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 6 rats/group was pooled and analyzed with the isobaric Tags for Relative and Absolute Quantitation(iTRAQ) 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≤0.05, error factor≤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 up-regulated vitamin D binding protein, gelsolin, and 14-3-3 sigma, reported to have tumor suppressive effects, whereas A1BG, 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 ITIH4. Ingenuity Pathway Analysis determined that the trends of specific proteins were related to lipid metabolism in the 25:1 n-3:n-6 group, while the 25:1 n-3:n-6 plus Tamoxifen group included proteins involved in cancer and inflammation. Our results demonstrate 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.
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 anti-estrogens, 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, anti-estrogens are only able to inhibit the development of estrogen receptor positive tumors, while they are totally ineffective at inhibiting estrogen receptor 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 anti-estrogens. The combined approach may allow the use of lower doses of anti-estrogens 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 utilized 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 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 in order to provide a robust evaluation of the role of this ratio in affecting the post-initiation phase of 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis in the rat (4). Our laboratories were the first to demonstrate 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 the present study, a proteomics approach utilizing 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/IgG removal kits were purchased from EMD Millipore (Billerica, MA). iTRAQ reagents were obtained from Applied Biosystems Inc. (ABI, Foster City, CA). DC Protein Assay Reagents for protein quantification were obtained from Bio-Rad Laboratories (Hercules, CA). Reagents for Western blots were obtained from Bio-Rad Laboratories (Hercules, CA) and primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, MA), and Cell Signaling (Davers, MA). Antibodies against vitamin D binding protein, VDBP, (Santa Cruz Biotechnology, SC-32899), 14-3-3 sigma (Abcam, ab14123), alpha-1-b-glycoprotein, A1BG, (Santa Cruz Biotechnology, sc-132613), gelsolin (Abcam, ab134183), apolipoprotein E (Abcam, ab20874), haptoglobin (Abcam, ab117316), and inter-alpha inhibitor H4 heavy chain, ITIH4 (Abcam, ab118283) were used. Chemiluminescent immunodetection reagents and autoradiography film were obtained from GE Healthcare (Piscataway, NJ).

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 i.p. at 21 days of age with 50 mg of MNU/kg body weight as previously described (4). Seven days post-administration 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 eight 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 six 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% sodium dodecyl sulfate (SDS)-polyacrylamide gel 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 200µl. After the precipitation 150µl of MilliQ water, 33µl of 6M urea, and 10µl 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 four groups (1:1 n-3:n-6, 10:1 n-3:n-6, 25:1 n-3:n-6, 25:1 n-3:n-6 plus Tamoxifen) were assigned an isobaric tag for a 4-plex experiment as described in Figure 1. A total of six 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, 5mM tris-(2-carboxyethyl) phosphine, 84mM iodoacetamide (Sigma-Aldrich, St. Louis, MO), and trypsin (5:1, Promega, Madison, WI), respectively. The samples were dried under vacuum for two 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 7.0-8.0. 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 above.

**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 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
based on simultaneously searching a Decoy database comprised of the exact reverses of each protein sequence in the normal forward database, were used to independently estimate the False Discovery Rate. All proteins considered confidently identified had a Local False Discovery Rate 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) utilizing a Student’s 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 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 10-20% acrylamide (Bio-Rad Laboratories) utilizing 14-35 µg protein per well. They were transferred to PVDF (Bio-Rad Laboratories), blocked with 5% milk, and probed over-night 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 utilized 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 utilized 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.

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 ≤0.05) and with an error factor (EF) less than or equal to 2 (standard deviation ≤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 (Supplement Table I).

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 up-regulated in plasma of the Sprague Dawley rats (p ≤0.05, EF≤2) given a dietary ratio of 10:1 n-3:n-6 fatty acids compared to the control group given the 1:1 ratio (Supplement Table II).

The plasma from rats administered a diet of 25:1 n-3:n-6 fatty acids had 12 known proteins up-regulated and 2 proteins down-regulated according to our criteria (p ≤0.05,
EF≤2) as illustrated in Supplement Table III. Proteins such as VDBP and A1BG were differentially altered in a manner consistent with chemoprevention (13-16). VDBP was found to be up-regulated, while A1BG was down-regulated in response to increasing levels of n-3 fatty acids.

Table Supplement IV illustrates 19 proteins were regulated by the dietary intervention of 25:1 n-3:n-6 fatty acids with Tamoxifen according to our criteria ($p≤0.05$, EF≤2); 11 proteins were up-regulated and 8 proteins were down-regulated. The statistically significant changes in observed iTRAQ ratios demonstrated that gelsolin was up-regulated, while A1BG was down-regulated further.

