

Metabolic and Molecular Regulation of Dietary Polyunsaturated Fatty Acids on Prostate Cancer

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Running title: Quantitative proteomic analysis of prostate cancer cells treated with polyunsaturated fatty acids

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Abbreviations:

AR, androgen receptor; **AUC**, area under the curve; **CE**, collision energy; **CM**, conditioned media; **COX2**, cyclooxygenase 2; **DHA**, docosahexaenoic acid; **DP**, declustering potential; **EPA**, eicosapentaenoic acid; **FA**, fatty acid; **FASN**, fatty acid synthase; **FO**, fish oil; **LC-MS**, liquid chromatography mass spectrometry; **MRM**, multiple reaction monitoring; **OA**, oleic acid; **PCa**, prostate cancer; **PUFA**, polyunsaturated fatty acid; **SREBP-1c**, sterol regulatory binding protein 1c;

Clinical Relevance

Fish oil, rich in n-3 polyunsaturated fatty acids (PUFAs), is a potential dietary supplement to lower prostate cancer risk. The mechanism of action of fish oil on prostate tumor growth inhibition remains largely unknown. While the n-3 fatty acids may exert important anticancer effects, the effects of other types of fatty acids such as oleic acid, a n-9 fatty acid and the main fatty acid in olive oil, are much less known. Roles of these PUFAs in prostate cancer have been investigated previously, however, conflicting results and the limitations in the experimental design make it clear that additional work needs to be conducted, especially at the cellular level, to better understand the pathways by which FO help reduce prostate cancer risk.

Abstract

Purpose: The aim of this study is to investigate the role of n-3 and n-9 fatty acids in crucial processes involved in prostate cancer cell growth through a large-scale proteomic analysis.

Experimental design: We used a label-free protein quantification method to profile global protein expression of fish oil and oleic acid treated PCa cells and validated a panel of differentially expressed proteins by either Western blot or multiple reaction monitoring. Bioinformatic analysis was also performed to uncover the pathways involved in fatty acid metabolism.

Results: Fish oil, not oleic acid, suppresses prostate cancer cell viability. Assessment of fatty acid synthesis pathway activity also shows that oleic acid is a more potent inhibitor than fish oil on *de novo* fatty acid synthesis. Although fatty acid synthase activity decreases with fish oil treatment, the inhibition of its activity occurs over time while reduction in viability occurs within 24 hours. Bioinformatic analysis revealed the pathways altered by these fatty acid treatments.

Conclusions and clinical relevance: This study suggests that suppression of cell viability by fish oil is independent of fatty acid synthase and fish oil regulates prostate cancer cells through activation of other pathways depending upon length of exposure to fish oil.

1 Introduction

Prostate cancer (PCa) is the second most common cancer in men [1]. The risk of PCa is currently one in six men (16.7%) and the overall risk of death due to metastatic PCa is about 2.8% with the disease progressing slowly to advanced stage [2-4]. To date little is known about the etiological factors associated with PCa development and progression. There is increasing evidence suggesting that diet and lifestyle play a crucial role in PCa tumorigenesis [5, 6]. However, a common consensus on which nutrients may be beneficial and which could be harmful is lacking, and this is further complicated by the contradictory reports about the role of different dietary components and nutrients on PCa prevention and progression [7-10].

Although two recent studies failed to support the role of fish oil (FO), which is rich in n-3 polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid (DHA, C22:6, n-3) and eicosapentaenoic acid (EPA, C20:5, n-3), in protecting men against developing PCa [10, 11], it has long been viewed as a beneficial dietary supplement to lower the risk of PCa through suppressing PCa cell growth and inducing cell apoptosis, strengthening the effect of anti-tumor drug treatments to PCa cells [12-16]. These conflicting results and the limitations in the design of previous studies, make it clear that additional work needs to be conducted, especially at the cellular level, to better understand the pathways by which FO help reduce PCa risk.

Roles of other PUFAs in PCa have also been studied previously [17-20]. For example, an epidemiological study suggests that high ratio of n-6/n-3 PUFAs uptake is positively associated with PCa risk [17]. Besides n-3 and n-6 FAs, other fatty acids (FAs) such as oleic acids (OA, C18:1, n-9), an n-9 monosaturated FA component, may also

play important roles in reducing cancer risk. Unlike n-3 PUFAs, which are derived from limited sources (flaxseed, certain fish), n-9 fatty acids (such as OA) are one of the dominant components in most daily cooking oil. Results from studies investigating OA and cancer, however, are controversial. Some studies indicate OA has inhibitory effects on PCa cells [18], while others observed a higher OA/stearic ratio in untreated and more advanced PCa patients [19, 20]. However, whether the high level of OA/stearic ratio is due to more endogenous OA production, dietary OA consumption, or the decrease level of stearic acid remains to be investigated. In the present study, we selected OA, a commonly consumed fatty acid, to be used in comparison with FO to identify the pathways activated or suppressed in PCa cells, specifically by FO and/or by OA. One of the downstream targets of dietary FAs is fatty acid synthase (FASN) [21], which is associated with development of multiple cancers and is currently being pursued as a therapeutic target [22]. Dietary PUFAs have been shown to suppress *de novo* synthesis of fatty acids via FASN through inhibition of the sterol regulatory binding protein 1c (SREBP-1c) [23, 24]. Prostate tumor cells show markedly higher FASN expression compared to adjacent tissues [25, 26]. FASN regulates a series of genes and pathways [27], some of which play critical roles in cell viability and its inhibitors have been reported to have anti-cancer effects [26, 28, 29]. N-3 PUFAs also modulate other molecules in PCa. One study suggests androgen receptor (AR) protein expression may be down-regulated by DHA treatment [30]. N-3 PUFAs also regulate enzymes involved in n-3 PUFA processing, for instance, cyclooxygenase 2 (COX2) [31].

