# ADDITION OF PHOSPHOLIPIDS TO DIET TO ENHANCE THE BIOAVAILABILITY AND INCORPORATION OF FISH OIL - OMEGA-3-FATTY ACIDS.

By

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## ABSTRACT

Long-chain (LC) omega-3 polyunsaturated fatty acids (LC omega-3 PUFA) are important in regulating inflammation, maintaining cell membrane integrity, and are required for healthy growth and development. For omega-3 fatty acids to perform their biological function, they must first be bioavailable to the target tissues. Studies have shown that numerous factors affect their bioavailability, such as the molecular carrier (ethyl ester (EE), triacylglycerol (TAG), phospholipid (PL), positional distribution, and the amount and type of fat consumed in the diet. While fatty fish is the best dietary source of LC omega- 3 PUFA, fish oil supplements are a common source for many people. Phospholipids, such as phosphatidylcholine, may improve the efficiency of the omega-3 fatty acids in target tissues when added to a diet containing added fish oil. Twenty-four adult Yucatan miniature pigs (females, 6-8 months old) were used in this study and divided into three iso-caloric diet groups (Western diet (WD), Western diet plus fish oil (FO), and Western diet plus fish oil and phospholipid (FO-PL)); they were fed ad-libitum for four hours per day. The first objective of this study was to determine the distribution of fatty acids in various organs of pigs consuming a diet containing FO (EPA+DHA, 2.7% of total fat) compared to pigs consuming a diet without fish oil (WD). The second objective of this study was to determine if PL added to a diet containing fish oil increased the tissue content of omega-3 fatty acids compared to a FO diet without added PL. The third objective of this study was to determine if PL added to a diet containing fish oil decreased the concentration of serum pro-inflammatory cytokines compared to FO diet. After four months of feed intake, various tissue samples were collected to analyze different fatty acid distributions. Fatty acid and lipid analysis were assessed in various organs and tissues. The total omega-3 fatty acid level was significantly higher in the FO group compared to the WD group.

Interestingly, PL added to the diet increased the incorporation of the total omega-3 fatty acids measured in the brain (17.0%±8.0%) and heart (13.4% ±6.6%) compared to the other diet groups ($p<0.05$). For the distribution of omega-3 fatty acid in tissue PL, I found a significant increase in the distribution of total omega-3 fatty acid in the brain (9.7% ±4.1%) and retina (9.9 %±3.1%) in the FOPL group compared to FO group. Surprisingly, FOPL had a significantly higher serum concentration of tumor necrosis factor (TNFα) (0.098±0.067 ng/ml) compared to the FO group, and there was no significant effect observed for serum concentration of interleukin (IL-6) ($p<0.05$) across the dietary groups. It could be concluded that dietary FO and FOPC had a negligible effect on serum pro-inflammatory cytokines levels. However, adding phospholipid to the diet with fish oil improved the bioavailability of omega-3 fatty acids in most tissues.

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## TABLE OF CONTENTS

**List Of Tables**

**List of Figures**

**List of Abbreviations**

| | |
|---|---|
| 18-HEPE | 18-hydroxyeicosapentaenoic acid |
| ALA | Alpha-linolenic acid |
| Apo | Apolipoproteins |
| ARA | Arachidonic acid |
| COX-1 | Cyclooxygenases-1 |
| COX-2 | Cyclooxygenases-2 |
| cPLA2 | Calcium-dependent cytosolic phospholipase |
| CPT | Carnitine palmitoyl transferase I |
| CRP | C-reactive protein |
| CVD | Cardiovascular diseases |
| DGAT | Diacylglycerol acyltransferase |
| DHA | Docosahexaenoic acid |
| EE | Ethyl ester |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| FABP | Fatty acid-binding protein |
| FAS1 | Fatty acid synthetase 1 |
| FFA | Free fatty acid |
| FO | Fish oil |
| FOPC | Fish oil and phosphatidylcholine |
| GMP | Cyclic guanosine 3′,5′-monophosphate |
| HDL | High-density lipoprotein |
| HP | Horseradish peroxidase |
| IL | Interleukin |
| iPLA2 | Calcium-independent phospholipase A2 |
| LA | Linoleic acid |
| LCPUFA | Long-chain- polyunsaturated fatty acid |
| LDL-R | Low-density lipoprotein receptor |
| LPCAT | Lysophosphatidylcholine acyltransferase |
| LPL | Lipoprotein lipase |
| MFSD2A | Multi-facilitated super-family domain 2A |
| MTTP | Microsomal triglyceride transport protein |
| MUFA | Monounsaturated fatty acid |
| NA | Nervonic acid |
| NEFA | Non-esterified fatty acid |
| PKG | Protein kinase G |
| PL | Phospholipid |
| PPARγ | Peroxisome proliferator-activated receptor gamma |
| PUFA | Polyunsaturated fatty acid |
| SDA | Stearidonic acid |
| SFA | Saturated fatty acid |

| | |
|---|---|
| SNP | Single nucleotide polymorphisms |
| TAG | Triacylglycerol |
| THA | Tetracosahexaenoic acid |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| TNFα | Tumor necrosis factor α |
| VLCPUFA | Very long-chain polyunsaturated fatty acid |
| VLDL | Very-low-density lipoprotein |
| W | Western diet |

# CHAPTER ONE: LITERATURE REVIEW

## 1.0 LOW OMEGA-3 FATTY ACIDS INTAKE

The low intake of omega-3 polyunsaturated fatty acids (n-3 fatty acids) in the diet of North Americans has become a nutritional concern, as most individuals fail to meet their daily dietary requirements of n-3 fatty acids (Simopoulos *et al*., 2008). Adequate intakes of plant oils containing alpha-linolenic acid (ALA, 18:3n-3) or one to two servings of fatty fish per week containing eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (22:6n-3) are recommended for an individual to achieve their recommended intake of n-3 fatty acids, but approximately 50% of people living in Canada do not meet this recommendation (Simopoulos *et al*., 2008). The consumption of omega-6 polyunsaturated fatty acids (n-6 fatty acids) in North America is high, and the ratio of n-6/n-3 fatty acids is also high due to the low level of n-3 fatty acid consumption in a typical North American diet (western diet). Therefore, the overall profile of the typical North American diet (Western diet) is usually a high proportion of the PUFA content being n-6 fatty acid, a high n-6/n-3 fatty acid ratio, and a low absolute content and proportion of PUFA coming from n-3 fatty acids. It is speculated that the high intake of n-6 fatty acids and low levels of n-3 fatty acid intake may be linked to the rates of various chronic diseases in North America. One dietary strategy to address the low intakes of n-3 fatty acids is nutritional supplementation with fish oil supplements (Petersen *et al*., 2014).

Since n-3 fatty acids are well known to reduce inflammation, improve visual acuity, and regularise the blood lipid profile, there is a surge in the intake of nutritional supplements enriched with n-3 fatty acids (Akil and Ahmad, 2011). As of 2020, the global n-3 fatty acids supplements market is worth more than $5.6 billion, and North America dominates the market and accounts for 37.7% of the total global revenue (Nevigato *et al.,* 2021). Studies have shown fish oil supplements

alone are not as effective as a diet rich in fish, as fish oil supplement is made up of only 30% n-3 fatty acids (Wang *et al*., 2006), and most supplemental sources are composed of ethyl ester (EE), which is poorly digested and absorbed (Chevalier *et al*., 2021). Also, fish oil supplements are prone to oxidation, as they are chemically unstable and rapidly oxidized into lipid peroxide (Visioli *et al*., 2003). Preservatives and deodorizing agents may cause the oxidation of n-3 fatty acids. Typically, the shelf-life of most fish oil is about 2- months and can rapidly oxidize. However, the oxidative stability of these oils can be improved by using the encapsulation method to conceal the off-flavor and increase oxidative stability (Rahim *et al*., 2022). Taken together, this may limit the beneficial effect of n-3 fatty acids, thereby triggering potential consumers' safety with long-term usage. On the other hand, various components in the fish meal contribute to its effective utilization of n-3 fatty acids. Fish meals are known to increase the bioavailability of n-3 fatty acid ten folds compared to supplemental sources (Visioli *et al*., 2003).

Krill oil is an alternative source of n-3 fatty acids bound to a phospholipid (PL). Phospholipids are known to be effectively absorbed and utilized because of their amphiphilic structure, and krill oil has been reported to be rich in antioxidants like astaxanthin (Cunningham, 2012). However, krill oil is ten times more expensive than fish oil because of the high cost of harvesting and the processing method. There are much cheaper plant-based sources of PL, but unlike krill oil, most contain high levels of n-6 fatty acids. For example, soy lecithin, from soybeans, was first isolated by French chemist Theodore Gobley in 1846 (Thies et al., 1994). It is characterized as a mixture of naturally occurring lipids made up of more than 50% of PL, and phospholipid is an amphiphilic molecule that acts as a good emulsifier that improves the solubility of the fatty acids and further influences the rate of absorption and greater bioavailability (Robert *et al*., 2020). Studies have shown that adding PL increased the bioavailability and accretion of n-3 fatty acids, even when n-

6 fatty acids were present at a higher percentage in the diet (Van Wijk *et al*., 2016; Chen *et al*., 2015). Therefore, this suggests that adding PL to an n-3 fatty acid-rich diet could improve the overall bioavailability of n-3 fatty acids in Western diet consumers.

