University of Nevada, Reno

The Effects of Omega-3 and Omega-6 Fatty Acids on the Epigenetic Landscape of Human Mammary Carcinoma (MDA-MB-231)

A thesis submitted in partial fulfillment
of the requirements for the degree of

BACHELOR OF SCIENCE, BIOCHEMISTRY AND MOLECULAR BIOLOGY

by

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THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

Tayeb Abbas

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Ronald S. Pardini, Ph.D., Thesis Advisor

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Tamara Valentine, Ph.D., Director, Honors Program
[May, 2018]

Abstract:

Breast cancer is the most common form of cancer among women, with over 250,000 new diagnoses in the United States this year alone. In addition, breast cancer has been the cause of death for more than 40,000 women in the United States in 2017. While there are many genetic and environmental effects that play a role in cancer formation, the role of epigenetics and dietary habits have been shown to have varying effects as well. Previous research in the Pardini Lab has shown that omega-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have anti-inflammatory activity and depress tumorigenesis, while high amounts of omega-6 PUFAs such as linoleic acid (LA) and arachidonic acid (AA) increase tumor proliferation. Epigenetic processes have been reported to regulate cancer proliferation. Previous studies have shown that increased methylation of oncogenes and tumor suppressor drugs have decreased and increased tumor formation, respectively, which favors tumor proliferation. Ultimately, the focus of this study is to examine the effects of PUFAs on epigenetic patterns on the breast cancer cell line, MDA-MB-231. We hypothesize that DHA, EPA, Azacytidine, and Panobinostat treatments will decrease methylation levels of DNA, while omega-6 PUFAs such as linoleic acid (LA) and arachidonic acid (AA) will promote methylation.

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Introduction:

In the United States, breast cancer is the second most prevalent form of cancer in women. Although there are various subtypes of breast cancer, the three most common forms that appear are: progesterone receptor breast cancer (PRBC), estrogen receptor breast cancer (ERBC) and human epidermal factor receptor 2 breast cancer (HER2BC), which appear via distortions in molecular signaling. While PRBC and ERBC are manifested due to malfunctions in hormone receptor pathways, positive HER2BC results from abnormal growth factors. If detected early, these forms of cancer can be treated easily. Triple negative breast cancer (TNBC) on the other hand, is another common form of breast cancer among women, that makes up nearly twenty percent of all breast cancer diagnoses in the United States [1]. With over 250,000 new diagnoses in the United States in 2017, TNBC is more difficult to treat due to an absence in ER, PR, and HER2 [1], which makes this cancer resistant to hormone treatment. Consequently, women who suffer from TNBC have an increased risk of death because they are less susceptible to hormone therapy.

UV radiation, carcinogen exposure, and the Western lifestyle, coupled with one's own genetic predispositions, contribute to the level of high level of breast cancer in the United States. Invasive breast cancer accounts for 29.5% of all female cancers [2], and about half the women that were diagnosed with TNBC in the United States had no family history of cancer, with the only risk factor for breast cancer being aging [2]. Aside from environmental factors, epigenetic predispositions may play a key role in breast cancer development. Although DNA is replicated on its own, DNA along with other proteins are

subject to different types of modifications where different chemical groups can be attached. These chemical groups (i.e.: hydroxyls, phosphates, acetyl, and methyl groups) can then dictate DNA activity. Any alterations to the DNA from the modifications can result in variable expression levels of numerous oncogenes and tumor suppressor genes. In particular, studies have shown that cancer initiation and proliferation occurs when tumor suppressor genes are hypermethylated [3]. When certain genes undergo post-translation modifications such as hypermethylation (Figure 1), the genetic expression of those genes is down-regulated. The post-translational modifications of the cancer genome are a potential target when treating TNBC.