To date the mechanism of FO's effects on PCa remains unknown. There is increasing evidence suggesting that FO may have multiple targets and thus its

regulation may also be multi-level. This makes global proteome comparison an attractive approach. This study evaluated FO and OA's effects on PCa cell viability and FASN activity and sought to answer how the protein profiles are being regulated by FO or OA in a longitudinal manner with an LC-MS/MS method.

2 Materials and methods

2.1 Cell line

The PCa cell line, PC3 (ATCC), was grown in RPMI-160 medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza, Walkersville, MD, USA) in 37°C with 5% CO₂. For the androgen receptor positive PCa cell line, LAPC4, IMDM medium (HyClone, Logan, UT, USA) was used.

2.2 FA treatment, chloroquine treatment and conditioned media collection

FO and OA were purchased from KD Pharma Bexbach GmbH (Bexbach, Germany). FO capsules contained 90% n-3 fatty acid ethyl ester (40% EPA, 40% DHA and 10% other n-3 PUFAs). OA capsule contained 90% oleic acid ethyl ester. 100 mM stock was made by dissolving fatty acids (FAs) in ethanol. When treating the cells, FAs-ethanol stock solution was mixed into complete medium at a 1:1000 ratio to a final concentration of 100 µM, while 0.1% ethanol was used in controls. For day-6 treatment samples, the medium was changed four days after the treatment with a fresh medium containing FAs. In autophagy suppression assay, chloroquine diphosphate salt (Sigma-Aldrich, St. Louis, MO, USA) was diluted to 8 µM in medium. To harvest conditioned medium (CM), the growth medium was replaced with serum-free medium, CM was harvested 24 hrs later

and cell debris was removed with centrifugation at 1,500 rpm. CM volume was normalized based on cell count with fresh serum-free medium to reach a same volume/cell number ratio among different treatments.

2.3 Clonogenic cell survival assay

Cells were seeded in 6-well plates (1,000 PC3 cells or 3,000 LAPC4 cells per well, respectively) and grown in complete medium with different FA treatments. Colonies formed at approximately 1 week for PC3 cells and 1.5 weeks for LAPC4 cells. Colonies were fixed and stained with crystal violet, washed and counted.

2.4 Cell proliferation assay

Cells were seeded in 96-well plates (1,000 PC3 cells or 6,700 LAPC4 cells per well, respectively) and grown in complete medium with different FA treatments. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was carried out by incubating cells with MTT (Sigma-Aldrich, St. Louis, MO, USA) for 4 hrs. DMSO was used to dissolve the formazan. Absorbance was measured at 570 nm.

2.5 Western blot

Cell pellets were lysed by NP-40 lysis buffer supplemented with 1% protease inhibitor (Thermo-Fisher Scientific, Waltham, MA) and phosphatase inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Laemmli buffer (Bio-Rad, Hercules, CA, USA) containing 5% 2-mercaptoethonal (Fisher Scientific, Pittsburgh, PA, USA) was added to cell lysate supernatant and the mixtures were boiled to denature. Proteins were separated by SDS-PAGE gel then transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA). Blocking was done in 5% BSA (Roche, Indianapolis, IN, USA). PVDF membrane was incubated with primary antibodies anti-MSMP (Abnova, Walnut, CA, USA), anti-

sequestosome-1 (Novus Biologicals, Littleton, CO, USA), anti-beta-actin (Fisher Scientific, Pittsburgh, PA, USA) and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Cell Signaling Technology (Danvers, MA, USA) sequentially. To detect LC3 I/II, cells were lysed by lysis buffer (1% Triton-X100, 150 mM NaCl, 50 mM Tris pH7.6), LC3 antibody were purchase from Thermo-Fisher Scientific (Waltham, MA, USA). Bands were detected by ECL™ Western Blotting Detection Reagents (Amersham™, GE Healthcare, Piscataway, NJ, USA). Quantification was carried out using ImageJ, an open source image processing program.

