Discovery, The Student Journal of Dale Bumpers College of Agricultural, Food and Life Sciences

Volume 20 Article 8

Fall 2019

The Effects of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) on Brown Adipogenesis in Stem Cell Culture

Darynne A. Dahlem
University of Arkansas, Fayetteville, darynnedahlem@gmail.com

Yan Huang
University of Arkansas, Fayetteville, yxh010@uark.edu

The Effects of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) on Brown Adipogenesis in Stem Cell Culture

Cover Page Footnote
Darynne Dahlem is a senior animal science majoring in Pre-professional Animal Science in the Department of Animal Science Dr. Huang is an assistant professor in the Department of Animal Science.

The effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on brown adipogenesis in stem cell culture

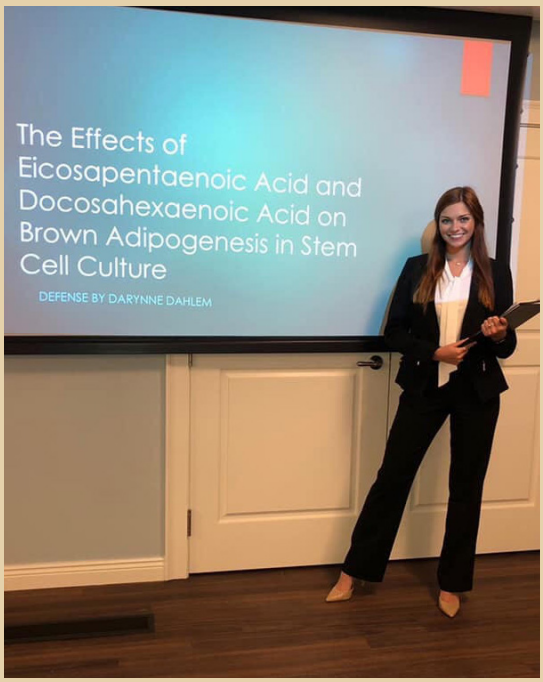
Meet the Student-Author

Darynne Dahlem

I am originally from Greenwood, Arkansas. I am a senior Animal Science major in the Bumpers College of Agricultural, Food, and Life Sciences. While studying at the University of Arkansas I have had the opportunity to be a part of Greek life as an active member of Delta Delta Delta, serve as an Associated Student Government Senator, and hold executive positions on the Bumpers College Honors Student Board. After graduation, I plan on getting a Master of Science degree at the University of Arkansas and then attend medical school to become a physician. In June, I was crowned Miss Arkansas 2019 and I will be competing in the Miss America competition on December 19th. I would like to thank Dr. Yan Huang for serving as my honors mentor and advisor for this project and I wish to recognize his constant support and fantastic teaching skills that helped me learn as much as possible throughout this experience. I would also like to thank Dr. Jason Apple, Dr. Charles Rosenkrans, and Dr. Jamie Baum for serving as committee members. I would also like to acknowledge Saeed Hemza for his help and technical assistance in completing this project.

Research at a Glance

- The objective of this study was to measure the effect of fish oil supplements on brown fat development.
- Stem cell culture was used as a model for this study.
- As a prenatal supplement, fish oil ingredients have a positive influence on fetal growth.
- The cell culture study indicated that fish oil inhibits muscle formation and promotes fat growth through genetic regulation.



Darynne at her thesis defense.

The effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on brown adipogenesis in stem cell culture

Darynne Dahlem and Yan Huang†*

Abstract

Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are major maternal dietary supplements due to their positive benefits on neurological tissue growth during the first 12 weeks of gestation. Previous studies show that EPA and DHA inhibit muscle formation but promote fat growth (adipogenesis). However, no research has addressed the question of whether a high intake of EPA and DHA affects brown fat development during gestation. The objective of this study was to measure the effect of EPA and DHA supplementation on brown adipogenesis and potential pathways related to mitochondrial biosynthesis using fibroblasts as an in vitro model. Using Oil-Red-O staining and polymerase chain reaction (PCR) testing, lipid droplet formation and six genes were examined. Results indicated that *PGC1α* gene expression was affected by EPA and DHA treatment. Mitochondrial biosynthesis can potentially be promoted by increased PGC1α gene expression with EPA and DHA supplementation. However, lipid droplet accumulation observed in the PUFAS-treated group indicated a previously unknown effect of n-3 PUFA supplementation on adipogenesis that needs to be further investigated.