**Verification by Western Blot Analysis of Proteins Identified by iTRAQ**

Next, we performed 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 up-regulated in the 10:1 n-3:n-6, but significantly higher yet in the 25:1 n-3:n-6 group (Figure 3A). A similar trend was seen with VDBP (Figure 3B). However, the protein A1BG was found to be down-regulated in the 10:1 n-3:n-6, and was significantly lower in the 25:1 n-3:n-6 group (Figure 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 (Figure 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 Figures 4A, 4B, and 4C, respectively.

**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 (Protein ANalysys THrough Evolutionary Relationships). 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, alpha-1-macroglobulin), proteolysis (complement C3, inter-alpha-trypsin inhibitor heavy chain H3, inter-alpha-inhibitor H4 heavy chain, alpha-2-antiplasmin, alpha-1-macroglobulin) and blood coagulation (fibrinogen gamma chain, fibrinogen alpha chain isoform 1, coagulation factor XIII B chain, fibrinogen beta chain). Additional biological processes that were determined by the PANTHER database are summarized in Figure 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 alpha chain isoform 1, coagulation factor XIII B chain, fibrinogen beta chain), proteolysis (inter-alpha-inhibitor H4 heavy chain), complement activation (complement C3, complement component C9, alpha-1-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 Figure 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 beta chain, fibrinogen alpha chain isoform 1, leukemia inhibitory factor receptor, serum amyloid A-4), transport (VDBP, serotransferrin), proteolysis (plasma kallikrein, alpha-2-antiplasmin, serine protease inhibitors A3N and A3M), complement activation (C4b-binding protein alpha chain, alpha-1-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 Figure 5C.

**Ingenuity Pathway Analysis (IPA) 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. Utilizing 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 error factor 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 hematological 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, while 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, since it is a minimally-invasive, and consequently a practical method to measure biomarkers in clinical chemoprevention studies.

The results of the present study demonstrate 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 demonstrated (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 the present report (plasma proteins) and those of our previous study (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 Ingenuity Pathway Analysis 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 up-regulated 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 down-regulated in fetuin B knockdown cells (20). Therefore, the up-regulation of fetuin by high n-3:n-6 fatty acid ratios 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 Ingenuity Pathway Analysis were A1BG, fibronectin, gelsolin, fibrinogen alpha chain, fibrinogen beta 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 alpha chain, fibrinogen beta chain, complement C3, complement component C9, serotransferrin, and VDBP.

VDBP is involved in calcium homeostasis, immunity (21, 22), and anti-angiogenic (23) as well as anti-proliferation 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 beta-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 down-regulated 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 up-regulated 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 utilizing MMTV-Her2/neu transgenic mice have shown down-regulation of gelsolin at the mRNA level in primary mammary tumors in comparison to the normal mammary gland (31). Furthermore, it was demonstrated that gelsolin expression was reduced in human mammary lesions as the disease progressed; atypical ductal hyperplasia \( \rightarrow \) ductal carcinoma in-situ \( \rightarrow \) 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 the present study we showed that gelsolin was up-regulated in plasma of the rats given high n-3:n-6 ratios. The role of n-3 fatty acids in the up-regulation 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 down-regulated 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 up-regulated 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 down-regulated 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 down-regulation 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 up-regulate 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 anti-estrogen) 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 two different interventions, i.e. fatty acids vs. fatty acids plus Tamoxifen. We showed in the present 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, while 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 down-regulated 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.

Inter-alpha inhibitor H4 heavy chain (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
up-regulated 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 120kda (data not shown) and 100kda. 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 four 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 up-regulated in breast cancer in pre-diagnostic 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, i.e. 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.
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References


Figure Legend

**Figure 1**- Experimental set-up of 4-plex iTRAQ analysis. Plasma from six 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, utilized in iTRAQ analysis.

**Figure 2**- Identification of gelsolin. MALDI-TOF MS (A) for gelsolin and its tandem MS (B) demonstrating 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.

