; *, $P \leq 0.05$.

### Biological significance of proteins modulated by n-3:n-6 fatty acid ratios of 10:1 and 25:1 and 25:1 plus Tamoxifen

All biological processes were defined through data according to PANTHER Classification System (*P*rotein *AN*alysis *TH*rough *E*volutionary *R*elationships). The 10 proteins identified in the 10:1 n-3:n-6 group are known to be involved in several biological processes that include complement activation (complement C3, haptoglobin, α1-macroglobulin), proteolysis (complement C3, inter-α-trypsin inhibitor heavy chain H3, ITIH4, α2-antiplasmin, α1-macroglobulin) and blood coagulation (fibrinogen γ chain, fibrinogen α chain isoform 1, coagulation factor XIII B chain, fibrinogen β chain). Additional biological processes that were determined by the PANTHER database are summarized in Fig. 5A.

In the 25:1 n-3:n-6 group, the biological processes of the 14 proteins identified include involvement with transport (VDBP, ceruloplasmin, serotransferrin), lipid transport and metabolism (apolipoprotein E), blood coagulation (fibrinogen α chain isoform 1, coagulation factor XIII B chain, fibrinogen β chain), proteolysis (ITIH4), complement activation (complement C3, complement component C9, α1-macroglobulin), and responding to certain stimuli (A1BG). Fetuin B did not have a noted biological process according to PANTHER. A hypothetical protein with accession number 293348303 was also identified but its function still has to be determined. Additional biological processes that were determined by the PANTHER database are summarized in Fig. 5B.

The plasma from rats administered a diet of 25:1 n-3:n-6 fatty acids plus Tamoxifen had 19 proteins modulated in response to high n-3 fatty acids and the anti-estrogen. These proteins are known to be involved in immune system processes (fibrinogen β chain, fibrinogen α chain isoform 1, leukemia inhibitory factor receptor, serum amyloid A-4), transport (VDBP, serotransferrin), proteolysis (plasma kallikrein, α2-antiplasmin, serine protease inhibitors A3N and A3M), complement activation (C4b-binding protein α chain, α1-macrogloblin), blood coagulation (kininogen-1 isoform 1, coagulation factor XIII B), cell adhesion (fibronectin), responding to stimuli (A1BG), and cell component morphogenesis (gelsolin). Fetuin B did not have a noted biological process according to PANTHER. A hypothetical protein with accession number 293348303 was also identified but its function still has to be determined. Additional biological processes that were determined by the PANTHER database are summarized in Fig. 5C.

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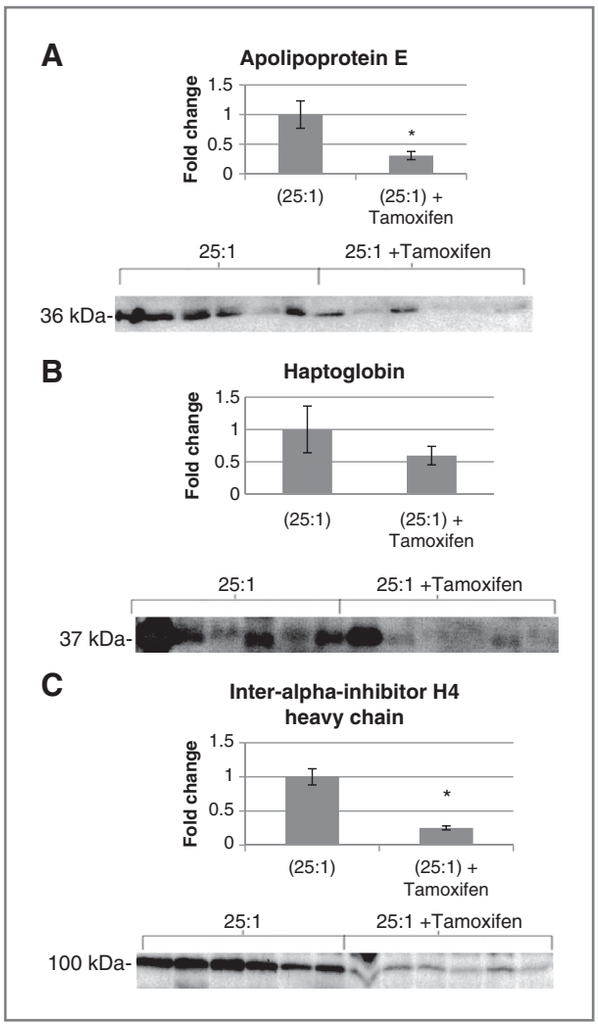