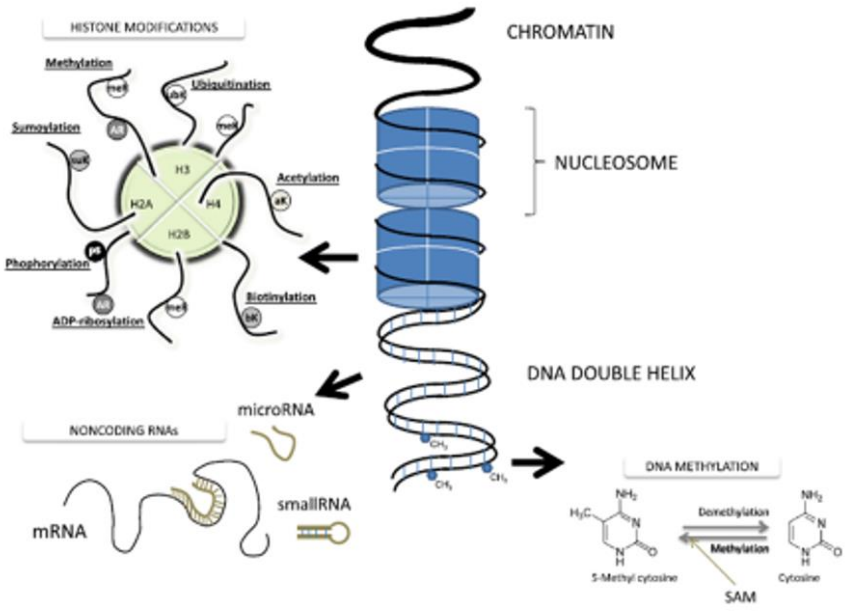

Figure 1: The Various Post-Translational Modifications of DNA. The cancer genome can undergo various types of DNA modifications. In this experiment, methylation levels of MDA-MB-231 DNA will be examined [5].

Although cancer used to be thought of as a disease that was inherited through one's family lineage as well as the exposure to environmental factors over time, studies

are now finding that epigenetic changes can also induce certain cancers [4]. In particular, DNA methylation levels play a critical role in whether certain genes will be expressed or inhibited [5]. As is the case with TNBC, when there is an increase in DNA methylation, there is a decrease in gene expression. Consequently, many tumor suppressor genes have been found to be hypermethylated while oncogenes have been found to be hypomethylated in TNBC. Studies have also shown that specific promoter sequences can also alter the methylation levels of CpG islands, which lead to the up-regulation of tumorigenesis [6].

While cancer can be prevented by decreasing the rates of exposure to environmental mutagens, many studies are now suggesting that one's diet also has a role in cancer formation. More specifically, dietary fatty acids have shown to have varying effects on both cancer prevention and formation. Many nutritional cancer reports have shown that the incorporation of polyunsaturated fatty acids (PUFAs) into the diet can not only modulate gene expression by inducing local and global effects in DNA, but also inhibit cancer as well [7]. Aside from improving neural and cardiovascular health and regulating immune response [8, 9, 10] docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), both omega-3 PUFAs (Figure 2) inhibit cancer growth [11]. On an molecular level, both DHA and EPA have been shown to possess anti-inflammatory properties [12]. On the other hand, some omega-6 PUFAs (Figure 3) such as linoleic acid (LA) and arachidonic acid (AA) have been shown to promote tumorigenesis, depending on the type of cancer [13].

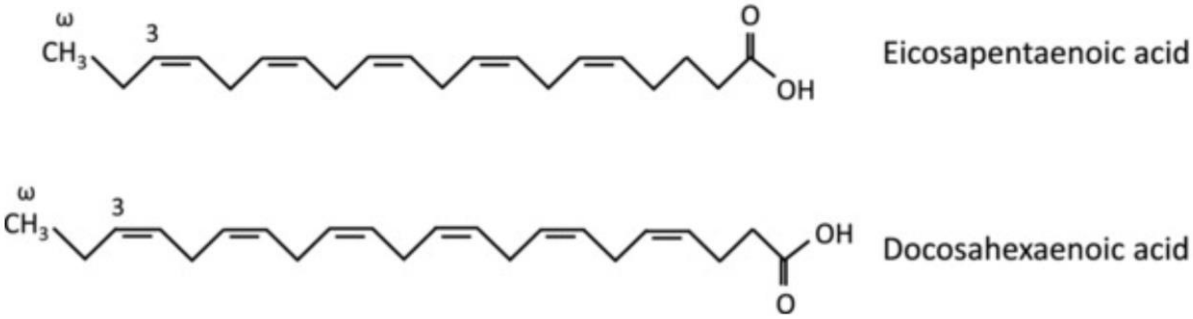

Figure 2: Diagram of Omega-3 Polyunsaturated Fatty Acid Structure. DHA and EPA can be found from leafy green vegetables, as well as marine sources like salmon [18].