### 1.1 CLASSIFICATION OF FATTY ACIDS.

Generally, fatty acids are classified into three sub-groups based on the absence or presence (number) of double bonds and the position of these double bonds in the fatty acid chain. The scientific abbreviation describes the number of carbon atoms, the number of double bonds, and the type of PUFA in the chemical structure. For example, in DHA (22:6n-3), the number 22 denotes that the fatty acid has 22 carbon atoms, 6n indicates six double bonds, and 3 represents an n-3 fatty acid. Saturated fatty acid (SFA) predominantly has a single bond in their carbon chain, e.g., palmitic acid (16:0), lauric acid (12:0), and stearic acid (18:0). Examples of foods that are high in SFA are lard, butter, whole milk, red meat, and chocolate. Studies have shown that excessive consumption of SFA can lead to an increased risk of dyslipidemia and coronary heart disease. Monounsaturated fatty acids (MUFA) have a single, double bond near the center of the fatty acyl chain (e.g., palmitoleic acid (16: 1n-7) and oleic acid (18:1n-9). Examples of foods high in MUFA include nuts, avocados, seeds, and canola oils (Burdge *et al*., 2002). Polyunsaturated fatty acids (PUFA) have two or more double bonds, throughout the acyl chain (e.g., alpha-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6)). In n- 3 fatty acids, there is the presence of a double bond between the third and fourth carbon atoms counting from the terminal methyl group of the carbon chain (Suito *et al*., 2018). Omega 3 fatty acids (e.g. DHA, EPA, and DPA) are metabolically related and synthesized from their dietary precursor ALA (Shahidi and Ambigaipalan, 2018).

Most vegetable oil or plant-based oils, like soy, canola, flax, and sunflower oil, are high in

PUFA (Suito *et al*., 2018). Peanuts, corn, and soybeans are also high in PUFA, and they are commonly referred to as the specific crop they originate from or as vegetable oils. The levels of n-6 or n-3 in oils are not always clearly labelled on the product packaging. They are also classified based on their degree of saturation, ability to synthesize them, and chain length. Fatty acids are mainly used as energy for the muscles, heart, and other organs and as building blocks for cell membranes. Fatty acids not used as energy are converted into triglycerides and stored in adipose tissue, while some fatty acids are metabolized into cell signalling molecules or other compounds (Burdge *et al*., 2002).. Both ALA and LA are essential long-chain polyunsaturated fatty acids (LCPUFA) because the human body and higher animals cannot synthesize them due to a lack of the delta (Δ) 12 and Δ15 desaturase enzymes. These desaturases insert a *cis* double bond at the n-6 or n-3 position of fatty acid; therefore, both ALA and LA are essential and should be obtained from the diet (Covington, 2004).

**1.2. SOURCES OF OMEGA-3 FATTY ACIDS.**

Most seafood and fatty fish such as salmon, tuna, and mackerel are rich sources of DHA and EPA, while seeds and nuts such as flaxseed, green leafy vegetables, and walnuts are rich in ALA (Rizos *et al*.,2012). Human breast milk, most synthetic infant formulas, supplements like cod liver oil, fish oil, and concentrated pharmaceutical- preparations (mostly in ethyl ester), such as Epanova® and Lovaza®, contain significant DHA and EPA (Harris et al.,2007). Wild marine fish contain higher n-3 fatty acids than farmed fishes (cultivated) because they feed on the phytoplankton that consumes the microalgae, accumulating n-3 fatty acids in their tissues.

In contrast, cultivated fish generally consume feeds made of cereal and vegetable oil richer in n-6 fatty acids than n-3 fatty acids (Saini and Keum, 2018). Farmed fish are sometimes portrayed

negatively and are considered to be exposed to environmental pollution, having a higher rate of contamination, and lower nutritional value (high in SFA). However, mercury (Hg), which has numerous health-deteriorating effects, primarily on children and pregnant women, is highly accumulated in most top-level marine predators, e.g., whales, sharks, and tuna. Bioaccumulation of Hg in fish is a significant concern, Annibaldi and colleagues (2019) reported that farmed tuna has a health benefit value compared to wild fish due to a higher Hg content in wild tuna (1.7 ± 0.6 mg/kg) compared to farmed tuna (0.6 ± 0.2 mg/kg) (Annibaldi et al., 2019). Microalgae (e.g., *Crypthecodinium cohnii* and *Schizochytrium* spp) and other microorganisms are the two significant sources of n-3 fatty acids in the marine food chain, containing high levels of DHA (40% -55% of total lipid) (Janaka Senanayake & Fichtali 2006). Hence, consuming fish and other seafood from marine sources enriched with n-3 fatty acids is an important source of n-3 fatty acids in human diets. Nevertheless, due to the purported health benefits of n-3 fatty acids, some plants, like the flax and *Brassica* species, are now genetically modified to incorporate genes that allow EPA and DHA to be produced by the plants (Shahidi & Miraliakbari 2004). Similarly, eggs can be enriched with n-3 fatty acids by feeding poultry birds diets rich in n-3 fatty acids (most notably ALA).

### 1.3 METABOLISM OF OMEGA-3 FATTY ACIDS

The conversion of essential fatty acid LA to ALA is impossible in humans, but this process occurs in plants. Although LA and ALA share the same pathway, they are not interconvertible, and their physiological and metabolic functions are distinct. After digestion, the forms in which very long chain PUFA exist in the plasma pool determine their absorption rate, metabolic fate, and functionality (Hamilton *et al*., 2007). Like other fatty acids, LCPUFA is transported into the bloodstream as components of chylomicrons via the lymphatic system and eventually esterified into triacylglycerol (TAG), phospholipids (PL), and cholesteryl esters (CE). Unlike SFA and

MUFA, n-3 PUFA do not tend to be found in adipose at high levels rather, DHA is found in high concentration in the rod segment of the retina, the grey matter of the brain, while EPA is present at a high level in the heart. Several plasma pools of fatty acids have been identified, including nonesterified fatty acid (NEFA), TAG, PL, and a minimal amount of ethyl ester (EE) in the plasma (if consumed as a supplement) (Chouinard-Watkins et al., 2015). However, EE has low intestinal absorption due to the impaired intestinal hydrolysis and re-esterification process (Hamilton *et al*., 2007; Mitchell *et al*., 2011). The synthesis rate of EPA from ALA is low in humans; the conversion process occurs in a two-step reaction that is catalyzed by delta-6 desaturase, elongase-5, and delta-5 desaturase, which takes place in the endoplasmic reticulum (Pender-Cudlip *et al*., 2013).

On the other hand, the conversion rate of ALA to DHA is much lower, which involves complex reaction steps (Figure 1). First, two carbon atoms are added to EPA by ELOVL fatty acid elongase 5 to yield DPA (Covington, 2004). DPA further undergoes a second chain elongation reaction, giving rise to tetracosahexaenoic acid (THA) 24:6n-3. Finally, via oxidation and desaturation by the enzyme delta-6-desaturase, the 24-carbon chain is then reduced to DHA via the removal of 2 carbon atoms, which occurs in the peroxisome (Covington, 2004).

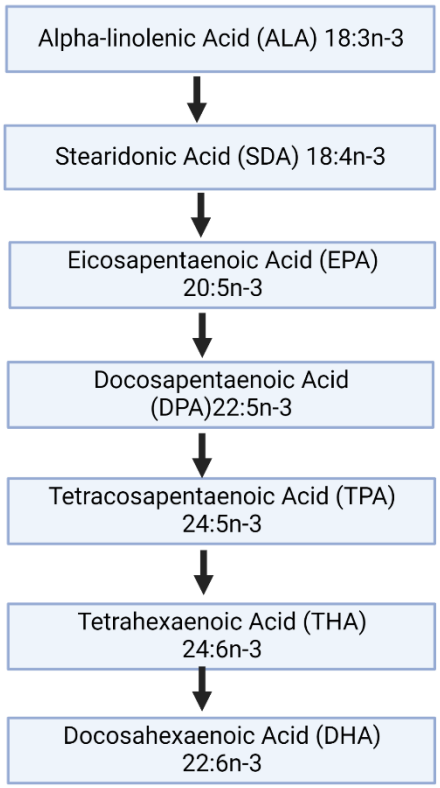


**Figure 1.** Metabolism of omega-3 fatty acids.

The conversion rate from ALA to EPA is approximately 5-8 % and to DHA 0-4% in men (Burdge et al., 2002). In women, the conversion rate from ALA to EPA is 20-22%, and about 7-9% is converted to DHA (Burdge *et al*., 2002). The higher conversion rate in women is due to hormonal differences; women need more n-3 fatty acids during pregnancy and lactation (Burdge *et al*., 2002). However, some factors also influence the conversion rate, including age, genetics, and disease. Additionally, the dietary precursor of n-6 fatty acids GLA and LA acids compete with ALA for the same enzymatic pathway. The enzyme gives preference to n -3 fatty acids first (i.e., it is the first preferred substrate), followed by n-6 fatty acids. However, in the Western diet, when there is a high level of LA compared to ALA, the pathway favors the conversion of LA to Arachidonic acid (ARA) and various biological derivatives. Thus, the conversion rate of ALA to

EPA and DHA will be limited. Intake of SDA has been shown to increase the level of EPA and DHA because the conversion rate is low with ALA (Covington, 2004).