Figure 4. Western blot analysis of specific proteins for validation of iTRAQ analysis (comparison of 25:1 n-3:n-6 with 25:1 n-3:n-6 plus Tamoxifen). A, apolipoprotein E expression; B, haptoglobin expression; C, inter-α-inhibitor H4 heavy chain expression; *, $P \leq 0.05$.

### Ingenuity pathway analysis of proteins quantitated via iTRAQ

A means of determining the significance of the proteins identified by iTRAQ analysis would be advantageous in understanding how their regulation is being controlled by the various dietary interventions. Using the program ingenuity pathway analysis (IPA) allows for such relationships to be further investigated. Molecules that resulted in a *Z*-score higher than 3, an indicator signifying high confidence in the pathways linked to our proteins determined by mass spectrometry identification and iTRAQ, contributed to the pathway analysis. Figure 6 illustrates the proteins that are unique and in common to 10:1, 25:1 n-3:n-6, and 25:1 n-3:n-6 plus Tamoxifen groups identified by iTRAQ that have a *P*-value less than or equal to 0.05 and an EF less than or equal to 2 when compared to 1:1 n-3:n-6.

IPA determined that the trend of regulation seen in some proteins in the 10:1 n-3:n-6 group were related to hematologic system development and function. Specifically, the fold change seen in all proteins was related to this associated network function. IPA determined that the trends of specific proteins were related to lipid metabolism in the 25:1 n-3:n-6 group, whereas the 25:1 n-3:n-6 plus Tamoxifen intervention included proteins involved in cancer and inflammation. Specifically, it determined that the increase in apolipoprotein E and VDBP were reported to be linked to lipid metabolism. In the 25:1 n-3:n-6 fatty acids plus Tamoxifen group, the decrease in A1BG and increase in gelsolin were linked to cancer.

## Discussion

To our knowledge this is the first study that examined the influence of effective chemopreventive amounts of n-3 relative to n-6 fatty acids on protein expression using iTRAQ method in a well-defined animal model of chemically induced mammary carcinogenesis (4). Our goal is to understand how ingestion of high amounts of n-3 fatty acids in the absence and presence of Tamoxifen influence the plasma proteome in a way that can provide insights into the mechanism of chemoprevention (4). Furthermore, we focused on the plasma proteome, because it is a minimally invasive, and consequently a practical method to measure biomarkers in clinical chemoprevention studies.

The results of this study show that changes in plasma protein expression in rats that received high n-3:n-6 ratios were consistent with chemoprevention (4). In a recent study, we showed (12) that the mechanism that can account for the chemopreventive activity of the high ratio of n-3:n-6 (25:1) against the development of mammary cancer is due to the inhibition of cell proliferation and more so the induction of apoptosis in the adenocarcinomas. In this previous report (12) we showed that in the target tissue (mammary adenocarcinomas) from the same animals from which plasma samples were collected using Western blot analysis changes in proteins involved in apoptosis, transcription regulation, growth factor regulation, and lipid metabolism were consistent with cancer prevention. Taken together, the results of this report (plasma proteins) and those of our previous study (ref. 12; target organ proteins) provide a plausible justification of the potential utility of some of these proteins to monitor efficacy of n-3 fatty acids as chemopreventive agents in clinical trials.