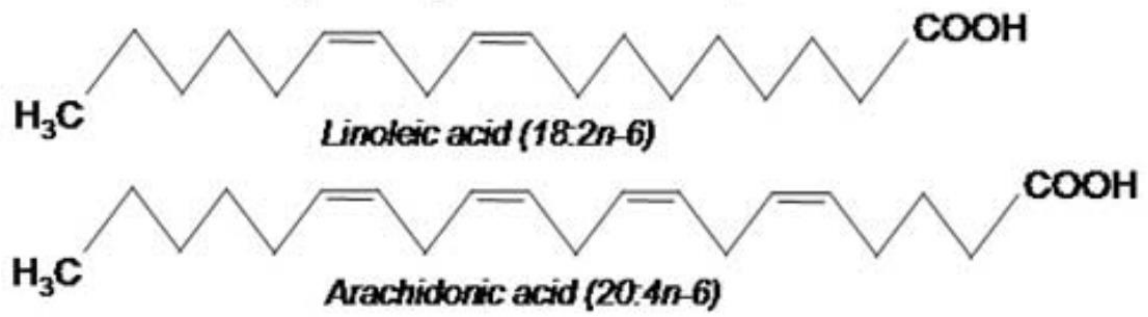

Figure 3: Diagram of Omega-6 Polyunsaturated Fatty Acid Structure. LA and AA can both be found in corn oil, canola oil, and nuts [19].

A previous study compared the Western diet (high omega-6: omega-3 ratio) to diets of the Eskimo-Inuit tribe and traditional Japanese diets (high omega-3: omega-6 ratio, respectively). Researchers found lower rates of breast cancer among women of Japan [14] and the Eskimo-Inuit tribe, compared to women in the United States. Further epidemiological studies showed that when Japanese women immigrate to the United States and adopt the Western diet into their own lifestyle, then they become susceptible to an increased risk of breast cancer. These studies suggest that different ratios of omega-3 and omega-6 fatty acids have a critical role in cancer. Consequently, many studies are now looking at the potential benefits of coupling PUFAs such as DHA and EPA with

anti-cancer drugs [15]. In addition, other reports are examining whether omega-6 PUFAs play an antagonistic role when paired with different chemotherapies. Ultimately, the focus of this study is to examine the effects that PUFAs play on the epigenetic landscape in the TNBC breast cancer cell line MD-MDA-231. We hypothesize that DHA, EPA, Azacytidine (AZA-DNA methyl transferase inhibitor), Panobinostat (histone deacetylase inhibitor) and C646 (inhibitor of histone deacetylase) treatments will decrease methylation levels of DNA, while omega-6 PUFAs such as LA and AA will promote methylation which will ultimately increase tumorigenesis.

Materials and Methods:

Reagents and Cell Lines

The MDA-MB-231 cell line was purchased from the American Type Culture Collection. All chemicals and fatty acids (FAs) were purchased from Sigma Aldrich. DHA, EPA, LA, and AA were separately mixed with bovine serum albumin (BSA), then dissolved in phosphate buffered saline (PBS). Lastly, anti-cancer drugs AZA, Panobinostat and C646 were dissolved in DMSO.

Cell Culture

The MDA-MB-231 cell lines were treated with RPMI-1640 media in addition with 10% fetal bovine serum (FBS). The cells were incubated in 37°C with a 5% CO_2 environment. After 24 hours of initial cell seeding, cells were treated with different concentrations (75 nmol and 250 nmol) of DHA, EPA, LA, and AA. Then, assays were conducted at 1, 3, 6, 12, 24, and 48 hours post treatment.