* Darynne Dahlem is a senior animal science majoring in Pre-professional Animal Science in the Department of Animal Science.
† Yan Huang, the faculty mentor, is an assistant professor in the Department of Animal Science.

Introduction

In the United States, rates of childhood and adolescent obesity have been on the rise for years and have nearly tripled since the 1970s. The Centers for Disease Control and Prevention (CDC) reports data taken from 2015–2016 show that nearly 1 in 5 school-age children and young people (6 to 19 years) in the United States are obese. There is increasing evidence that infants exposed to obesity-induced diabetes in utero have an increased incidence of childhood obesity and diabetes (Feig and Moses, 2011). Understanding the mechanism of the relationship between maternal and infant obesity is an urgent task in the study of childhood obesity.

Epidemiological and experimental studies show that food supplements, such as fatty acids, supplied to the fetus during pregnancy and to the newborn immediately after birth, can have long-term health effects on the development of metabolic diseases. These diseases include cardiovascular diseases, Type 2 diabetes, hypertension, and obesity. (Kabaran and Besler, 2015). Growing bodies of experimental studies indicate that reducing the risk of a variety of obesity-related diseases is strongly linked to an increase in the dietary supplementation and consumption of n-3 fatty acids (Seo et al., 2005). While a substantial number of studies have delineated many differences between the biological effects of saturated versus polyunsaturated fatty acids (PUFAs), less is known about the long-chain n-3 fatty acids commonly present in certain fish oils (Seo et al., 2005), such as eicosapentaenoic acid (EPA, 20:5,n-3) and docosahexaenoic acid (DHA, 22:6,n−3). Fish oil components, particularly two key biological regulators, EPA and DHA, appear to have the ability to modulate both cellular metabolic functions and gene expression. The synthesis of EPA and DHA from their 18:3 precursor α-linoleic acid is relatively inefficient, so meeting the body's need of n-3 fatty acids depends to a significant degree on the direct delivery of EPA and DHA with diet particularly from marine or industrial sources, such as fish oils. (Qi et al., 2002). Clinical research also showed that EPA and DHA supplemented during pregnancy accumulates in fetal tissues and cause a longer gestation.

Our previous studies showed that EPA and DHA inhibit muscle formation but promote fat growth, also called adipogenesis. However, no research has addressed the question of whether a high intake of EPA and DHA affects brown fat development during gestation. Brown adipose tissue (BAT) is an essential target in obesity prevention as well as treatment due to its ability to utilize fatty acids and glucose to generate heat by a mechanism not requiring muscle contraction, also called non-shivering thermogenesis. Most brown adipocytes originate from precursor cells in the embryonic stage that express skeletal muscle marker genes and have similar mitochondrial features with muscle (Seo et al., 2005; Ong and Muhlhausler, 2011). In most eukaryotes, mitochondria are primary organelles that are responsible for energy metabolism derived from the breakdown of carbohydrates and fatty acids. It was reported that the n-3 PUFAs could cause higher oxidation levels of mitochondrial fatty acids in the myocardium (Flachs et al., 2005; Martins et al., 2012; Anderson, et al., 2014; Cavaliere et al., 2016). We hypothesize that EPA and DHA treatment impacts the brown adipogenesis of BAT precursor cells via metabolic changes in mitochondria. The objective of the current study is to measure the effect of maternal EPA and DHA supplementation on brown adipogenesis and potential pathways related to mitochondrial biosynthesis using fibroblasts as an in vitro model.

Materials and Methods

Cell Culture

National Institutes of Health (NIH) 3T3 fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 atmosphere. When cells confluency reached 90%, cells in the control group wells (Con, n = 6) were induced to brown adipocyte differentiation by switching to the differentiation medium 1 (DM1) containing 10% FBS, 1% penicillin-streptomycin, 170 nM insulin, 1 uM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 nM 3, 3',5-triiodo-L-thyronine sodium salt (T3), while 50 μM EPA and 50 μM DHA were added to DM1 in the fatty acid treatment group (FA, n = 6) for 3 days. Then Con cells were introduced to DM2 which only contained 10% FBS, 170 nM insulin, and 1nM T3. The DM2 in the FA group contained 50 μM EPA and 50 μM DHA. The DM2 with or without fatty acids was changed every 24 hours for 3 days.