We used the IPA to investigate the biological role of the proteins altered by n-3:n-6 fatty acid ratios according to iTRAQ. We found that high ratios of n-3:n-6 fatty acids with and without Tamoxifen affected plasma levels of proteins involved in cancer and lipid metabolism. It is proposed that n-3:n-6 fatty acid ratios influence the synthesis of lipids which is correlated to mammary tumor growth in MNU-treated rats (12). For example, we showed that n-3:n-6 ratios of 10:1 and 25:1 upregulated Fetuin B. Fetuin B, a glycoprotein of the fetuin family, has been shown to be involved in fatty acid metabolism in human liver cell lines as supported by the observation that pACC and pAMPK were downregulated in fetuin B knockdown cells (20). Therefore, the upregulation of fetuin by high n-3:n-6 fatty acid ratios

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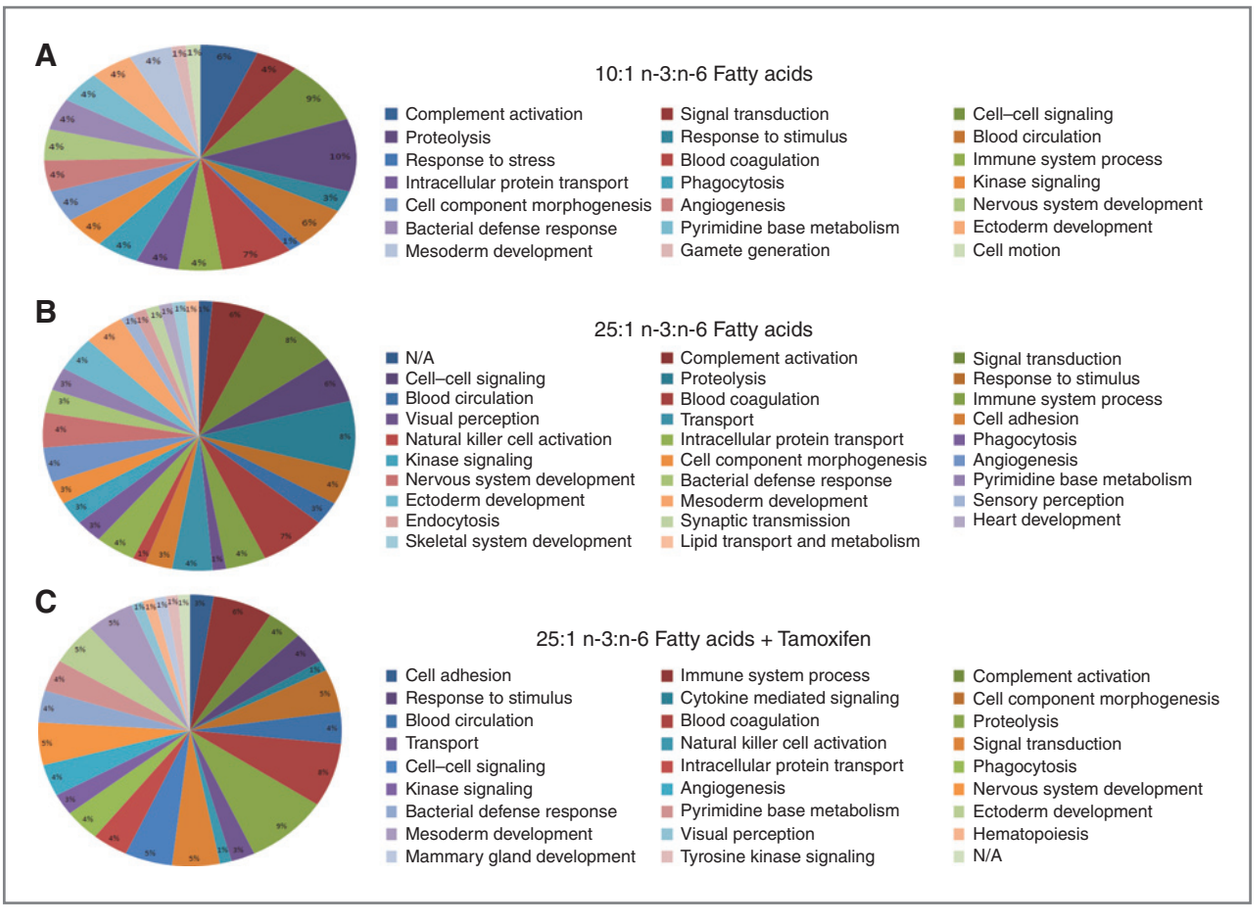