Further, the conversion rate from ALA to EPA is about 6%, while from SDA to EPA is approximately 14-20% (Lefort *et al.* 2016). Therefore, high consumption of SDA increases the plasma and tissue levels of EPA and DHA more efficiently than ALA (Kuhnt et al. 2014). SDA is present in ahiflower, echium oil, blackcurrant oil, soybean, hemp seed oil, among others.

Hence, direct consumption of SDA may provide an alternative source to meet the dietary recommendation level of health promoting LCPUFA, mainly EPA. When SDA was supplemented, it was shown that there was no increase in the SDA status, but the level of EPA was increased, which indicates there is a reasonable conversion rate (Pender-Cudlip *et al*. 2013).

VLCPUFA undergo enzymatic and non-enzymatic conversion into their bioactive mediators. The non-enzymatic conversion with oxygen free radicals (ROS) produces 4hydoxyhexenal (4-HHE), an aldehyde derivative that forms an adduct with DNA, proteins, and lipids, resulting in several health-deteriorating effects (Juan *et al*., 2021). When VLCPUFA is required to carry out biological functions in the cell membranes, they are first released from the membrane phospholipid by calcium-dependent cytosolic phospholipase A2 (cPLA2, E.C 3.1.1.4) and further hydrolyzed into bioactive eicosanoid mediators. ARA and EPA are parent compounds in generating pro-inflammatory eicosanoids that lead to inflammation, vasoconstriction, and platelet aggregation. However, the production of pro-inflammatory eicosanoids from EPA is minimal compared to ARA because EPA also participates as an anti-inflammatory mediator. The enzymes necessary to convert ARA to prostaglandins, leukotriene, epoxyeicosatrienoic acids, thromboxanes, and lipoxins are cyclooxygenases-1 (COX-1, EC 1.14. 99.1), 5-lipoxygenases (5LOX, EC 1.13.11.34), cytochrome P450 (CYP) oxygenase, cyclooxygenases-2 (COX-2, EC 1.14.99.1), and

lipoxygenases (15-LOX, EC 1.13.11.34), respectively. On the other hand, EPA is also converted to pro-inflammatory eicosanoids such as prostaglandins, leukotriene, and thromboxanes by COX-2 as well as synthesized by 5-LOX to resolvins (Alashmali *et al*., 2016). The latter involves anti-inflammatory responses, vasodilatation, and anti-aggregation (Saini and Keum, 2018).

The release and metabolism of DHA from the cell membrane are regulated by DHA selective phospholipase, called calcium-independent phospholipase A2 (iPLA2). Once it is released, it is metabolized by two different enzymes, 12 and 15-lipoxygenase (or in the presence of aspirin by acetylated COX-2), to its natural derivatives. (Alashmali *et al*.,2016). However, as stated earlier, n-3 and n-6 fatty acids compete for the synthesis of eicosanoids; when there is a high intake of n-3 fatty acids, it favors the synthesis or production of anti-inflammatory mediators. When there is a high intake of n-6 fatty acids, as occurs in the Western diet, the synthesis of anti-inflammatory mediators from n-3 fatty acids is inhibited. Thus, the production of proinflammatory eicosanoids is also inhibited. Studies have shown that a lower level of dietary n-6 fatty acids in rats increases the expression of DHA-metabolizing enzymes (iPLA2 and 15lipoxygenase), which are accompanied by a decrease in the expression of ARA-metabolizing enzymes (cPLA2 and COX-2) (Alashmali *et al*., 2016).

A balanced ratio of n-6/n-3 fatty acids in the diet should be maintained for an individual's healthy growth and development, leading to improved cardiovascular, neurological health, and visual acuity (Bazinet and Laye, 2014). The ratio of n-6/n-3 fatty acid is a major contributing factor to an individual's inflammatory and chronic disease status. The estimated dietary ratio of n-6/n-3 fatty acids for health benefits is 1:1–2:1 (Simopolous, 2002). But recently, studies have shown that the ratio of n-6/n-3 fatty acids has increased from (ancestral diet) 1:1 to 25:1 in the diets of many developed and developing countries due to modern agribusiness and the widespread use of soybean

oil in processed foods (Simopoulos *et al*., 2016). A study by Weber and colleagues (2007) reported that when the ratio of n-6/n-3 increases in platelets, the death rate from cardiovascular diseases and type 2 diabetes increases. The ratio of n-6/n-3 fatty acids is also essential for pregnant women, newborns, and infants for optimal brain and retina growth and development. A 300mg/ day intake of n-3 fatty (DHA) acids is recommended for pregnant women and breastfeeding mothers (Simopoulos, 2002). The high n-6/n-3fatty acids ratio has also been associated with an increased prevalence of overweight and obesity. In mature adipocytes, the lipogenic gene regulation is highly dominated by ARA, which may initiate chronic inflammatory reactions. This reaction may be due to the low accumulation of n-3 fatty acids. Thus, increasing n-3 fatty acids will promote adipocyte fat metabolism (Serhan and Levy, 2018). Aside from the level of n-3 and n-6 fatty acids, gene alterations of enzymes involved in metabolism can also influence the occurrence of chronic diseases. Single nucleotide polymorphisms (SNP) of fatty acid synthetase 1 (FAS1), FAD2, 5LOX, and COX-2 can impact plasma, serum, and membrane phospholipid concentrations of PUFA mostly during pregnancy and lactation (Simopoulos, 2002). These influence an infant's neuronal development; when n-3 fatty acids intake is low, it has been documented that the SNPs at 5-LOX and COX-2 increase the risks of cardiovascular diseases and prostate carcinoma, respectively.

## 1.4 DIGESTION AND ABSORPTION OF DIETARY FAT

Lipids are hydrophobic compounds that cannot dissolve in water. The fat in food comprises triglycerides, phospholipids, and cholesterol esters, the majority being triglycerides. Lipids are a source of energy and a structural component of the cell membrane. Triglycerides consist of three fatty acids esterified to a glycerol molecule, primarily long-chain fatty acids (16-22 carbon atoms) at the sn-1, sn-2, and sn-3 positions of the glycerol backbone. It has been reported that LCPUFA tends to be preferentially located in the sn-2 (Lee Chang et al., 2021). Phospholipid has a similar

structure as TAG but with the difference being two fatty acids at the sn-1 and sn-2 positions are esterified to the glycerol molecule and phosphate group when esterified to an organic molecule at the sn-3 position. The phosphate group is attached to a hydrophilic group, such as choline, serine, or inositol, via an ester bond. The positional distributions of the FA in the TAG and PL molecules significantly affect their physiological functions (Linderborg and Kallio *et al*., 2005).

### 1.4.1 DIGESTION OF TRIGLYCERIDES

Several essential steps of lipid digestion and absorption occur within the small intestine; however, the metabolism of lipids begins in the mouth by the enzyme produced and secreted by serous lingual glands called lingual lipase (E.C 3.1.1.3). Minimal amounts of fat are broken down by lingual lipase because it has a slower enzyme activity than other enzymes involved in lipid digestion (Scorletti *et al*., 2013). Lingual lipase is not particularly important for healthy adults but is essential for infants whose pancreatic lipase is still not matured (Valentini, 2018).

After swallowing, muscle contractions called peristalsis move the lipids through the esophagus and into the stomach. In the stomach, lipids are further hydrolyzed by a second acid lipase, called the gastric lipase, released from the chief cells of the gastric mucosa in response to the gastrointestinal hormone cholecystokinin (Valentini, 2018). About 30% of TAG are preferentially hydrolyzed by gastric lipase at the sn-1/sn-3 position of TAG, generating mainly fatty acids and 1, 2-diacylglycerides. The action of the gastric lipase results in forming an emulsion containing small lipid particles (micelles) 0.5 nm in diameter that can then be delivered from the stomach into the first part of the small intestine (duodenum). There is partial TAG hydrolysis in the stomach. It should be noted that the rate of hydrolysis at the sn-2 position remains intact as 2-MAG during digestion and absorption (Kohan *et al*., 2011).