2.6 FA synthesis measurement

Cells were incubated with complete culture medium with 1 $\mu\text{Ci/ml}$ 1,2-¹⁴C-acetic acid sodium salt (PerkinElmer, Waltham, MA) was added to complete medium and cells were incubated for 2 hrs. The medium was discarded. For lipid extraction, 0.9 ml chloroform/methanol (2:1, v/v) mixture and 0.7 ml 4 mM MgCl₂ were added to the cells. The mixture was vortexed thoroughly and centrifuged at 10,000 rpm. The upper aqueous layer was removed and the lipid was maintained in bottom layer which is the chloroform fraction. Extraction was repeated for 3 times. Every sample was air dried in fume hood, then re-dissolved in 200 μl chloroform and finally transferred to 10 ml scintillation fluid Ultima Gold™ F (PerkinElmer). Disintegration Per Minute (DPM) value was measured by TRI-CARB 2100TR liquid scintillation analyzer (Packard Instrument Company, Kennesaw, GA).

2.7 Real-time PCR

Cells were harvested and RNA extraction was carried out using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using

QuantiTect Reverse Transcription kit (Qiagen). Realtime PCR was performed on QuantStudio 12K Flex (Applied Biosystems, Foster City, CA, USA) and the protocol was designed according to Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA, USA). Sequestosome-1 (SQSTM1) was labeled with reporter dye FAM. Glucuronidase β (GUSB), which serves as internal standard was labeled with reporter dye VIC. Realtime PCR primers and probes targeting SQSTM1 (assay ID Hs01061917_g1), GUSB (assay ID: Hs00939627_m1), as well as Mastermix (the reaction solution), were designed and purchased from Applied Biosystems.

2.8 Mass spectrometry

For global unbiased proteomic analysis, cell pellets were lysed in 8 M urea and 10 mM dithiothreitol (DTT) and then sonicated on ice. Equal amount of chicken lysozyme was spiked into each sample at a final concentration of 0.5% (w/w), serving as a quality assurance (QA)/quality control (QC) reference. Reduction/alkylation of cysteine residues was done by incubating the cell extracts with acetonitrile (ACN)/iodoethanol/triethylphosphine (195:4:1, v/v/v) mixture. Samples were dried in a speed vacuum concentrator (Genevac, NY, USA) and resuspended in 310 μ l of 100 mM ammonium bicarbonate, followed by overnight trypsin (Worthington, Lakewood, NJ) digestion (trypsin:protein=1:25). Desalting was carried out by repeating ACN-H₂O with 0.1% formic acid wash in Silica C18 MacroSpin Column (The Nest Group, Southborough, MA, USA). The resulting peptides were eluted by 80% ACN in last step and speed-vac dried. Samples were resuspended in 5% ACN in water with 0.1% formic acid and filtered through Ultrafree-MC Centrifugal filters (EMD Millipore, Billerica, MA). Peptides were eluted with a linear gradient from 5% to 80% ACN developed over 120

min at a flow rate of 50 μ l/min, and effluent was electro-sprayed into the LTQ Orbitrap Velos Pro mass spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA). Two injections of each sample were carried out randomly. X!tandem algorithms were applied to search the acquired data against UniProt human database (released in April 2013). Peptides and proteins were validated using PeptideProphet and ProteinProphet in the Trans-Proteomics Pipeline (TPP, v.4.6). All the proteins listed in this study have protein probability >99.8% and peptide probability >80%. Label-free quantification was carried out using IdentiQuantXL™ [32].

2.9 MRM-based validation

Sample preparation, including cell lysis, reduction/alkylation and digestion was carried out the same way as that in proteomic discovery. Mass spectrometric analyses were performed on an AB/SCIEX 4000 Qtrap mass spectrometer (Sciex, Framingham, MA, USA) interfaced with a Dionex U-3000 UHPLC system (Thermo-Fisher Scientific, Waltham, MA, USA). Peptides were injected on an ODS-100V C18 column (1.0mm x 150mm, Tosoh Biosciences, South San Francisco, CA, USA) and eluted with a linear gradient from 5 to 80% acetonitrile over 32 min at a flow rate of 50 μ l/min. The parameters were as follows: curtain gas of 40.0 psi, collision gas of 7 psi, ion spray voltage of 5500 V, temperature set at 500 °C, ion source gas 1 of 50.0 psi, ion source gas 2 of 40.0 psi. Entrance potential is set on 10.0 V; collision cell exit potential is 12.0 V. Data acquisition was carried out using the AB/SCIEX Analyst® software. For MRM development, the Skyline software was first used to generate candidate peptides and theoretical transitions for each peptide. PC3 cell lysate was tested to select the best experimental peptide and transitions, which could be stably detected and also had high

peak intensity. One specific transition was monitored for each target peptide, which represents one specific protein. Each monitored transition was confirmed by spiking in varied concentration of the corresponding synthetic peptides. For FSCN1, a transition for target peptide $^{23}\text{YLTAEAFGFK}^{32}$ was monitored: m/z 573.8 ($M+2H^+$) \rightarrow m/z 870.4. For CANX, a transition for target peptide $^{78}\text{GTLSGWILSK}^{87}$ was monitored: m/z 531.3 ($M+2H^+$) \rightarrow m/z 790.4. For MSMP, a transition for target peptide $^{52}\text{YFTLGESWLR}^{61}$ was monitored: m/z 636.3 ($M+2H^+$) \rightarrow m/z 747.4. For SCP2, a transition for target peptide $^{48}\text{IGGIFAFK}^{55}$ was monitored: m/z 426.8 ($M+2H^+$) \rightarrow m/z 739.4. A transition for chicken lysozyme, which serves as external standard, target peptide $^{40}\text{GYSLGNWVCAAK}^{61}$ was monitored: m/z 656.8 ($M+2H^+$) \rightarrow m/z 892.4. We also monitored a transition for RPL6, which serves as the internal standard, target peptide: $^{211}\text{HLTDAYFK}^{218}$, m/z 497.8 ($M+2H^+$) \rightarrow m/z 744.4. Declustering potential (DP) and collision energy (CE) were optimized for each peptide. Scan time for each peptide was 120 msec and total scan time was 0.875 sec. Quantification of target peptide was performed using Skyline software to calculate area under curve (AUC) of extracted ion chromatograms.