**Figure 5.** Panther biological processes according to dietary supplementation. The proteins are identified by the percentage of biological processes shared. A, 10:1 n-3:n-6 fatty acids; B, 25:1 n-3:n-6 fatty acids; and C, 25:1 n-3:n-6 fatty acids plus Tamoxifen.

may provide a mechanism for the induction of pACC and pAMPK by high n-3:n-6 ratios as recently reported (12).

Some proteins that were associated with cancer included in the IPA were A1BG, fibronectin, gelsolin, fibrinogen α chain, fibrinogen β chain, VDBP, plasma kallikrein, leukemia inhibitory factor receptor, kininogen, serum amyloid A4, serine protease inhibitors A3N/M, and serotransferrin. Proteins involved in associated networks with lipid metabolism include apolipoprotein E, ITIH4, A1BG, ceruloplasmin, fibrinogen α chain, fibrinogen β chain, complement C3, complement component C9, serotransferrin, and VDBP.

VDBP is involved in calcium homeostasis, immunity (21, 22), and antiangiogenic (23) as well as antiproliferation processes (24). The protein is primarily synthesized in the liver and secreted into the plasma, where it binds to vitamin D and known to be taken up by human mammary cells via endocytosis (25). With regards to immunity, VDBP is involved in macrophage activation by having its branched trisaccharide complex hydrolyzed by β-glactosidase on B cells and salidase from T cells forming the macrophage activating factor (GcMAF). In patients with metastatic breast cancer it was found that administration of VDBP in the form of GcMAF decreased Nagalase activity, an indicator of macrophage activation that can lead to cancer cell death (21).

A major role of VDBP is to transport vitamin D to peripheral tissues and to facilitate the tissue uptake of vitamin D (25). This protein has been noted as having a protective effect in breast cancer by facilitating transport of vitamin D metabolites (13) and has been shown to be downregulated in the serum of breast cancer patients (26). Some studies suggest that specific genetic variants of VDBP influence a woman's risk of developing breast cancer (27). In the aggregate, our results show an increase in plasma levels of VDBP in rats fed a high n-3:n-6 fatty acid ratio which provides support for an additional possible mechanism of chemoprevention by n-3 fatty acids.

Another protein found in our study to be significantly upregulated by high n-3:n-6 fatty acid ratio was gelsolin. Its expression has been shown to be reduced in various human cancer cell lines and tumors including breast (28–30). *In vivo* studies using MMTV-*Her2*/neu transgenic mice have shown downregulation of gelsolin at the mRNA level in primary mammary tumors in comparison to the normal mammary gland (31). Furthermore, it was showed that