In the duodenum, the hydrolysis of TAG continues, where the chyme is mixed with bile. Bile is secreted by the liver cells, stored in the gallbladder, and released into the duodenum when stimulated by cholecystokinin. It is released in response to the ingestion of a meal, particularly if it is high in fat (Clement, 1976). Therefore, the mechanism of the emulsifying agent ensures the formation of micelles, which also increases the available surface areas for pancreatic enzyme activities (Sung *et al*., 2017). Short-chain and medium-chain fatty acids can be hydrolyzed and absorbed without bile salts; however, their presence increases the absorption of these fatty acids.

The pancreatic lipase catalyzes the digestion of most ingested TAG, mainly in the jejunum's upper part, and the pH ranges from 7.0 - 8.8. Pancreatic lipase is irreversibly inactive at acidic pH (less than pH 4.0). Bicarbonate in pancreatic secretions then neutralizes the acid effect initiated by the lingual and gastric lipase to preserve the pancreatic enzyme activity (Valentini, 2018).). The lipid droplets are coated on their surfaces with bile salts, phospholipids, and other compounds that are negatively charged and, thus, inhibit the binding of pancreatic lipase. To achieve a productive pancreatic lipase activity on the oil droplet surface, a protein co-factor is secreted by a pancreatic fluid called a colipase that binds on the oil droplet (lipase and colipase bind in a 1:1 molar ratio) (Kohan *et al.,* 2011). Pancreatic lipase preferentially hydrolyzes FAs in the sn-1 and sn-3 position of the TAG, forming free fatty acids (FFA) and 2-monoacylglycerol.

### 1.4.2 DIGESTION OF PHOSPHOLIPIDS

Similar to the process described for TAG, PL are digested by phospholipase A1 by cleaving the sn-1 acyl chain in the glycerol backbone and phospholipase A2, which cleaves the sn-2 acyl chain in the glycerol backbone. When secreted by the pancreas, the phospholipases are zymogens which need to be activated by trypsin (Kohan *et al.,* 2011). As mentioned earlier, like pancreatic lipase, phospholipase A1 and A2 cleave the fatty acid at the sn-1 and sn-2 positions of the

phospholipids, respectively. Phospholipids form micelles with cholesterol and bile salts; in the intestinal lumen, they are distributed among the lipid droplets and these micelles. In the micelles, phospholipids are hydrolyzed by phospholipase A2 to free fatty acid (FFA) and lysophosphatidylcholine (a lysophospholipid-a PL that has one FA removed) (Martin *et al*., 1993).

## 1.5 ABSORPTION OF LIPIDS

Bile salts are essential for effectively absorbing TAG and PL hydrolytic products. At concentrations of l–5 mmol/L, bile salts form micelles, TAG and PL are incorporated between the bile salts and the inner core of these structures, thereby forming mixed micelles, and this initiates the emulsification of fatty acids, monoglycerides, and other lipids in the aqueous environment of the luminal contents (Scorletti *et al*., 2013). The mixed micelles are more water-soluble, allowing them to cross the unstirred water layer to the brush border of enterocytes for absorption.

Enterocytes, or intestinal absorptive cells, have micro-villi that increase the surface area and facilitate the transport of lipids into the enterocyte from the intestinal lumen. Specific binding proteins carry FFA, MAG, and LPC to the intracellular site, where they are used for TAG biosynthesis. The two major fatty acid-binding proteins (FABP) found in the enterocytes are the liver FABP and intestinal FABP. The TAG and PL are resynthesized within the smooth endoplasmic reticulum (SER) (Mitchell *et al*., 2011). The process to resynthesize (reassemble) TAG within the enterocyte proceeds by one of two pathways: the sn-2 MAG pathway (majority) and glycerol-3-phosphate pathway (minor pathway) followed by chylomicrons delivering the dietary lipids into the circulation (Khatun et al., 2016). Newly synthesized TAG and PL were transverse to the SER membrane bound to microsomal TAG transport protein (MTTP), which transports the lipids to the Golgi apparatus. Nascent chylomicron particles leave the rough endoplasmic reticulum (RER) and are transported to the Golgi, and the lipids are packaged into

CM (Martin et al., 1993). The CM leaves the basolateral membrane of the enterocyte via exocytosis and enters the lymphatic capillaries (the lacteals). Short-chain fatty acids (such as butyric acid) with relatively small sizes are absorbed by simple diffusion into the enterocytes, delivered into the blood, and is transported in the circulation bound to the protein albumin. LCPUFA (i.e. DHA, EPA) are too large to fit through the pores in the blood capillaries; however, they can go through the larger fenestrations in the lacteal (Dixon, 2010). In the lymphatic system, the lymph (a fluid that flows through the lymphatic system) travels through various segments of the lymphatic vessels and lymphatic ducts. The particular one it gets to is the thoracic duct, located at the left jugular vein, where it meets with the left subclavian vein (where the CM is delivered into the systemic circulation).

Chylomicrons, now in the bloodstream, enter the heart via the vena cava, pumped out of the heart via the aorta to the general circulation (Dixon, 2010). After CM has distributed the TG to the adipose tissue and muscle, it is removed from circulation as a CM remnant particle, which requires apolipoproteins (Apo) (Apo E) to be cleared by the liver. CM remnants are proatherogenic because they are more enriched with cholesterol than CM (Dixon, 2010). The CM (phospholipids, cholesterol, and triglycerides) are catalyzed by lipoprotein lipase (LPL). Various lipoproteins like the very-low-density lipoprotein (VLDL) produced in the liver acts as a means of transporting lipids (cholesterol, cholesterol esters, PL, and TG) from the liver to peripheral tissues (adipose tissue and muscle). Apolipoproteins E stimulates the low-density lipoprotein receptor (LDL-R), and LPL binds to Apo C-II. Once the TAG content is low enough, and the VLDL has lost several Apo C-II, it is called the VLDL remnant, commonly known as intermediate-density lipoprotein (IDL) enriched with cholesterol and is known to be pro-atherogenic. The IDL also goes through a similar mechanism as VLDL after depositing TAG to various organs and tissues.

In circulation, the half-life of CM is about 30 minutes (Sugasini et al., 2017). On the other hand, DHA absorbed as PL is incorporated rapidly into HDL either through enzyme activity or via coupling in the mucosal cells, which has a longer half-life of about 12-24 hours (Sugasini *et al*., 2017). TAG-DHA is a common molecular carrier of DHA, frequently used as an ingredient in infant formula. However, researchers are now focusing on DHA esterified to LPC because of LPC antioxidant properties, emulsifying properties, importance in CM formation, increased bioavailability of EPA and DHA, and LPCs are suitable for use in infant formulas as well as other consumers (Sugasini *et al*., 2017).

The bioavailability of dietary lipids, in simple terms, can be explained as the amount of lipids consumed that reaches the systemic circulation or site of physiological activity. Bioavailability depends on the molecular carrier or chemical binding form. It has been reported that PL is more bio-available than the other molecular carrier esterified to DHA (Kohan *et al*., 2011).

## 1.6 REGIO-ISOMERIZATION OF OMEGA-3 FATTY ACID

Regiospecificity can be described as when a single fatty acid is attached to the sn- position of the glycerol backbone of TAG and PL (Ruiz-Lopez *et al*., 2015). After ingestion of n-3 fatty acids, several factors influence their nutritive value and metabolic fate that will enable them to elicit their physiological and biological function, such as the positional distribution of the fatty acid (regiospecificity), degree of saturation, and chain length (Amate, Gil, and Ramírez 2002). Fatty acid esterified in the sn-2 position is retained in its native form after absorption, reesterification, and incorporation into CM. Further, it is also more stable for oxidation and thermal degradation (Wang and Shahidi, 2011). However, fatty acids esterified to sn-1 and sn-3 positions are released by the action of sn-1 and 3 specific pancreatic lipase, which is later re-esterified back

to TAG in the enterocyte in a study by Zhang *et al*. (2018), it was shown that EPA was randomly distributed among the sn-1 position of the glycerol backbone. At the same time, DHA was attached preferentially at sn-2 in the TAG molecule (Zhang *et al*. 2018), which agreed with the findings of other researchers. He *et al*. (2016) also found that about 62% of EPA was attached to sn-1 and sn-3 positions, whereas approximately 71.3% of DHA was attached to sn-2 after re-esterification (Zhang *et al*. 2018). When DHA is linked to the sn-2 of TAG, it exhibits a serum triglyceride lowering effect. This may explain why cold-water fatty fish numerous therapeutic benefits have because DHA is preferentially esterified to the sn-2 position. The analysis of positional distribution in TAG and PL can be carried out by enzymatic hydrolysis, chemical, nuclear magnetic resonance (NMR) spectroscopic, and gas chromatography (Ruiz-Lopez *et al*., 2015). Therefore, when a diet is low in DHA and EPA and high in LA and ARA, there will be a higher level of LA attached to sn-2, which would be absorbed appropriately, incorporated into chylomicrons, and distributed into various tissues and may lead to undesirable health conditions, e.g., decreasing the antioxidants and immune system. As earlier described (structure of PL), when DHA is esterified in the sn-1 position, it may be good compared to when it is esterified to sn-2 because DHA in the sn-1 would escape the enzymatic hydrolysis of PLA2. Therefore, it will be retained in the PL during the incorporation into the lipoprotein as sn-1 LPC (Sugasini *et al*., 2017).