Treatment Dosage

DHA titration curve was used for PUFA dosage selection. Afterwards, all other PUFAs were treated in molar equivalents. AZA, Panobinostat, and C646 were also titrated to determine 50% of cell viability.

DNA Extraction and Methylation Detection

DNA was extracted from all cells using Qiagen DNeasy Blood and Tissue Kit. Cell samples were first centrifuged to isolate the nuclei from other cell fragments. 20 µL of Proteinase K was added to denature nuclei for DNA extraction. The samples were then transferred to a spin column and centrifuged at 8000 rpm for two minutes. After each

centrifuge, Buffer AW1 was added to ensure proper resuspension. The centrifugation and resuspension processes were repeated three times. Finally, the DNA was extracted and incubated at room temperature for one minute.

With the DNA properly extracted from the cell lines, ELISA experiments were conducted using the 5-mC DNA ELISA kit from the Zymo Company. 100 ng of DNA was mixed with 100 µL of 5-mC Coating Buffer. The samples were denatured for five minutes at 98°C and then immediately placed on ice for ten minutes. Each mixture was then transferred to a new plate and incubated for one hour at 37°C.

Following the first incubation step, the coating buffer was discarded, and the plate was washed three times with 200 µL of 5-mC ELISA Buffer. The plate was incubated for thirty minutes at 37°C. After the buffer was discarded from the wells, 100 µL of Anti-5-Methylcytosine and secondary antibody mix were added to each sample. Samples were then incubated for one hour at 37°C.

Finally, the antibody mixture was discarded from the wells and washed with 200 µL of 5-mC ELISA Buffer three times. 100 µL of HRP Developer was added to each sample. The plate was incubated at room temperature while the color development progressed. After one hour, the sample absorbances were measured at 450 nm using the SpectraMax M5 plate reader (Molecular Devices LLC, Sunnyvale, CA).

Results:

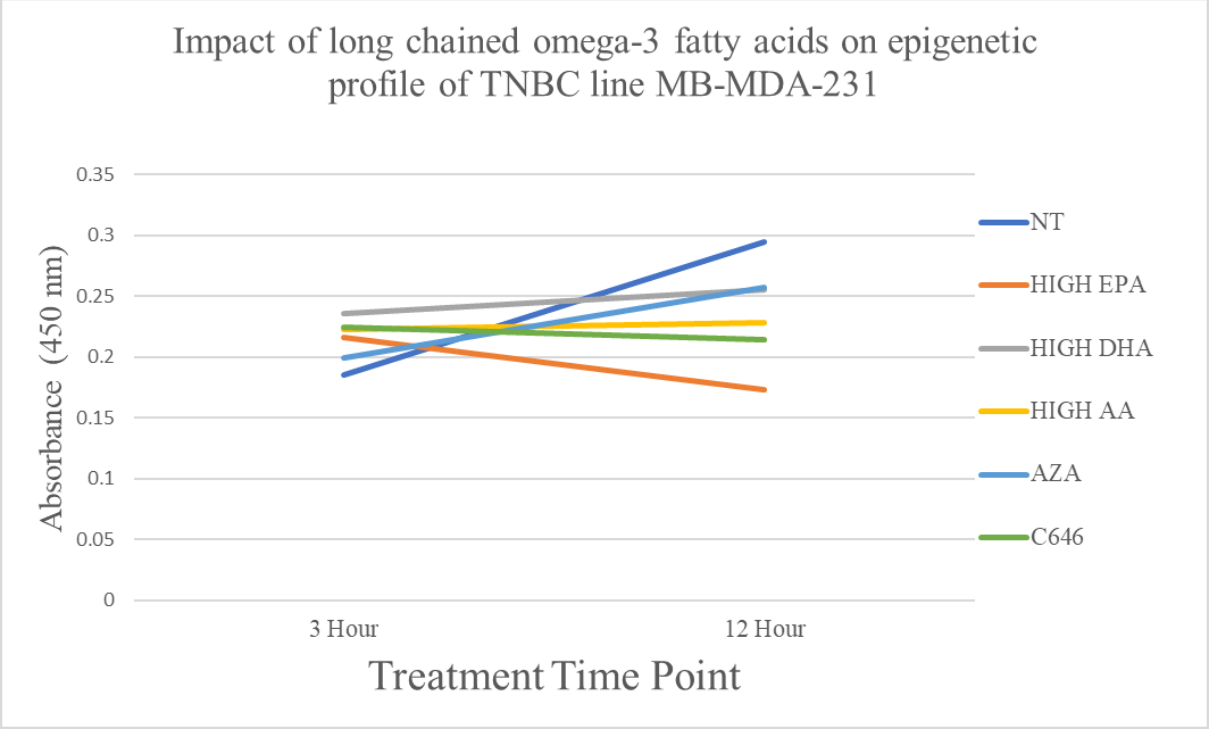

Figure 4: Absorbance values of 250 nmol PUFA and Epigenetic Inhibitors Treatments of MDA-MB 231 cell lines.

Absorbance values indicate the level of DNA methylation in each of the samples, with higher absorbance values resulting in increased methylation patterns in DNA. NT, High EPA, High DHA, High AA, AZA, and C646 treatments were plated in duplicate, respectively, on a clear-bottom 96-well plate. After incubation with HRP color development for 1 hour, absorbance values were measured on a SpectraMax M5 plate reader (Molecular Devices LLC, Sunnyvale, CA). *Abbreviations:* NT (No treatment), EPA (eicopentaenoic acid), DHA (docosahexaenoic acid), AA (arachidonic acid), AZA (5-Azacytidine), C646 (histone acetyltransferase inhibitor).