3 RESULTS

The aim of this study was to better understand the pathways involved in FO inhibition of PCa cell viability and to further investigate FO and OA's effects in global protein expression. We compared global protein expression profiles of PC3 cells treated with FO and OA at two different time points. Protein expression data presented here clearly demonstrate that FA-mediated molecular changes are not directly linked to FASN activity and the function of FAs may likely be mediated through multiple targets.

3.1 FO, not OA inhibits PCa cell growth

A MTT cell proliferation assay was performed to assess cell viability upon PUFA treatment. Shown in **Fig. 1A**, FO, not OA, suppresses cell viability. A significant difference in cell growth (FO=74.8%C, $p=0.0105$) was observed after 24 hours in the FO treated cells but not in OA group (OA=101.4%C, $p=0.5493$). This observation was confirmed by clonogenic assays, in which colony density was significantly lower with FO treatment (**Fig. 1B**). Clonogenic cell survival assays were also performed on androgen receptor (AR) positive LAPC4 cells (data not shown). FO treatment demonstrated significant inhibition of colony formation ($p<0.05$) with no LAPC4 colonies observed in FO treated group after 1.5 weeks. Again, the OA group showed no significant change as compared to control group ($p=0.2842$). During colony formation, vacuoles were observed in the FO treated cells, which remained single cells and stopped proliferating (**Fig. 1C**).

3.2 Both FO and OA inhibit FA synthesis and OA is a more potent inhibitor than FO

De novo FA synthesis was measured in the FO and OA treated groups after three days and six days treatment. At day three there was no change in fatty acid synthesis with fish oil treatment (**Fig. 2A**), while a 30.97% ($p=0.0001$) suppression was observed in the OA group. After six days of treatment, a significant FA synthesis suppression was observed in both FO (27.11%, $p<0.05$) and OA (49.21%, $p<0.0001$) groups (**Fig. 2B**). Additionally, OA's FA synthesis level was lower than FO (OA=69.7%FO, $p=0.0301$), which suggests that OA is a more potent FA synthesis inhibitor than FO. This

observation also implicates that the viability inhibition effect of FO on PC3 cells may not be directly linked to FA synthesis.

3.3 The differential proteome profile in prostate cancer cells treated with FO or OA

LC-MS-based label-free protein quantification was performed to investigate the longitudinal regulation of FO and OA on PCa cells. PC3 cells were plated at ~170,000 cells/plate on 100-mm plates with the complete medium with 0.1% ethanol, 100 μ M FO, or 100 μ M OA, respectively. Cells were harvested on day 1 and day 6 after treatment for MS analysis. A total of 1,478 proteins were identified with protein probability >99.8% and peptide probability >80%. Protein and peptide probabilities were produced through calculation by TPP. Random sequences were set up serving as decoy sequences. A protein identification probability >99.8% corresponds to a FDR of <1%, which is a commonly used cutoff in the proteomics field.

Protein expression comparison was conducted based on treatment conditions (control, FO, and OA) and time-course (day-1 and day-6). Three hundred and twelve proteins were positively identified based on p-values less than 0.05. As indicated in the Supplementary Table S1, majority of the technical variations are under 10% with a few outliers based on the internal control (chicken lysozyme) data and the overall %CV is below 13% (Supplementary Table S1). This is what we expected from this type of global protein profiling experiment with the platform we applied. The data was further filtered by FDR-adjusted p value (q value cut off=0.05) and the expression levels of 127 proteins were significantly changed upon treatment (Supplementary Table S2). Principal component analysis (PCA) was performed on this 127-protein dataset that consisted of