## 1.7 FACTORS THAT AFFECT THE BIOAVAILABILITY OF N-3 FATTY ACIDS.

With the numerous health benefits associated with the intake of n-3 fatty acids, consumers are increasingly interested in knowing which molecular carrier of n-3 fatty acids is more bioavailable to subsequently perform their biological functions; first, they must be bioavailable in the target site. However, several factors influence the bioavailability of n-3 fatty acids.

1. **Molecular carrier/ chemical binding form:** The most critical factor is the molecular carrier, and there are various molecular carriers of FAs, which include TAG, phospholipid (PL), and EE. The EE form is mostly present in drug formulation, despite EE having a poor rate of absorption because of a low level of intestinal hydrolysis. It has been reported that PL is more bio-available than the other molecular carrier esterified to DHA (Kohan *et al*., 2011). Lysophosphatidylcholine (LPC)-DHA increases the brain DHA more effectively than TAG-DHA and non-esterified (NE)-DHA because the NE-DHA is incorporated into CM, but LPC-DHA is absorbed as PC in HDL and CM. Therefore, LPC-DHA is easily transported to the brain compared to TAG-DHA, and the brain preferentially takes up DHA as LPC (Sugasini *et al*., 2017). However, it is due to a specific transporter called multifacilitated super-family domain 2 (MFSD2A) in the blood-brain barrier that transports LPC-DHA (Ben-Zvi *et al*., 2014). Therefore, the increase (bioavailability) of DHA in the brain explains the marked increase in spatial learning and memory in mice (Guemez-Gamboa *et al*., 2015).
2. **Positional distribution/Regio-isomer:** This influences the functionality, rate of lipid absorption, metabolic fate, and oxidative stability of n-3 fatty acids (Subbaiah *et al*., 2016). In TAG and PL structures, the fatty acids can be esterified in any of the glycerol backbone's stereo-numbering positions; they could be esterified to sn-1, sn-2, and sn-3. When n-3 fatty acids (e.g. DHA), are esterified in the sn-2 position in TAG, it may be advantageous compared to when it is esterified to sn-1/sn-3 because DHA in the sn-2 would escape the enzymatic hydrolysis of specific 1,3-pancreatic lipase. Therefore, it will serve as a native structure for the re-synthesis of TAG and be retained during the incorporation into the

lipoprotein as sn-1 LPC (Sugasini *et al*., 2017). Fatty acids in sn-2 are more easily absorbed, thus enhancing their bioavailability (Guemez-Gamboa *et al*., 2015).

3. **Presence of another micro/macro-nutrient**: When n-3 PUFAs are ingested with other nutrients, it may affect the rate of absorption and incorporation into target tissues. Studies have revealed that in the presence of high calcium, some FFA liberated during digestion forms insoluble soap calcium complex, likely leading to increased stool hardness and fecal lipid loss, resulting in increased fat malabsorption (Graham and Sackman., 1983).
4. **The health of an individual:** Numerous pieces of evidence has shown that an individual's health also affects the rate of digestion and absorption of fat and nutrients. In a randomized cross-over trial study, pre-emulsification increased the absorption of PUFAs in healthy participants compared to unhealthy participants with hypercholesterolemia (Garaiova *et al.,* 2007).
5. **Enzyme/substrate specificity:** Added phospholipid (i.e., (PC)) could increase the systemic availability of dietary n-3 fatty acids as they are known to be resistant to the enzymatic hydrolysis of pancreatic lipase. Additionally, TAG is highly hydrophobic, which affects absorbability. At the same time, PL is amphiphilic and possesses self-emulsifying features, decreasing the fatty acid's surface tension so it can be easily digested and absorbed (Pichot *et al*., 2013). Thus, it can increase their absorption rate and tissue accretion due to the interfacial-surfactant properties of PL when added to other forms of oil treatment (TAG, EE). Also, PL is a source of active compounds, such as choline, released from phosphatidylcholine.

   The enzyme monoacylglycerol acyltransferase (MGAT) catalyzes diacylglycerol (DAG) synthesis, a TAG synthesis precursor that plays a significant role in the absorption of dietary fat. Studies have shown its preferential selectivity for monounsaturated acyl species

over polyunsaturated fatty acid (Waters *et al*., 2002). On the other hand, diacylglycerol acyltransferase (DGAT) catalyzes the last and committed step of TAG biosynthesis and has two isomers: DGAT1 and DGAT2. DGAT1 prefers to transfer C16 and C18 saturated/monounsaturated fatty acids, and DGAT 2 preferential substrates are PUFAs, mainly n-6 PUFAs (e.g., linoleic acid), for TAG assembly (Waters *et al.,* 2002; Zammit *et al*., 2008). In contrast, lysophosphatidylcholine acyltransferase (LPCAT) prefers to add PUFAs, mostly EPA and DHA, to LysoPC sn-2 position (Zhao *et al*., 2008). Therefore, it would be interesting to evaluate the potential of supplementary PL with fish oil (TAG) to improve the uptake and distribution of n-3 fatty acids in animals.

## 1.8 OMEGA-3 FATTY ACIDS AND HEALTH BENEFITS

### 1.8.1 CARDIOVASCULAR DISEASES AND N-3 FATTY ACIDS

Cardiovascular diseases (CVD), also called heart and blood vessel diseases, are life-threatening. It has been postulated that n-3 fatty acids reduce the risk of most cardiovascular-related conditions, such as abnormal heart rhythms, myocardial infarction, cardiomyopathy, sudden cardiac death, hypertension, coronary heart disease, atrial fibrillation, and mortality from heart failure, which is now occurring in an increased rate. (Lavie *et al*., 2009). 31% of global death is linked to CVD, and 85% of CVD is mainly associated with a heart attack and stroke (WHO, 2017).

A comprehensive review study showed the anti-arrhythmic effect of increased intakes of DHA (Tribulova *et al*., 2017). Arrhythmia is when the heartbeat is irregular, either too fast or too slow. DHA is known to mitigate this condition by a rapid inhibition mechanism involving voltage dependent sodium channel, L-type calcium channel, and potassium channel ($K^+$) (Anand et al., 2008). $K^+$ depolarizes both the ventricular and atrial cardiac potentials, which release calcium and

causes the heart to contract. Therefore, the repolarization of atrial and ventricular tissues induced by DHA (by pushing the membrane action potential to become negative) provides a protective effect against dysrhythmias (Yasuma *et al*., 2004). Also, a high intake of omega-3 fatty acids by a higher intake of fish and seafood reduced the incidence of heart failure (HF) in the study reported on 60,000 Japanese women (Seikikawa *et al*., 2007). The result showed an inverse relationship between omega-3 fatty acids, marine fats, and CV mortality. Geelen et al. 2005 showed a decrease in the average heart rate of ventricular arrhythmias patients given a moderate dose of omega-3 fatty acids (1,260 mg/day EPA and DHA).

Furthermore, a combination of n-3 fatty acids and statin has been shown to be safe and effective in correcting high blood TAG and cholesterol profile (Yokoyama *et al*., 2007). Yokoyama and colleagues (2007) demonstrated that EPA (1,800mg/day) plus statin administered in a Japanese EPA lipid intervention study (18,645 hypercholesterolemia patients) reduced cardiovascular diseases compared to control by 19%. This agreed with Christensen and colleague's (1996) work; after 4.3g/day of EPA and DHA administration for 12 weeks, patients with post-MI and impaired systolic function showed an improved heart rate. Fish oil supplementation increased the level of omega-3 fatty acids in the ventricular membrane significantly compared to saturated fat, which led to gap junction (myocardial structure) remodeling, an increase in capillary density, and alkaline phosphatase activity both in spontaneously hypertensive rats (SHR) male and female rat (Mitasikova *et al*.,2008). The cellular membrane function, as well as membrane fluidity, was greatly improved by n-3 fatty acids.