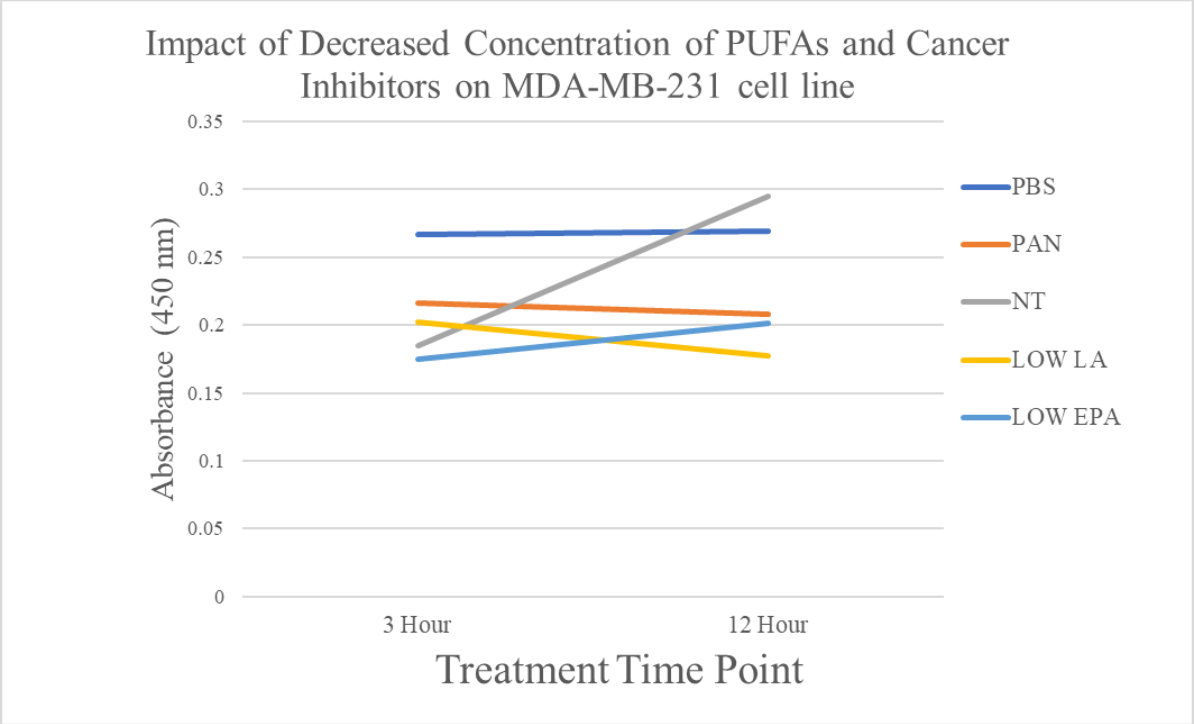

Figure 5: Absorbance values of 75 nmol PUFA Treatments and Epigenetic Inhibitors of MDA-MB 231 cell lines. Absorbance values indicate the level of DNA methylation in each of the samples, with higher absorbance values resulting in increased methylation patterns in DNA. PBS, PAN, NT, Low LA, and Low EPA treatments were plated in duplicate, respectively, on a clear-bottom 96-well plate. After incubation with HRP color development for 1 hour, absorbance values were measured on a SpectraMax M5 plate reader (Molecular Devices LLC, Sunnyvale, CA). *Abbreviations:* PBS (phosphate buffered saline), PAN (Panobinostat), NT (not treated), LA (linoleic acid), EPA (eicosapentaenoic acid).

Both experiments show that breast cancer cells that were not treated with any PUFA or anticancer drug have an increased absorbance, proving that MDA-MB-231 DNA becomes hypermethylated if there is no treatment involved. Cells treated with 250 nmol of EPA show a decreased absorbance from the three hour and twelve-hour timepoint compared to the NT [Figure 4]. However, cells treated with 250 nmol of DHA

show a slight increase in absorbance at the twelve-hour time point. Cancer inhibitor drug, Panobinostat, along with C646 and LA reveal a lower absorbance at the twelve-hour time point. Finally, low concentrations of EPA, along with AZA show an increased absorbance of when analyzed at the three-hour and twelve-hour time point, respectively.

Discussion:

While many studies are investigating the long-term effects of dietary fatty acids, these results show how different treatments at different time points can affect the epigenetics of the breast cancer cell line. Majority of the results show that DHA and EPA reduce the methylation levels of DNA, thereby increasing the expression levels of the DNA itself [15]. In the 3-hour time point, the lowest absorbance was observed in the cells treated with 250 nmol of EPA [Figure 4], indicating that EPA inhibits DNA methylation. In addition, at low levels of LA, there was a decreased absorbance, which indicates lower methylation levels in the DNA. However, it is still not understood why there was such a decreased absorbance in the samples treated with higher concentrations of AA, when it was expected to have higher methylation levels. The same speculations apply to the readings in the 12-hour time point, only this time, there was an increased methylation absorbance of DNA treated with 75 nmol of EPA compared to the absorbance at the three-hour time point [Figure 5]. Once again, omega-6 fatty acid treatments such linoleic acid shows a lower absorbance reading at the twelve-hour time point, while the absorbances were higher for cells that were treated with PBS. However, as expected, the levels were still higher for cells that were left untreated. Ultimately, this study shows that 250 nmol EPA is the most potent methylation inhibitor for MDA-MB-231 cell lines at the

twelve-hour time point, which is comparable to Panobinostat, while more effective than C646 and AZA.

Although the mechanism of DHA and EPA on the cancer genome inhibition is still under investigation, previous studies show consumption of foods rich in DHA and EPA decrease the rate of breast cancer proliferation [16]. However, increased ratios of omega-6 to omega-3 PUFAs result in an increased risk of cancer formation. Ultimately, on a global context, proper ratios of omega-3 to omega-6 fatty can inhibit tumorigenesis [17].

Further studies will continue to be conducted on the same samples to determine whether there is any reason for the unexpected results. In addition, according to the 5-mC ELISA protocol, the directions stated to measure the absorbance from 405 to 450 nm. While these experiments show the result of the readings at the 450 nm absorbance, in future experiments, absorbances will be measured at both 405 and 450 nm to account for any possible changes in the color development analysis. As of right now, we can infer that the time of treatment and the time of the treatment is crucial in terms of DNA expression.

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