6 treatment groups with 5 replicates in each group. Prolonged FO treated group (FO-6 day) was a distinct group indicated by the red circle, separated from the other treatments (**Fig.3A**). The blue and yellow circles indicate there were fewer protein changes among treatments in day-1 and between control and OA group in day-6. By comparing each FO or OA group to its same-day corresponding control group, 73 proteins showed significant changes ($p \leq 0.05$). $\text{Log}_2(\text{Ratio} \langle \text{FO}/\text{C} \rangle)$ and $\text{Log}_2(\text{Ratio} \langle \text{OA}/\text{C} \rangle)$ values of protein entries were clustered by one minus Pearson Correlation (Gene E online analysis platform, <http://www.broadinstitute.org/cancer/software/GENE-E/>). Three major clusters were generated (shown in **Fig.3B**). Cluster A contained 7 proteins (encoded by 6 genes) (**Table 1**), including SCP2 and acyl-coenzyme A oxidase which participate in fatty acid transport and metabolism [33, 34]; coactosin-like protein is reported to be involved in PUFA metabolism [35]. Cluster B showed that five of the six proteins (encoded by 4 genes) were significantly elevated in the FO group one day after treatment, and protein expression levels were higher than the OA group. Among these proteins, sequestosome-1 (SQSTM1) and heat shock 70 kDa protein 1A/1B (HSPA1A) were identified and have roles in the autophagy process [36, 37]. In cluster C, which consisted of 60 protein entries, no significant changes were observed in FO and OA groups in the first day of treatment. However, on day-6, FO-treated and OA-treated groups showed divergent protein expression changes. In the FO-treated group, 53 out of 60 proteins showed a significant decrease in expression while in the OA-treated group, the majority of differentially regulated proteins were elevated and eight of the changes were significant. Ingenuity[®] pathway analysis (IPA) indicated that cell

movement, growth and proliferation, cell development, cell death/survival, and carbohydrate metabolism were the most significant molecular and cellular functions involved (**Table 2**), while glycolysis and gluconeogenesis were the top canonical pathways. Four enzymes from cluster C: phosphoglycerate kinase 1 (PGK1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase isozymes M1/M2 (PKM) and its isoform M1 (PK) and glucose-6-phosphate isomerase (GPI) were all significantly decreased in expression in FO-6-day treated group, but have little change in the OA-6-day treated group (**Fig. 4**).

In combination with IPA, the proteins with significant expression changes were selected based on the following criteria: 1) protein probability >99.8%; 2) two or more unique peptide sequences identified with high confidence; 3) within every group, ANOVA p-value ≤ 0.05 , q-value ≤ 0.05 ; 4) multiple comparison test p-value $\leq 5/N_{\text{total tests}}$; 5) protein frequency (occurrence in all injections) >50%; 6) association with cancer, prostate, or lipid processing; 7) absolute fold change >1.3. Under these stringent criteria, we identified only one differentially regulated protein, sequestosome-1 (SQSTM-1), in the day-1 treatment group. SQSTM-1 was elevated (1.7-1.8 fold) in the FO group compared to control and OA group. In the 6-day treatment comparison, the expression levels of 4 proteins were significantly altered as compared to the controls (**Table 3**).

3.4 Key differentially expressed protein validation

Both Western blot and MRM-based methods were used for validation studies to confirm the differential protein expression observed in the global discovery study. Since the majority of these proteins were in the day-6 treatment group, we used an independent set of day-6 samples for validation studies. In MRM, for each selected

protein, one unique peptide and one pair of precursor and product ions (MRM transitions) were selected. Relative quantity and specificity of these MRM peptides/transitions are illustrated in Supplementary Figures S1, S2, and S3. Synthetic standard peptides were first tested to confirm the transition ion pairs derived from Skyline. Results showed that among the 4 proteins changed in the day-6 treatment group, prostate-associated microseminoprotein (MSMP) was found significantly decreased when the FO treatment group was compared to control (63.3% lower expression in the FO group, $p=0.0242$). Down-regulation of MSMP in the OA treated group was not significant (28.4% lower expression, $p=0.3474$), (**Fig. 5A**). Since MSMP is also a secreted protein, we tested expression in conditioned medium (CM) of PC3 cell grown in the presence of FO, OA or vehicle. Western blot results show that secreted MSMP in CM appears as a single band around 16kD. FO treated PC3 cells secreted less MSMP compared to control ($p=0.0096$) or OA group ($p=0.029$) (**Fig. 6F & 6G**).

The other protein, sterol carrier protein 2 (SCP2), was also found to be significantly up-regulated in the FO treated group compared to control (47.1% over-expressed in FO group, $p=0.001$), or OA (27.8%FO, $p=0.0113$) (**Fig. 5B**). However, unlike the global proteome results, there was no significant change between OA and C. No significant changes were observed in spiked-in chicken lysozyme and RPL6, which served as QA/QC samples (constant amount among three groups observed) (**Fig. 5C & 5D**).

Sequestosome 1 (SQSTM1), a protein involved in autophagy, was found to be over-expressed in FO group one day after the treatment in our global proteomic study. Western blot results were consistent with the LC/MS results that demonstrated SQSTM1 levels in the FO group were 2.1-fold higher than that in the control group

($p=0.042$) (**Fig. 6A & 6B**). Although the proteomic analysis also showed a higher level of expression in FO group when FO and OA groups were compared (fold-change=2.35), the change, however, was not statistically significant ($p=0.0867$) based on our $p=0.05$ cutoff. There was no significant difference between OA and control. Real-time PCR results showed that at the transcriptional level, mRNA expression of SQSTM1 in FO treated group was higher than both control (fold-change=2.16, $p=0.003$) and OA treated group (fold-change=2.26, $p=0.003$) (**Fig. 6C**). To further investigate the function of autophagy, autophagy inhibitor chloroquine (CQ) was applied to cells with different FA treatment, and LC3 I/II levels were measured by Western blot. The results indicated that, under the condition of autophagy suppression induced by CQ, LC3 II levels accumulated significantly (two-way ANOVA, $p=0.0005$) while LC3 I levels were not affected. However, no significant LC3 II changes were observed among FA treatment groups (two-way ANOVA, $p=0.7853$) (**Fig. 6D, 6E**), suggesting that autophagy may not be significantly inhibited under this experimental condition.