The National Cardiac Societies and American Heart Association recommend the daily intake of 1 g/kg of both EPA and DHA in the capsule or oil form for the treatment of secondary prevention of cardiovascular events (Baker *et al*., 2016). The American Food and Drug

Administration recommends 4.0g/d of n-3 fatty acids in ethyl ester form to prevent and treat high triglyceride levels. However, findings from some researchers suggest that at least 1-2 servings of oily fish per week may mitigate the occurrence of cardiovascular disorders. Supplementing 4.0g/d of n-3 fatty acids decreases the blood pressure of both old and young people with high blood pressure (Geleijnse *et al*., 2004). The major constraints of meeting the daily dietary recommendation are the intake of food and oil high in pro-inflammatory n-6 fatty acids in combination with little or no n-3 fatty acids, dietary preference, sustainable supply of marine sources, seafood pollution (mercury and polychlorinated biphenyls), socio-economic status and geographic location (Baker *et al*., 2016). The dosage and duration of treatment are significant as they elucidate the clinical effectiveness or relevance of n3 fatty acids, as a low dosage of n-3 fatty acids also has a beneficial effect (Innes, 2020). In one month study, 3.4g of n-3 fatty acids was administered to a patient with hypertriglyceridemia (TAG>500 mg/dL), and there was a 45% reduction in TAG levels (Harris *et al*., 1997).

A case-control study in Seattle showed that persons with 6.5% n- 3 fatty acids in their red blood cell membrane have a lowered risk for sudden cardiac death (SCD) by 90% compared with persons with 3.3% n-3 fatty acids. Therefore, a high level of n-3 fatty acids may prevent cardiovascular diseases (Siscovick *et al*., 1995). Likewise, people consuming high n-3 fatty acids had a lower heart rate, lower incidence of atrial fibrillation, and the risk of stroke declined in women (Mozaffarian, 2005). EPA and DHA stabilize plaques in the coronary circulation compared to ARA, which initiates inflammatory reactions, and causes oxidation of LDL, which later leads to atherosclerosis. Additionally, the histological assessment of plaque morphology and the presence of macrophages was performed on 188 patients (randomized control trial) and showed no inflammation when given EPA and DHA (Thies *et al*., 1994). Their TAG levels were

significantly reduced compared to those given sunflower oil (high in n-6 fatty acid) and control (palm and soybean oil) (Thies *et al*., 1994). However, n-6 fatty acids did not significantly affect carotid plaque and may be correlated with plaque instability.

In a Diet and Reinfarction Trial (DART) study, the participant (n=2033) had 357 mg/day of EPA for 42 days after the first myocardial infarction. It was observed that there was a 29% reduction in total mortality and a 32 % decrease in myocardial reinfarction (Burr et al.,1989). When the intake of n-3 fatty acids is low, the appearance of the tissue lipid and plasma will be significantly low and vice versa. Thies *et al*. (1994) experiment shows that EPA incorporation into plaque lipids has a linear relationship with time. Therefore, high consumption of n-3 fatty acids will increase incorporation into the cell membrane and tissue.

### 1.8.2 DYSLIPIDEMIA AND N-3 FATTY ACIDS

Poor lifestyle, such as dietary habits (high n-6 fatty acids), can lead to a condition known as dyslipidemia. This condition consists of high blood concentrations of TAG, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), or a combination of these abnormalities. It is usually accompanied by a low high-density lipoprotein (HDL). This condition leads to chronic inflammation, contributing to chronic disorders associated with cardiovascular, kidney, and neurological well-being (Peev *et al*., 2015). EPA and DHA are anti-inflammatory, and they mitigate the inflammation caused by dyslipidemia by downregulating the expression of the inflammatory genes through NF-kB inhibition and normalizing the lipid profile via decreasing TAG and LDL and subsequently increasing the level of HDL (Balk *et al*., 2006). Evidence shows that n-3 fatty acids plus Vitamin E (which has anti-inflammatory and antioxidant properties) were able to inhibit lipid peroxidation *in vivo* and *in vitro* by breaking the chain propagation reaction, suppressing atheroma plaque formation, and inhibiting oxidative stress induced by n-6 fatty acids

(Sepidarkish *et al*., 2019). The systematic review and meta-analysis by Sepidarkish *et al*. (2019) found that n-3 fatty acids and Vitamin E decreased the VLDL level. Still, no significant effect was observed in TAG, TC, LDL, and HDL levels. However, his findings did not correlate with Balk et al. systematic review and meta-analysis, which showed that the administration of fish oil alone showed a significant decrease in TG, and an increase in HDL and LDL but no significant effect on TC (Balk *et al*., 2006). The difference in results may be due to the dosage, duration of the studies included, or type of fish oil administered. However, the increase in the LDL level is associated with the buoyant, fast-floating LDL, while the denser and slow-floating LDL decreases (von Schacky, 2006).

Omega-3 fatty acids also decrease the amount of free fatty acid stored in the liver by upregulating beta-oxidation of fatty acid in tissues (e.g. adipose tissue, heart, liver, and skeletal muscle). This process causes fewer fatty acids to be stored in various tissues and organs or released into the general circulation. Omega-3 fatty acids, notably EPA, are known to have a fat and cholesterol-lowering effect and elicit a thermogenic impact in obese patients by increasing energy expenditure through activating brown adipose tissue via uncoupling protein 1 (UCP-1) and also by activating the enzymes involved in fatty acid oxidation (Parmentier *et al*., 2007).

### 1.8.3 STROKE AND OMEGA-3 FATTY ACID

Stroke can also be described when the brain is deprived of oxygen and nutrients. It is fascinating that there is no specific age limit for stroke conditions; even babies and children can have a stroke, but people over age 65 are profoundly affected (Boehme et al., 2017). This condition is prevalent in most low-income countries; however, in Canada, over 405,000 people are affected, and about 14,000 people die from stroke yearly **(**Katan and Luft, 2018). Diabetes, hypertension, and most cardiovascular diseases are the fundamental risk factors for stroke; high body mass,

unhealthy dietary lifestyle, low physical activities, and smoking can also contribute to this condition (Boehme *et al*., 2017). However, changes to dietary lifestyle, engaging in exercise, and reducing smoking are correlated with a decline in stroke incidence. There are different kinds of strokes depending on the part of the brain affected. Fish consumption (1 serving/per month) significantly reduces the occurrence of ischemic stroke. Greater than 2 servings/per week reduces lacunar stroke, but no effect was observed with hemorrhagic stroke (He *et al*., 2012). EPA and DHA metabolites such as protectin, maresins, and resolvin are anti-inflammatory, can regress stroke injuries, and improve neurogenesis in an experimental stroke model (Serhan and Levy, 2018).

The increased concentrations of inflammatory markers interleukin-6 (IL-6, IL-1) tumor necrosis factor α (TNFα) is directly proportional to an increased risk of cardiovascular diseases. IL-6 triggers the production of C-reactive protein (CRP) in the liver (Micallef *et al*., 2009). In a study reported by Ebrahimi et al., 2019, 60 subjects given 1g of fish oil (capsules) containing 180mg EPA and 120 DHA for six months experienced not only a decrease in the CRP level but also a total reduction in body weight, systolic blood pressure, TC, TG, and heat shock protein 27 compared to control subjects (Ebrahimi et al., 2019,). A higher intake of n-3 fatty acids lowers the stroke incidence in women compared to men in a prospective study (de Goede *et al*., 2012), which therefore shows that n-3 consumption may decrease stroke in humans. However, limited scientific data show the protective effect of n-3 fatty acid and stroke; more research needs to be carried out. Among the PUFAs, DHA is the most abundant PUFA in the CNS and is highly enriched in the grey matter of the brain and rod segment of the retina (Saini and Keum 2018). DHA phospholipid integration in the membrane modulates several membrane properties, including stabilization of the acyl chain, membrane permeability, fluidity, and enhancing flip-flop protein activity (Sugasini *et*

*al*., 2017). An alteration of the fatty acid composition of the membrane significantly affects a wide range of brain functions, e.g., neuronal plasticity, axon, and dendrite maintenance, lipid raft formation, G-protein signaling, hypothalamic, and glucose uptake regulation (Mozaffarian *et al*., 2005). These alterations may lead to the development of several neurological diseases, e.g., Alzheimer's conditions linked with the instability of the axons and dendrites, Parkinson's disease, low mood, and depression.

## 1.9 HYPOTHESES

I hypothesized that supplemental PL with fish oil would increase n-3 fatty acid bioavailability to target tissues through enzyme preference and enzyme-substrate specificity. Also, supplemental PL with fish oil would increase the levels of n-3 fatty acid in the PL of various tissues. My second hypothesis was that the increased tissue n-3 PL fatty acids would decrease proinflammatory cytokines compared to the fish oil group, and fish oil group would decrease proinflammatory cytokines compared to Western diet.

### 1.9.1 RATIONALE

Western diets are characterized by the excessive intake of refined and processed sugars, high saturated fat, and high sodium. These diets are high in saturated fatty acids, n-6 fatty acids, and low in n-3 fatty acids and are key contributors to cardiovascular (CV) abnormalities, metabolic syndrome, and neurological deficits. However, considerable evidence shows that n-3 fatty acids reduce the risk of CV and neurological diseases and reverse metabolic syndrome by stabilizing plaques in the coronary circulation, inhibiting inflammatory response progression, decreasing elevated lipid profile, and reducing fat accumulation. There are three major forms (molecular carriers) in which n-3 fatty acids are distributed into the systemic circulation, which are TAG, PL, and EE. Fish oils are a source of n-3 fatty acids, predominantly bound in TAG. These fishes have

significant n-3 fatty acids, notably EPA and DHA. Soy is a mixture of naturally occurring lipids composed of PL; this amphiphilic molecule acts as a good emulsifier that improves the solubility of the fatty acids and further influences the absorption rate and greater bioavailability.