Oil-Red-O Staining

Oil-Red-O staining was used to identify mature adipocytes. Cells were fixed in 10% formalin in phosphate-buffered saline (PBS) for 10 min at room temperature. Fixed cells were stained with the Oil-Red-O working solution for 7 min and rinsed with PBS to remove the excessive Oil-Red-O dye. The presence of Oil-Red-O dye in adipocytes was further quantified by measuring the optical absorbance at 520 nm.

Real-Time Polymerase Chain Reaction (PCR)

Gene expression related to brown adipogenesis, mitochondrial biosynthesis, and peroxisome biosynthesis was measured by quantitative real-time PCR. Total mRNA was extracted from cells with the TRIzol reagent (Fisher,

Pittsburgh, Pa.). The concentration of total RNA was assessed by Nanodrop OneC (Thermo Scientific, Waltham, Mass.), and quality was examined in the absorption ratio of OD260 nm/OD280 nm. The cDNA was synthesized from the RNA with iScript cDNA synthesis kit (Bio-Rad, Richmond, Calif.). Real-time PCR was carried out by using SYBR Green Supermix (Bio-Rad, Richmond, Calif.) on CFX Connect Real-Time PCR Detection System (Bio-Rad, Richmond, Calif.). The oligonucleotide primers used were designed with the NCBI database and Primer Quest (IDT.com). The primers (Table 1) were designed to target the genes: uncoupling protein 1 (UCP1), PR/SET domain 16 (PRDM16), iodothyronine deiodinase 2 (DIO2), peroxisome proliferator-activated receptor alpha (PPARα), carnitine palmitoyltransferase 1beta (CPT1β), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). Each reaction yielded amplicons from 80 to 250 bp. The PCR conditions were as follows: 30 s at 95 °C, 30 s at 55 °C, and 40 s at 72 °C for 40 cycles. After amplification, a melting curve (0.01 °C/s) was used to confirm product purity, and the PCR products were electrophoresed to verify the targeted sizes. Results were expressed relative to β-actin. Data were analyzed using the ΔΔCT method, and the 18S gene was the reference gene.

Statistical Analyses

Differences between groups were assessed for significance by the unpaired *t*-test with the assumption of equal variances, and arithmetic means ± SEM are reported. Statistical significance was considered as $P \leq 0.05$.

Results and Discussion

The Oil-Red-O staining showed lipid droplet accumulation in pre-adipocytes differentiated from 3T3 fibroblasts (Fig. 1A). The quantitative data showed that the red dye accumulated 20.04 ± 6.95% more ($P < 0.05$) in the FA group than in Con cells (Fig. 1B).

Table 1. List of primers.

Primers	Accession no.	Forward sequence	Reverse sequence
UCP1	NM_009463	CACGGGGACCTACAATGCTT	ACAGTAAATGGCAGGGGACG
PRDM16	NM_027504	AAGGAGGCCGACTTTGGATG	TTTGATGCAGCTCTCCTGGG
PPARα	NM_011144	TGGTGTTCGCAGCTGTTTTG	AGATACGCCCAAATGCACCA
CPT1β	NM_009948	TATAACAGGTGGTTTGACA	CAGAGGTGCCCAATGATG
PGC1α	NM_008904	TCCTCTGACCCCAGAGTCAC	CTTGGTTGGCTTTATGAGGAGG
18S	NR_003278	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