4 DISCUSSION

Saturated fatty acids, OA, and n-6 PUFA are main components of daily cooking oil, including corn, peanut or sunflower seed oil. Except for flaxseed oil (alpha-linolenic acid, ALA) and some fish (DHA and EPA), n-3 PUFA, one of the essential FAs, exist as a very small portion of dietary oil intake. Proteomic studies of this sort have been carried out to investigate the global protein level change under different dietary FA consumption. However, most of these studies used two-dimensional gel electrophoresis approaches with the study objectives varying from insulin resistance to colon inflammation [38-44].

Large-scale proteomic studies on dietary fish oil effect on PCa using the label-free protein quantification method have not yet been reported.

In this study, we investigated the different molecular pathways of OA and FO action in PCa cells. Previous investigations of n-3 PUFAs and PCa have pointed to some of the potential mechanisms of FO on PCa growth inhibition. One study detected down-regulated AR protein expression in LNCaP cells after 10 weeks continuous 20 μ M DHA treatment [30], while another study did not detect a AR protein change in LNCaP cells treated by DHA at concentrations as high as 180 μ M for 24 to 36 hrs [14], suggesting differential regulatory changes over time may have occurred during the treatment periods. One point worth noting is that the concentration of the fatty acids used for this study is based on our preliminary experiments and other reported studies [13, 45, 46]. At 100 μ M, FASN activity is significantly reduced by OA while cell viability remains unaffected. However, *in vivo* human exposure to this concentration of FAs may never reach 100 μ M due to its poor water solubility and lipid intake limitation. Thus the actual exposure of the cells to FAs may be much lower than the calculated 100 μ M concentration.

Our results suggest that only FO, not OA, suppresses PC3 cell viability, and this inhibition occurred as early as one day after the treatment. Hierarchy analysis of global proteomic data generated a group of proteins in which the majority of proteins were increased after 24-hr FO treatment. Specifically, both sequestosome-1 (SQSTM1), heat shock 70 kDa proteins A1A/A1B are connected to autophagy [36, 37] and both were significantly elevated one day after FO treatment but with no change in the OA treated group. This result indicates FO, not OA, triggers rapid stress-responding reactions,

which may explain the early event of viability inhibition. The elevated expression of SQSTM1, an autophagy associated protein, was also validated by Western Blot (**Fig. 6A & 6B**). Tumor cells activate autophagy in response to cellular stress and/or increased metabolic demands related to rapid cell proliferation. In the dynamic process of autophagy, SQSTM1 is required for the degradation of polyubiquitinated substrates, and SQSTM1 itself is also degraded during this process. The accumulating SQSTM1 level implicates a possible inhibition of autophagy, which in turn causes the accumulation of SQSTM1 that should be otherwise degraded in a normal autophagy process, suppressing the growth of the tumor cells. Consistent with our result, one study also found elevated SQSTM1 12-hr after the treatment of EPA in promyelocytic cells [47]. Conversely, Shin *et al.* showed that autophagy, in this case acting as a tumor suppressor, was activated by DHA and played a critical role in apoptosis of PC3 cells induced by DHA [48]. However, in investigating the cause of SQSTM1 elevation under FO treatment, we found this increased protein expression was most likely regulated by transcriptional mechanism rather than reduced post-translational degradation, implicating other transcription regulator(s) may be involved. It has been previously reported that oxidation stress can induce SQSTM1 at the transcriptional level, and SQSTM1 triggers antioxidant effects [49]. Since FO was found to induce oxidation stress and cytotoxicity [50], we suggest that the observed increase of SQSTM1 might take place at the stage when cells are starting to respond to oxidation stress.

Our study suggests that although OA suppressed FA synthesis rate to near 50% on day 6, FASN protein level showed no significant change in the OA group compared to control. One study in C6 glioma cells suggested OA was a stronger inhibitor than n-3

PUFA and other FAs in FA synthesis [51]. Consistent with their result and another study in 1999 [24], we found no protein level changes in FASN expression. Interestingly, they discovered that OA down-regulated the expression of another key enzyme in FA synthesis, acetyl-coenzyme A carboxylase, which is involved in the formation of malonyl-CoA. In contrast, FASN expression levels in FO treated group are decreased compared to OA group (t-test, $p=0.05$, multiple comparison test $p=0.1$), indicating additional transcriptional level modulation of FASN was triggered by FO.