Additionally, different enzymes are involved in the TAG and PL digestion because the lipids have different structural and chemical compositions. 1,3-pancreatic lipase catalyzes TAG digestion to yield 2-monoglyceride (2-MAG) and free fatty acid. On the other hand, phospholipase A2 catalyzes phospholipid digestion to lyso-phospholipid (LYSO-PL) and FFA (because the digestive capacity of these enzymes may determine the bioavalibility of n-3 fatty acid). With the numerous health benefits associated with the intake of n-3 fatty acids, consumers are increasingly interested to know which molecular carrier of n-3 fatty acids are more bioavailable. However, several factors influence the bioavailability of n-3 fatty acids. The most critical factor is the molecular carrier, for example, TAG, PL, and EE (primarily present in drug formulation). However, EE has a poor absorption rate because of impaired intestinal hydrolysis.

Furthermore, for n-3 fatty acids to perform their biological function, they must be bioavailable in the target site. Studies have shown that phospholipid increases n-3 fatty accretion to their target site either when ingested alone or co-ingested with other molecular carriers. Positional distribution is another crucial factor that affects the bioavailability of n-3 fatty, as different fatty acids can be attached to the various sn positions of the TAG and PL structure. For TAG, studies show that EPA is mainly bound to sn-1 and sn-3 positions. The pancreatic lipase will cleave off the fatty acid in sn-1 and 3 positions, which may not be re-coupled during the re-synthesis/reassembling of the new TAG. It may further affect the absorption and secretion into the circulation, possibly leading to lower secretion into target tissues.

In contrast to TAG, supplemental phospholipids could increase the systemic availability of dietary n-3 FAs, as they are known to be resistant to the enzymatic hydrolysis of pancreatic lipase. LPCAT specifically prefers re-esterification of LCPUFA to lyso-PC sn-2 position, leading to a higher probability for the n-3 fatty acids to be transported and incorporated as phospholipids in the circulation, resulting in increased bioavailability of n-3 fatty acids. On the other hand, MGAT-2 prefers the re-esterification of fatty acids to 2-MAG at the sn-2 position, while MGAT-3 prefers the re-esterification of fatty acids to the sn-1 and sn-3 positions. Additionally, TAG is highly hydrophobic, which affects absorbability. At the same time, PL is amphiphilic and possesses self-emulsifying features, decreasing the fatty acid's surface tension so it can be easily digested and absorbed. Thus, adding to other forms of oil treatment (TAG, EE) can increase their absorption and tissue accretion rate due to the interfacial-surfactant properties of PL. Also, PL is a source of active compounds, such as choline, released from phosphatidylcholine.

This study evaluated whether adding PL to the diet increases n-3 fatty acid accretion to target tissues. Therefore, adding fish oil (TAG) to the western diet would increase the pool's n-3 PUFA content. However, due to the preferential enzyme and substrate specificity, long-chain n-3 PUFAs incorporation into PC and n-3 rich tissues will be lower in fish oil-only animals versus fish oil + PL. Specifically, the addition of PL to the diet will increase n-3 PUFAs in target tissues because lysophosphatidylcholine acyltransferase 3 (LPCAT3) favors the transfer of n-3 PUFAs to lysoPC molecules and is rapidly available in the plasma for incorporation into tissues.

Yucatan miniature pigs were chosen as an excellent animal model for this study because of the similarities in gastrointestinal tract physiology between pigs and humans. A previous study from our laboratory showed that pigs are susceptible to developing metabolic syndrome in response to high dietary intake of sodium and fat, just like humans (Myrie *et al*., 2012). Also, some studies

showed that females are more susceptible to develop metabolic syndrome compared to males (Beigh and Jain *et al*., 2012; Moore *et al*., 2017; Tramunt *et al*., 2020). Hence, the reason our laboratory focused on using only female pigs for the present study that was completed with fellow MSc student Mr. Jagatheesan Manoharan. Mr. Manoharan focused on the metabolic disease outcomes of this 12-week pig feeding study, and this thesis focused on the n-3 fatty acid uptake and distribution outcomes of this study.

### 1.9.2 RESEARCH QUESTION

1. Would supplementary PL increase the amount of n-3 fatty acid esterified to PL versus TAG, and will this improve the tissue distribution of n-3 fatty acids into target tissues (various tissues analysed)?

2. Would fish oil intake decrease the level of pro-inflammatory cytokines compared to Western diet, and would any increase in tissue n-3 PL FA alleviate the level of pro-inflammatory cytokines when compared between the fish oil group and the fish oil plus PL group**?**

### 1.9.3 HYPOTHESIS

1. Supplemental PL with fish oil would increase the n-3 fatty acid bioavailability to target tissues.

2. Supplemental PL with fish oil would increase the levels of n-3 fatty acid in the PL of specific target tissues.

3. Lastly, the fish oil intake would decrease the level of pro-inflammatory cytokines compared to the Western diet control group, and fish oil plus PL intake would increase tissue n-3 PL fatty acids, thereby decreasing the levels of pro-inflammatory cytokines compared to fish oil intake alone.

### 1.9.4 OBJECTIVES

The primary objective of this study was to conduct a long-term feeding trial with diets containing no fish oil (WD), fish oil (FO), or fish oil with phospholipid FOPL, to determine tissue n-3 fatty acid compositions in target tissues, including pancreas, liver, adipose tissue, back fat, visceral fat, brain, and retina. Secondly, determine tissue n-3 fatty acid PL compositions in the retina, brain, and liver. Lastly, to determine if proinflammatory cytokine concentrations are different as a function of n-3 fatty acid in the serum.

# CHAPTER TWO

## 2.0 ANIMALS

The animal procedures were conducted following the approval of the Animal Care Committee (Memorial University of Newfoundland) and in accordance with the Canadian Council of Animal Care guidelines. Female Yucatan miniature pigs (N=24) obtained from the herd at the Memorial University Vivarium were included in our study. The piglets were housed with the sows and raised on sow milk from birth to 28 days. They were then separated from the sows and were weaned onto a grower diet (CO-OP Hog Grower Pellets, Atlantic Farm Services Inc, Moncton, NB). The pigs remained on the grower diet until the beginning of the experiment.

## 2.1 GROWER DIET

The grower diet (CO-OP Hog Grower Pellets, Atlantic Farm Services Inc, Moncton, NB) (Table 2.1) was introduced during the last week of suckling to transition to weaning slowly. The pigs were group-housed by size, regardless of age and sex, as part of standard Vivarium herd management. They were fed restricted amounts of grower diet appropriate for the growth phase (~3% of total body weights) by scattering pellets in a pen; they had free access to water. Pigs were fed a grower diet in their respective groups until the start of the experimental study.

**TABLE 2.1.** GUARANTEED ANALYSIS OF NUTRIENTS IN GROWER DIET BY WEIGHT PERCENTAGE (CO-OP HOG GROWER PELLETS, ATLANTIC FARM SERVICES, MONCTON, NB).

| **Nutrients** | **Guaranteed nutrient levels** |
|---|---|
| **Crude protein** | 16% |
| **Crude fat** | 3.4% |
| **Crude fiber** | 7% |
| **Calcium** | 0.7% |
| **Phosphorus** | 0.68% |
| **Sodium** | 0.2% |
| **Zinc** | 130 mg/kg |
| **Copper** | 20 mg/kg |
| **Vitamin A** | 8800 IU/kg |
| **Vitamin D3** | 1040 IU/kg |
| **Vitamin E** | 40 IU/kg |

### 2.2.2 EXPERIMENTAL DIET

**TABLE 2.2.** EXPERIMENTAL DIETARY GROUPING OF PIGS AND THEIR TOTAL NUMBER OF DAYS ON EXPERIMENTAL DIETS

| **Batch number** | **WD** (Pig ID number) | **FO** (Pig ID number) | **FOPL** (Pig ID number) | **Total number of days on experimental diets (n)** |
|---|---|---|---|---|
| 1 | 803 | 802 | 804 | 71 |
| 1 | 806 | 805 | 808 | 95 |
| 1 | 809 | 807 | 810 | 115 |
| 2 | 119 | 120 | 118 | 80 |
| 2 | 817 | 818 | 806 | 101 |
| 2 | 820 | 821 | 819 | 122 |
| 3 | 823 | 824 | 822 | 80 |
| 3 | 826 | 827 | 825 | 106 |

*(n=106 days on experimental diet) W = Western diet, FO = western diet + fish oil , FOPL = western diet + fish oil + phospholipid. Values in each column are pig identification numbers assigned by animal care staff at birth.