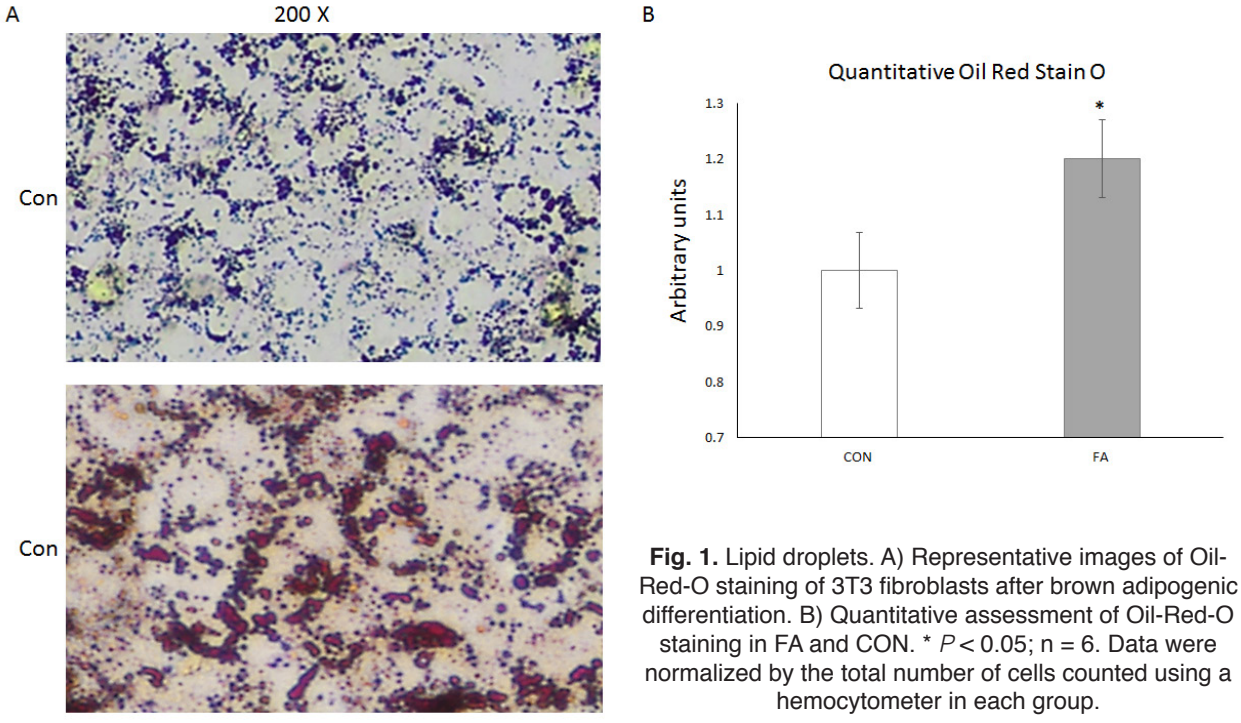


Fig. 1. Lipid droplets. A) Representative images of Oil-Red-O staining of 3T3 fibroblasts after brown adipogenic differentiation. B) Quantitative assessment of Oil-Red-O staining in FA and CON. * $P < 0.05$; n = 6. Data were normalized by the total number of cells counted using a hemocytometer in each group.

Among the brown and white adipogenic marker genes, the expression of PGC1α was greater 31.81 ± 5.17% ($P < 0.05$; Fig 2) in the FA group than in Con cells. Other gene expression including UCP1, PRDM16, PPARα, and CPT1β was not different.

Long chain fatty acids are known to activate brown adipocytes (Gonzalez-Hurtado et al., 2018). In this study, the expressions of six genes were measured: UCP1, PRDM16, DIO2, PPARα, CPT1β, and PGC1α. The UCP1 is known as uncoupling protein 1 and it works to separate oxidative phosphorylation from adenosine triphosphate (ATP) synthesis with energy dissipated as heat, it is also referred to as the mitochondrial proton leak and helps to reduce mitochondrial membrane potential in mammalian cells (Brondani et al., 2012). The PRDM16 is a protein-coding gene that has broad expressions in the stomach and thyroid among other tissues (Seale et al., 2008). Peroxisome proliferator-activated receptor alpha (PPARα) increases the size and number of peroxisomes, which are subcellular organelles found in plant and animal cells that contain enzymes for respiration and for cholesterol and lipid metabolism (Choi et al., 2015). Carnitine palmitoyltransferase1β (CPT1β) is a protein-coding gene that encodes a protein that is the rate-controlling enzyme of the long-chain fatty acid beta-oxidation pathway in muscle mitochondria (Choi et al., 2015). The protein coded by PGC1 α is a transcriptional cofactor that regulates genes involved in energy metabolism (Austin and St-Pierre, 2012). Of these six genes, only one was affected by EPA and DHA supplementation. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) is a transcription cofactor that functions as a master regulator for many metabolic and physiological processes such as adaptive thermogenesis, glucose and fatty acid metabolism, muscle fiber type, and mitochondrial biogenesis (Flachs et al., 2005; Austin and St-Pierre, 2012). Overexpression of this transcription coactivator could improve mitochondrial function. It also could increase oxidative stress resistance. This upregulation could be an indicator that these long-chain fatty acids can increase the speed of the metabolic pathways when introduced to fibroblasts. However, it has also been recognized in recent studies that 3T3 cells are insensitive to both fatty acid and beta-adrenergic agonist stimulation. The 3T3 cells are the most commonly used because they have a high affinity for harboring lipids into the cytoplasm when stimulated. The understanding that they are insensitive to the treatment of long-chain fatty acids helps to explain the lack of statistical differences between the control group and the treatment group (Shin and Ajuwon, 2018). The results collected are a helpful piece in the equation that is prenatal nutrition. A limitation of this study is that only PCR and staining results could be presented. The results could be fortified by further testing the cell line for oxygen consumption rates, conducting Western Blot tests, and additional PCR analysis of genes involved with thermogenesis, mitochondrial biosynthesis, and protein synthesis. Another limitation was the sample size that survived until final testing.