**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≤0.05

**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-Alpha-Inhibitor H4 Heavy Chain expression. *P≤0.05

**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 plus Tamoxifen.

**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≤0.05, ER ≤2).
Figure 1

Depletion of IgG, and Albumin

1:1

Depletion of IgG, and Albumin

10:1

Depletion of IgG, and Albumin

25:1

Depletion of IgG, and Albumin

25:1 + T

Rat Plasma (n=6)

Alkylate, & Trypsin Digest

Peptide

114 → 191

Peptide

115 → 190

Peptide

116 → 189

Peptide

117 → 188

Peptides

Isobaric tag-balance group-peptide

2D-LC, MALDI-TOF/TOF

Mix

Reduce,

Denature,
Figure 2

A Gelsolin

Precursor MS Region

MS/MS

B

R W I Q L G P E K G A

y1 y2 y3 y4 y5 y6 y7 y8 y9 y10 y11

0.0e0 5.0e3 1.0e4

Intensity

178.14 396.28 545.40 674.47 862.87 941.70 1069.70 1366.88 1421.84

C

Reporter Ions

0 1000 2000 3000 4000

Intensity

112.1243 114.1419 115.1299 116.1431 117.1396

ms/ Da
Figure 4

A. Apolipoprotein E

B. Haptoglobin

C. Inter-Alpha-Inhibitor H4 Heavy Chain

Fold Change

(25:1) (25:1) + Tamoxifen

36kDa-

37kDa-

100kDa-
5. A 10:1 n-3:n-6 Fatty Acids

- Complement Activation
- Proteolysis
- Response to Stress
- Intracellular Protein Transport
- Cell Component Morphogenesis
- Bacterial Defense Response
- Mesoderm Development
- Signal Transduction
- Response to Stimulus
- Blood Coagulation
- Phagocytosis
- Angiogenesis
- Pyrimidine Base Metabolism
- Gamete Generation
- Cell-Cell Signaling
- Blood Circulation
- Immune System Process
- Kinase Signaling
- Nervous System Development
- Ectoderm Development
- Cell Motion

B 25:1 n-3:n-6 Fatty Acids

- N/A
- Cell-Cell Signaling
- Blood Circulation
- Visual Perception
- Natural Killer Cell Activation
- Kinase Signaling
- Nervous System Development
- Ectoderm Development
- Endocytosis
- Skeletal System Development
- Complement Activation
- Proteolysis
- Blood Coagulation
- Transport
- Intracellular Protein Transport
- Cell Component Morphogenesis
- Bacterial Defense Response
- Mesoderm Development
- Synaptic Transmission
- Lipid Transport and Metabolism
- Cell Adhesion
- Phagocytosis
- Angiogenesis
- Pyrimidine Base Metabolism
- Sensory Perception
- Heart Development

C 25:1 n-3:n-6 Fatty Acids + Tamoxifen

- Cell Adhesion
- Response to Stimulus
- Blood Circulation
- Transport
- Cell-Cell Signaling
- Kinase Signaling
- Bacterial Defense Response
- Mesoderm Development
- Mammary Gland Development
- Immune System Process
- Cytokine Mediated Signaling
- Blood Coagulation
- Natural Killer Cell Activation
- Intracellular Protein Transport
- Angiogenesis
- Pyrimidine Base Metabolism
- Visual Perception
- Tyrosine Kinase Signaling
- Complement Activation
- Cell Component Morphogenesis
- Proteolysis
- Signal Transduction
- Phagocytosis
- Nervous System Development
- Ectoderm Development
- Hematopoiesis
- N/A
Figure 6

- **10:1**
  - Fibrinogen Gamma Chain
  - Inter-Alpha-Trypsin Inhibitor Heavy Chain H3
  - Haptoglobin

- **10:1 and 25:1**
  - Complement C3
  - Inter-Alpha-Inhibitor-H4 Heavy Chain

- **25:1**
  - Apolipoprotein E
  - Ceruloplasmin
  - Complement Component C9

- **10:1 and 25:1 + Tamoxifen**
  - Alpha-2-Antiplasmin

- **10:1, 25:1, and 25:1 + Tamoxifen**
  - Fibrinogen Alpha Chain Isoform 1
  - Coagulation Factor XIII B Chain
  - Alpha-1-Macroglobulin
  - Fibrinogen Beta Chain

- **25:1 + Tamoxifen**
  - Serine Protease Inhibitor A3M
  - Kininogen-1 Isoform 1
  - Serine Protease Inhibitor A3N
  - Plasma Kallikrein
  - Gelsolin
  - Leukemia Inhibitory Factor Receptor
  - C4b-Binding Protein Alpha Chain
  - Serum Amyloid A-4 Protein
  - Fibronectin

- **25:1 and 25:1 + Tamoxifen**
  - Fetuin-B
  - Vitamin D Binding Protein
  - Serotransferrin
  - Alpha-1B-Glycoprotein
  - Hypothetical Protein
Proteomic Changes Induced by Effective Chemopreventive Ratios of n-3:n-6 Fatty Acids and Tamoxifen against MNU-Induced Mammary Cancer in the Rat

Christine G. Skibinski, Henry J. Thompson, Arunangshu Das, et al.


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