Our quantitative global proteomic study also revealed that a group of glycolysis/gluconeogenesis enzymes were changed in FO treated cells and four of the enzymes were significantly down-regulated in the 6-day treatment group. Although most of the decreased enzymes are shared by both glycolysis and gluconeogenesis, PKM specifically catalyzes a rate-limiting step in glycolysis. In PCa, glucose is not a major bioenergy source as in many other cancer types [52], therefore rendering FDG-PET imaging ineffective for PCa tumor imaging [53]. However, a study based on NIH dbEST database showed PCa tissue has 9 out of 10 glycolysis genes overexpressed compared to normal tissue [54]. Additionally, some glycolytic enzymes, PKM and PGAM1, are expressed differently between PCa and normal tissue by isoform or post-translational modification (PTM) level [55]. PCa cell sensitivity to stress is enhanced when glycolysis is suppressed [56]. This suggests glycolysis still plays a role in PCa even though the glucose uptake is low. Expression of hepatic pyruvate kinase [57] and glucose-6-phosphate dehydrogenase [58] were also reportedly blocked by n-6 or n-3 PUFAs. One of the possible mechanisms is through prevention of chREBP translocating to nuclei [59]. In the context of cancer cells, the inhibition may be mediated by liver kinase B1

(LKB) [60]. Effects of PUFAs on glycolysis are tissue specific so that in skeletal muscle cells, the process is reversed [45]. Our study indicates FO may suppress glycolysis by decreased multiple enzyme expression, suggesting that in the PC3 cell line, *de novo* FA synthesis decreases in later time points may be due to the lower pyruvate supplied by glycolysis. To the contrary, prolonged OA treatment does not suppress glycolytic enzyme expression and we therefore conclude that FO and OA may inhibit FA synthesis through different pathways, which are dependent or independent of glycolysis down-regulation, respectively.

Besides involvement of glycolytic enzymes, additional potential targets of FO were investigated and validated in independent sets of samples. Among the five candidate proteins in the 6-day treatment arm, the increase of SCP2 and decrease of MSMP was validated by MRM. In the global study, SCP2 was elevated in both FO and OA, with the FO group demonstrating significantly higher than OA and control. This result was confirmed by Western Blot analysis, although the SCP2 increase in OA group was not significant. Functionally it is expected that this lipid carrier protein expression would increase when exogenous FAs are added to the medium. The other protein, consistent in validation experiments, MSMP, is a highly conserved protein and belongs to beta-microseminoprotein family and its expression was observed in both benign and tumor tissues although immunohistochemical analysis demonstrated that tumor MSMP expression is different from the pattern in benign tissue [61]. As a recently identified protein, MSMP has not been well studied. One report suggested MSMP's role in inflammation as a ligand binding to CC chemokine receptor 2B (CCR2B) and acts as a chemoattractant, which induces the migration of peripheral blood monocytes (PBM) and

peripheral blood lymphocyte (PBL) cells but not polymorphonuclear neutrophils (PMN) cells [61], indicating a pro-inflammation role of MSMP. MSMP was also found to stimulate p-ERK, which plays a central signaling role in cancer progression [62, 63]. Our results demonstrate that FO can lower MSMP levels, offering an additional explanation for the anti-inflammation action of n-3 FAs in prostate cancer. Taken together, FO and OA both suppress FA synthesis activity but only FO inhibits cell survival. Global proteomic studies and following validation demonstrated that FO modulates protein levels of SQSTM1, MSMP, and SCP2 in PCa cells, suggesting a possible longitudinal regulation by FO: inducing cytotoxicity after short treatment; suppressing inflammation pathways and inhibiting the glycolytic pathway in prolonged treatment.

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Table 1 Differentially expressed proteins in cluster A and B. (*: t-test $p \leq 0.05$, **: t-test $p \leq 0.01$, $n=5$)

Genes	Protein name	Log2 FO/CN (day1)	Log2 OA/CN (day1)	Log2 FO/CN (day6)	Log2 OA/CN (day6)	ANOVA p-value	q-value
Cluster A							
SCP2	Non-specific lipid-transfer protein	-0.12	-0.10	0.75**	0.36**	1.08E-04	4.22E-03
CD9	CD9 antigen	0.39	-0.10	0.81**	0.12	7.58E-05	3.43E-03
ACOX	Isoform 2 of Peroxisomal acyl-coenzyme A oxidase 1	0.25	-0.29	0.52**	0.14	8.71E-05	3.80E-03
ACOX1	Acyl-coenzyme A oxidase	0.30	-0.28	0.55**	0.12	7.29E-05	3.43E-03
SORD	Sorbitol dehydrogenase	0.11	-0.09	0.59**	0.09	1.97E-07	1.16E-04
COTL1	Coactosin-like protein	0.11	0.05	0.31*	0.19	6.32E-05	3.43E-03
KPNA2	Importin subunit alpha-2	0.08	0.02	0.29*	0.17	6.70E-04	1.34E-02
Cluster B							
APEH	Acylamino-acid-releasing enzyme (Fragment)	0.11	-0.07	-0.67*	-0.28	5.33E-03	4.94E-02
TSTA3	GDP-L-fucose synthase	0.50*	0.12	-0.33*	0.12	1.05E-03	1.85E-02
SLC3A	Isoform 2 of 4F2 cell-surface antigen heavy chain	0.59**	-0.02	-0.26	-0.18	1.20E-04	4.44E-03
SQSTM1	Sequestosome-1	0.76**	-0.06	0.24	0.18	1.10E-06	3.23E-04
HSPA1A	Heat shock 70 kDa protein 1A/1B	0.53**	-0.05	-0.21	0.01	3.11E-03	3.59E-02

Table 2. IPA pathway analysis of cluster C.