Conclusions

Results indicate that mitochondrial synthesis has the ability to be induced through the introduction of certain

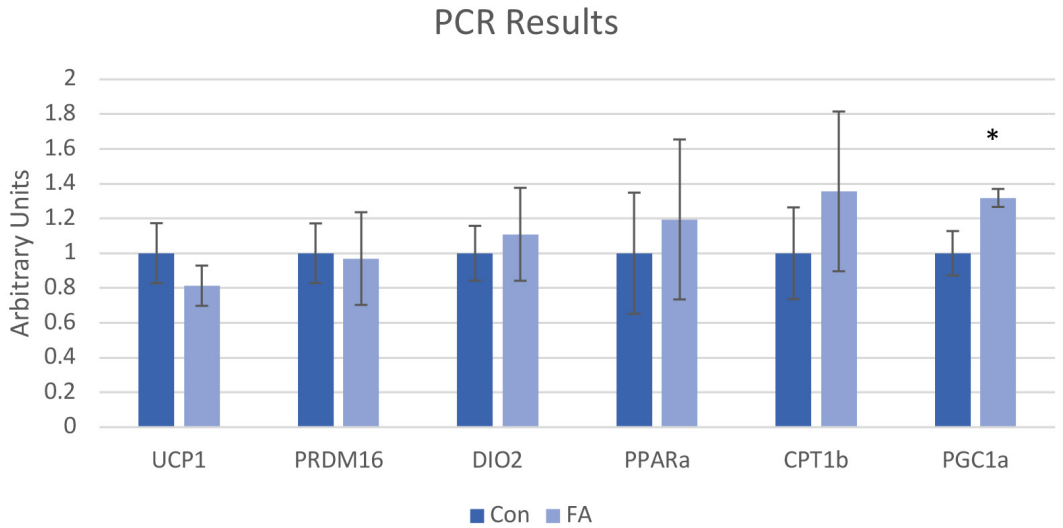


Fig. 2. Gene expression analysis by RT-qPCR of key genes related to adipogenesis, mitochondrial biosynthesis, and metabolism. Data are expressed as mean + SEM. The relative expressions were calculated in arbitrary units. * $P < 0.05$; n = 6.

long-chain fatty acids. This would usually be paired with smaller lipid droplets since higher mitochondrial counts allow for more rapid lipid degradation. However, the results of this study show greater lipid concentrations in the cells while also having greater mitochondrial counts. For this reason, additional studies are needed to understand the reason behind this discrepancy and to eventually realize the effect of EPA and DHA on adipogenesis in relation to thermogenesis and increase of obesity post-partum when introduced to fibroblasts in-vitro. The researchers suggest the use of a sturdier cell line that is easily stimulated by fatty acid treatment and to run more diagnostic testing focusing on mitochondrial biosynthesis.

Acknowledgements

This research was made possible by the Bumpers College Honors Faculty Committee's generous funding of this project.

Literature Cited

Anderson, E.J., K.A. Thayne, M. Harris, S.R. Shaikh, T.M. Darden, D.S. Lark, J.M. Williams, W.R. Chitwood, A.P. Kypson, and E. Rodriguez. 2014. Do fish oil omega-3 fatty acids enhance antioxidant capacity and mitochondrial fatty acid oxidation in human atrial myocardium via PPARγ activation? Antioxidants & redox signaling. 21(8):1156–1163.