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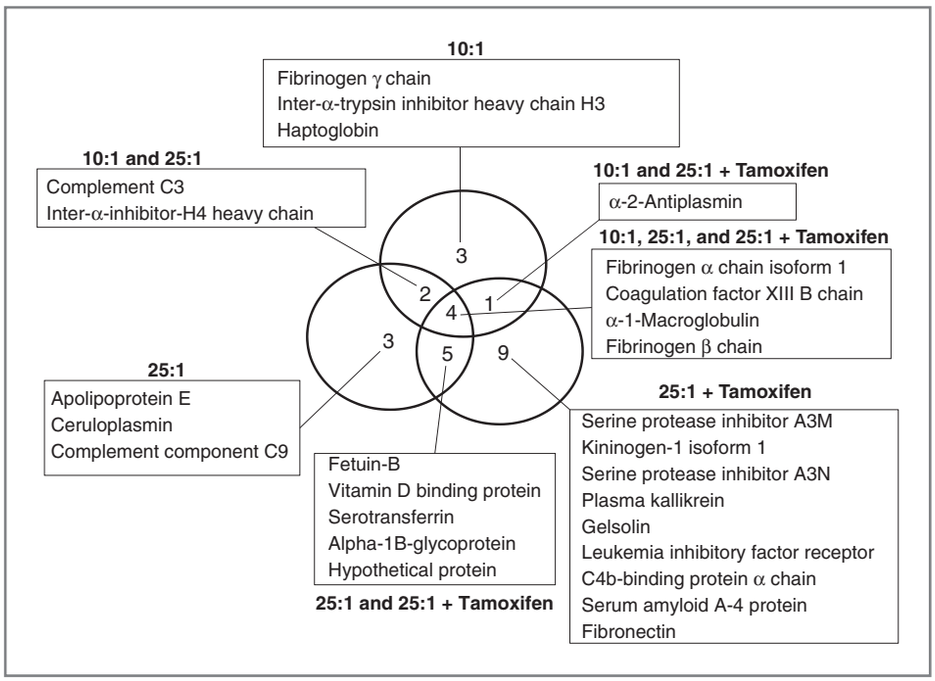


**Figure 6.** Proteins significantly modulated by dietary ratios of n-3: n-6 fatty acids in the presence and absence of Tamoxifen according to iTRAQ analysis ($P \leq 0.05$, ER $\leq 2$).

gelsolin expression was reduced in human mammary lesions as the disease progressed; atypical ductal hyperplasia→ductal carcinoma *in situ*→invasive carcinoma (32–34). However, a previous report indicated that gelsolin levels were increased in the plasma of breast cancer patients (35), suggesting the role of gelsolin in carcinogenesis needs to be further investigated. In this study we showed that gelsolin was upregulated in plasma of the rats given high n-3:n-6 ratios. The role of n-3 fatty acids in the upregulation of gelsolin is also supported by the observation that restriction of n-3 fatty acids leads to gelsolin cleavage by caspases (36).

Our studies also confirmed that A1BG was downregulated in a manner consistent with chemoprevention. A1BG is known to bind to CRISP-3 (37) and is partially similar in amino acid sequence to the opossum protein oprin, a metalloproteinase (38). In the serum of breast cancer patients, A1BG was shown to be slightly increased in expression in comparison to healthy controls (15). In pancreatic juice and pancreatic tissue of pancreatic cancer patients, A1BG was upregulated in comparison to cancer-free controls (39). A1BG has also been shown to be elevated in the urine of bladder cancer patients compared to controls (16).

Although not detected by iTRAQ, Western blot analysis clearly showed that a high n-3:n-6 ratio increased the plasma level of 14-3-3 sigma, a well-established tumor suppressor gene (17). This protein has been shown to be downregulated in many types of cancer including breast cancer (18, 40). It is thought that hypermethylation at CpG islands at the gene promoter of the 14-3-3 sigma causes the downregulation of the protein in breast cancer patients (19). It was recently determined that docosahexaenoic acid increased the amount of 14-3-3 sigma protein expressed in colon cancer cells (41). Collectively, these data raise the possibility that n-3 fatty acids may upregulate 14-3-3 sigma through an epigenetic mechanism.

Proteins altered by a high ratio of n-3:n-6 fatty acids and Tamoxifen in manner consistent with chemoprevention include haptoglobin, ITIH4, and apolipoprotein E. Considering the multiple pathways which can be targeted by n-3 fatty acids alone (function as PPARγ agonist) and Tamoxifen (function as antiestrogen) and the known crosstalk between PPARγ and ER receptors, it would not be unrealistic to expect that the type of proteins determined by iTRAQ may be regulated differently following the 2 different interventions, that is fatty acids versus fatty acids plus Tamoxifen. We showed in this study that differential expression of several proteins (VDBP, gelsolin, 14-3-3 sigma) is consistent with the chemopreventive effects of the high n-3:n-6 ratio, whereas the combination of Tamoxifen with high n-3:n-6 ratio unfavorably alters their expression. However, such a combination favorably altered other proteins (haptoglobin, ITIH4, and apolipoprotein E) in a manner that is consistent with chemoprevention. This suggests that the combination of fatty acids plus Tamoxifen exerted a different mechanism of action from that of the fatty acids alone.