Molecular and Cellular Functions		
Name	p-value	Number of Molecules
Cellular Growth and Proliferation	6.61E-03 – 2.26E-08	28
Cellular Movement	6.61E-03 – 1.45E-06	19
Cellular Development	6.61E-03 – 2.10E-06	23
Carbohydrate Metabolism	6.13E-03 – 4.16E-06	8
Cellular Death and Survival	8.17E-03 – 4.42E-05	22
Top Canonical Pathways		
Name	p-value	Overlap
Glycolysis I (GAPDH, GPI, PGK1, PKM)	1.86E-07	4/25 (16%)
Gluconeogenesis I (GAPDH, GPI, PGK1)	1.78E-05	3/25 (12%)
Remodeling of Epithelial Adherens Junctions (TUBB3, TUBB4B, ZYX)	3.65E-04	3/68(4.4%)
EIF2 Signaling (EIF3H, PABPC1, PPP1CB, RPL36)	5.49E-04	4/185 (2.2%)
Breast Cancer Regulation by Stathmin1 (TUBB3, TUBB4B, PPP1CB, GNAI2)	6.19E-04	4/191 (2.1%)

Table 3 Candidate targets of FO or OA treatment

Protein ID	Gene Name	Protein name	FC OA/CN	MP OA/CN	FC FO/CN	MP FO/CN	FC FO/OA	MP FO/OA	ANOVA q Value
Day 1									
E7EMC7	SQSTM1	Sequestosome-1	-1	0.7	1.7	0.0001	1.8	0.0001	0.0003
Day 6									
P22307	SCP2	Non-specific lipid-transfer protein	1.3	0.005	1.7	0.0002	1.3	0.003	0.004
Q16658	FSCN1	Fascin	1.1	0.08	-1.2	0.05	-1.4	6.00E-05	0.009
B4DGP8	CANX	Calnexin	1.1	0.3	-1.3	0.02	-1.4	0.001	0.004
Q1L6U9	MSMP	Prostate-associated microseminoprotein	-1	1	-2	0.0009	-2	0.0008	2.12E-05

FC: fold change

MP: p value of multiple comparison test

Figure Legends

Figure 1: Effects of FO and OA on PCa cell viability. A: MTT assay was measured 24-hr after PC3 cells were treated by control (0.1% ethanol), 100 μ M fish oil or 100 μ M oleic acid (n=3). B: PC3 cells were seeded at 1000 cell/well in 6-well plates, treated with control (0.1% ethanol), 100 μ M FO, or 100 μ M OA, respectively. Cells grew for approximately one week until the colonies were visible. Cells were fixed by methanol and stained with crystal violet (n=3). C: PC3 cell images captured during colony formation.

Figure 2: Effects of FO and OA on FASN activity in PC3 cells. Cells were seeded in 24-well cluster plates, treated with vehicle (0.1% ethanol), 100 μ M FO or 100 μ M OA for 3 days or 6 days. FASN activity was measured by 14 C-acetate incorporation assay, in which the scintillation counts were normalized by cell counts in parallel treated wells (n=3).

Figure 3: Principal component analysis (PCA). A: PCA analysis of 127 significantly changed proteins (ANOVA $p \leq 0.05$, $q \leq 0.05$, n=5) shows graphic distribution of 30 samples (6 groups and 5 replicates in each) on axes of component 1 (F1) and component 2 (F2). B: By one minus Pearson Correlation, three major clusters were generated out of 73 proteins selected based on 127 proteins in (A) and has at least one significant change (t-test $p \leq 0.05$, n=5) in FO1/CN1 (fish oil vs. control, one-day), OA1/CN1 (oleic acid vs. control, one-day), FO6/CN6 (fish oil vs. control, six-day), or OA6/CN6 (oleic acid vs. control, six-day).

Figure 4. Enzyme functions in glycolysis/gluconeogenesis and its relative pathways are changed. Differentially expressed glycolysis/gluconeogenesis proteins in cluster C (*: t-test $p \leq 0.05$, $n=5$)

Figure 5: MRM validation of expression changes of MSMP (A), SCP2 (B) in FO or OA group compared to control, with internal control RPL6 (C) and spiked-in external control chicken lysozyme (D) ($n=6$).

Figure 6: Western Blot validation of expression changes of MSMP, SQSTM1 and autophagy marker LC3 I/II in FO or OA group. (A) SQSTM1 and β -actin; **(B)** Bar graph quantification of (A), $n=3$; **(C)** SQSTM-1 mRNA level by RT-PCR (GUSB mRNA serves as internal standard), $n=5$; **(D)** Western blot of LC3 I/II from cells treated with different FAs in the presence of $8 \mu\text{M}$ CQ for 1 day; **(E)** Bar graph quantification of (D), $n=3$; **(F)** secreted MSMP in medium; **(G)** Bar graph quantification of (F), $n=3$.