Austin, S. and J. St-Pierre. 2012. PGC1 and mitochondrial metabolism–emerging concepts and relevance in ageing and neurodegenerative disorders. J. Cell Sci. 125:4963-4971.

Brondani, L.D., T.S. Assmann, G.C. Duarte, J.L. Gross, L.H. Canani, and D. Crispim. 2012. The role of the uncoupling protein 1 (UCP1) on the development of obesity and type 2 diabetes mellitus. Arquivos Brasileiros De Endocrinologia & Metabologia. 56(4):215-225.

Cavaliere, G., G. Trinchese, P. Bergamo, C.D. Filippo, G.M. Raso, G. Gifuni, R. Putti, B.H. Moni, R.B. Canani, R. Meli, and M.P. Mollica. 2016. Polyunsaturated Fatty Acids Attenuate Diet Induced Obesity and Insulin Resistance, Modulating Mitochondrial Respiratory Uncoupling in Rat Skeletal Muscle. PloS one. 11(2):e0149033.

Choi, S.H., S.K. Park, B.J. Johnson, K.Y. Chung, C.W. Choi, K.H. Kim, W.Y. Kim, and B. Smith. 2015. AMPKα, C/EBPβ, CPT1β, GPR43, PPARγ, and SCD Gene Expression in Single- and Co-cultured Bovine Satellite Cells and Intramuscular Preadipocytes Treated with Palmitic, Stearic, Oleic, and Linoleic Acid. Asian-Australasian J. Anim. Sci. 28(3):411–419.

Feig, D.S. and R.G. Moses. 2011. Metformin therapy during pregnancy: Good for the goose and good for the gosling too? Diabetes care. 34:2329-2330.

Flachs, P., O. Horakova, P. Brauner, M. Rossmeisl, P. Pecina, N.F. Hal, J. Ruzickova, Z. Sponarova, Z. Drahota, C. Vleck, J. Keijer, J. Houstek, and J. Kopecky. 2005. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. Diabetologia. 48(11):2365-2375.

Gonzalez-Hurtado, E., J. Lee, J. Choi, and M.J. Wolfgang. 2018. Fatty acid oxidation is required for active and quiescent brown adipose tissue maintenance and thermogenic programing. Molecular Metabolism. 7:45-56.

Kabaran, S. and Besler, H.T. 2015. Do fatty acids affect fetal programming? J. health, population, nutrition. 33:14.

Martins, A.R., R.T. Nachbar, R. Gorjao, M.A. Vinolo, W.T. Festuccia, R.H. Lambertucci, M.F. Curry-Botaventura, R.C. Silveira, and S.M. Hirabara. 2012. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: Importance of the mitochondrial function. Lipids in Health and Disease. 11(1):30.

Ong, Z.Y. and B.S. Muhlhausler. 2011. Maternal "junk-food" feeding of rat dams alters food choices and development of the mesolimbic reward pathway in the offspringFASEB journal: official publication of the Federation of American Societies for Experimental Biology. 25(7):2167-2179.

Qi, K., M. Hall, and R.J. Deckelbaum. 2002. Long-chain polyunsaturated fatty acid accretion in brain. Current opinion in clinical nutrition and metabolic care. 5:133-138.

Seale, P., B. Bjork, W.L. Yang, S. Kajimura, S. Chin, S.H. Kuang, A. Scime, S. Devarakonda, H.M. Conroe, H. Erdjument-Bromage, P. Tempst, M.A. Rudnicki, D.R. Beier, and B.M. Spiegelman. 2008. PRDM16 Controls a Brown Fat/Skeletal Muscle Switch. Nature. 454(7207):961-967.

Seo, T., W.S. Blaner, and R.J. Deckelbaum. 2005. Omega-3 fatty acids: Molecular approaches to optimal biological outcomes. Current opinion in lipidology. 16(1):11-8.

Shin, S. and K.M. Ajuwon. 2018. Divergent Response of Murine and Porcine Adipocytes to Stimulation of Browning Genes by 18-Carbon Polyunsaturated Fatty Acids and Beta-Receptor Agonists. Lipids. 53(1):65-75.