Haptoglobin is an acute-phase protein synthesized and dispersed by the liver, muscle, and adipose tissue and has a role in inflammatory states, such as obesity (42). Some studies (43) but not others (44, 45) have reported an increase in the level of this protein in the serum of breast cancer patients. Consistent with the iTRAQ analysis, haptoglobin was downregulated in response to an increase in n-3 fatty acid administration, with a more pronounced effect in the group given high n-3 fatty acids plus Tamoxifen.

Published OnlineFirst July 23, 2013; DOI: 10.1158/1940-6207.CAPR-13-0152



ITIH4, a protein secreted by the liver in times of trauma and involved in the acute-phase response, was shown to have a downward trend as the amount of n-3 fatty acids increased in the dietary groups. In serum from breast cancer patients it was shown that this protein as peptide fragments was upregulated in comparison to healthy controls (46, 47). ITIH4 has also proposed to be cleaved by plasma kallikrein in cancers such as ovarian and breast (47, 48) which could possibly explain the bands seen at 120 kDa (data not shown) and 100 kDa. Thus, the downward trend of the expression is consistent with chemoprevention.

Apolipoprotein E, responsible for fat and cholesterol transport, was found to be down-regulated in the serum of rats given an n-3:n-6 dietary ratio of 25:1 in combination with Tamoxifen, when compared to rats given only the 25:1 n-3:n-6 ratio, thus independently validating the iTRAQ results. There are 4 allelic variants of the apolipoprotein E gene (ε1, ε2, ε3, ε4,) with the ε4 gene being associated with breast cancer among Asian population (49). Apolipoprotein E has been shown to be upregulated in breast cancer in prediagnostic serum (50). Thus, it seems that the combination of high amounts of n-3 and Tamoxifen down-regulated apolipoprotein E in the plasma of rats, which could contribute to the increased efficacy of the combination in comparison to the individual interventions.

In summary, changes in the levels of plasma proteins identified via iTRAQ and further validated by Western blot analysis, that is VDBP, gelsolin, A1BG, 14-3-3 sigma, haptoglobin, apolipoprotein E, and ITIH4 are known to be involved in lipid metabolism and carcinogenesis, thus providing some insight on how high amounts of n-3 fatty acids in the absence and presence of Tamoxifen may inhibit breast carcinogenesis as seen in our previous study (4). However, better understanding of the defined role of these proteins in the molecular signature that can fully explain inhibition of mammary cancer by high n-3:n-6 ratio (12) and more so in the presence of Tamoxifen (4) requires further investigation.

## 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.):** H.J. Thompson, A. Stanley, B.A. Stanley, K. El-Bayoumy
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C.G. Skibinski, A. Das, B.A. Stanley, K. El-Bayoumy
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**Study supervision:** A. Das, A. Manni, K. El-Bayoumy

## Grant Support

This work has been funded by Susan G. Komen for the Cure, KG081632.

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Received April 24, 2013; revised July 10, 2013; accepted July 12, 2013; published OnlineFirst July 23, 2013.

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Published OnlineFirst July 23, 2013; DOI: 10.1158/1940-6207.CAPR-13-0152



Cancer Prevention Research

# Proteomic Changes Induced by Effective Chemopreventive Ratios of n-3:n-6 Fatty Acids and Tamoxifen against MNU-Induced Mammary Cancer in the Rat

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*Cancer Prev Res* 2013;6:979-988. Published OnlineFirst July 23, 2013.