This animal feeding and grouping was completed by Jagatheesan Manoharan (MSc student) in our laboratory group. Individual housing for pigs was unavailable at the Vivarium Memorial University of Newfoundland, so we fed each experimental diet to small groups of 2 or 3 per group containing pigs of similar size (Table 2.2). The pigs were six months old when assigned to each experimental diet batch. Experimental diet groups (n=8 per group) were comprised of 3 of these batches (n=2 or 3 pigs per group) (Manoharan, 2022). A maximum of 3 pigs was housed in each

experimental pen to provide sufficient space for mobility and socialization. These batch pens were used consecutively, so a new set of pigs was introduced after removing the previous set.

For pig surgery and surgical recovery, individual pigs were removed from each batch to individual cages. The largest pig was removed each time, so the surgery age/size would be similar, but this meant that some pigs were on the experimental diets longer than others. Studies in our lab have shown that adult Yucatan minipigs were fed a similar Western diet for a minimum of 4 weeks and displayed metabolic factors resembling metabolic syndrome (Myrie *et al*., 2012. Therefore, we used a minimum of two and a half months on diet as adequate to adapt to a Western diet and develop metabolic disease risk factors (Manoharan, 2022).

## 2.3 STUDY PROTOCOLS

### 2.3.1 EXPERIMENTAL DIETS

**TABLE 2.3.** WEIGHT COMPOSITION OF INGREDIENTS IN EACH EXPERIMENTAL DIET

| Feed Ingredient | WD | FO | FOPL |
|---|---|---|---|
| **Grower Diet (g/kg)** | 660 | 660 | 660 |
| **Sugar (g/kg)** | 100 | 100 | 100 |
| **Salt (g/kg)** | 40 | 40 | 40 |
| **Beef tallow (g/kg)** | 120 | 120 | 120 |
| **Safflower oil (g/kg)** | 80 | 30 | 25 |
| **Fish oil (g/kg)** | - | 50 | 50 |
| **Soy lecithin (g/kg)** | - | - | 5 |
| **omega-6/omega-3 ratio** | 45.5/1 | 2.02/1 | 2.25/1 |
| **Total kcal/kg** | 4097 | 4097 | 4091 |

W = Western diet, FO = western diet + fish oil Western diet, =FOPL = western diet + fish oil and + phospholipid.

**TABLE 2.4.** CALORIC COMPOSITION OF INGREDIENTS IN EACH EXPERIMENTAL DIET (KCAL/G).

| Components | WD | FO | FOPL |
|---|---|---|---|
| **Grower diet** | 1918 | 1918 | 1918 |
| **Sugar** | 379 | 379 | 379 |
| **Salt** | 0 | 0 | 0 |
| **Beef tallow** | 1080 | 1080 | 1080 |
| **Safflower Oil** | 720 | 270 | 225 |
| **Fish Oil** | - | 450 | 450 |
| **Soy Lecithin** | - | - | 38 |
| **Total kcal/kg** | 4097 | 4097 | 4091 |

W = Western diet, FO = western diet + fish oil Western diet, =FOPL = western diet + fish oil and + phospholipid. Total calories contributed per kg of diet (kcal/kg) = (ingredient weight per kg of diet (g/kg) total calories per kg of component (kcal/g).

**TABLE 2.5.** PERCENT COMPOSITION BY WEIGHT (%, W/W) OF FATTY ACIDS (ONLY ADDED FAT CONTENT) OF EACH EXPERIMENTAL DIET + SAFFLOWER OIL + FISH OIL + PHOSPHOLIPID).

| | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| **SFA** | 32.6 | 36.0 | 36.2 |
| **MUFA** | 55.2 | 50.5 | 48.9 |
| **PUFA** | 7.5 | 8.2 | 9.0 |
| **LA** | 5.5 | 6.0 | 7.0 |
| **ALA** | 0.12 | 0.49 | 0.62 |
| **DHA** | 0 | 1.1 | 1.1 |
| **EPA** | 0 | 1.6 | 1.6 |

W = Western diet, FO = western diet + fish oil Western diet, =FOPL = western diet + fish oil and + phospholipid. Percentage (%) = (fat component by weight/ total fat content) *100

These levels were chosen to simulate the 95 percentiles of saturated fat, sugar, and salt consumption of North Americans. This composition is plausible and consumed by at least 5% of the North American population. The ingredients used to formulate the base grower diet was requested from the 25 company but was not disclosed. Previous data obtained from the distributor (Eastern Farmer's Co-op, Newfoundland) ~10 years earlier was used to estimate the composition of the experimental diets in the absence of new data (Table 1). The caloric composition of each diet is summarized in Table 4. The three experimental Western diets (WD) were formulated by adding 10% sugar (Redpath$^{TM}$ 20 kg/bag), 4% salt (Windsor$^{TM}$ hi-grade, granulated sodium containing sodium salt, and sodium ferrocyanide decahydrate and yellow prussiate soda as anticaking; 20 kg/bag) and 20% fat (see below) by weight. In the grower diet, sugar, salt, and total

fat contents remained similar by weight percentage in all three diets. The added fat composition varied among the diets (Table 4), except for the beef tallow (Tenderfry$^{TM}$, Bunge Loders Croklaan, product code: 32752), which remained equal (12% of diet) in all three diets. The fat content that varied among diets was added through high-oleic safflower oil (President's Choice, Loblaws), fish oil (crude fish oil, Barry Group, Inc, Corner Brook, NL), and phospholipid as soy lecithin (Bulk Barn, Loblaws, product code: 000943). The 20% of added fat by weight in the WD diet was composed of shortening oil (12%) and safflower oil (8%). The 20% of the added fat by weight in the FO diet was composed of shortening oil (12%), safflower oil (3%), and fish oil (5%). The 20% added fat by weight in the FOPC diet was composed of safflower oil (2.5%), fish oil (5%), and soy lecithin (0.5%). The fatty acid composition of the added fat content by weight is listed in Table 5. The experimental Western diets were isocaloric (Table 4). The diet was mixed using a horizontal ribbon mixer at the Marine Bioprocessing Plant (Marine Institute, Memorial University of Newfoundland). The processed diet was immediately stored in a -20°C freezer until used for feeding.

### 2.3.2 SURGICAL PROCEDURES AND NECROPSY

The surgical and necropsy procedure was carried out by a previous master's student (Manoharan, 2022after the necropsy, all the organs were harvested and stored at -80°C. Jagatheesan Manoharan work focused on the In vivo metabolic tests (intravenous glucose tolerance test, insulin sensitivity test and oral fat tolerance test), and these outcomes were not documented in this thesis.

#### 2.3.2.1 SURGICAL PREPARATION AND MAINTENANCE

The pigs were fasted 12 hours before surgery. A mixture of analgesic (buprenorphine, 0.03 mg/kg), analgesic (meloxicam, 0.4 mg/kg), sedative (azaperone, 0.2 mg/kg) was injected intramuscularly as pre-anesthetic medication. Anesthetic (alfaxalone, 1 mg/kg) and analgesic (dexmedetomidine, 0.02 mg/kg) were administered intramuscularly after 15-20 minutes following the pre-anesthetic medication to achieve deep sedation. Inhalational anesthetic (isoflurane, 1.5%-2%) was administered along with a mixture of oxygen (1-2 L) and nitrous oxide (1 L) via intubation and the pig was completely anesthetized. Lactated Ringer's solution was administered at the rate of 510 mL/kg/h intravenously via ear vein. The vital parameters associated with the cardiovascular and respiratory systems were monitored at 10-minute intervals throughout the surgery using an automated monitoring system. The rectal temperature was monitored via a probe which was connected to an automated monitoring system. The surgery was completed within 2-2.5 hours.

#### 2.3.2.2 IMPLANTATION OF BLOOD SAMPLING CATHETERS AND TELEMETERS

An incision was made at the left femoral triangle. Two catheters (Tygon™ Flexible Plastic Tubing, Saint Gobain PPL Corp, ND-1000-80) each measuring a total length of 50 cm, were inserted in parallel into the left femoral vein and were advanced towards the inferior vena cava; the catheters were inserted 15 cm apart, so the tips were staggered in the vena cava. The external end of the catheters were attached to a sterile metal rod which was tunneled beneath the skin, in the fat layer, along the side of the animal and exteriorized by a dorsal incision dorsally in between the shoulder blades. Infusion ports were installed on catheter ends and the catheters 28 were coiled and secured on the back using medical adhesion tape (Elastoplast™). Sterility was maintained by capping and wiping the catheter ends with isopropyl alcohol before and after each use. A blood pressure telemeter with pressure-sensitive catheter was also installed into the femoral artery

(PhysioTel Digital M10; Data Sciences International, St. Paul, MN); the transmitter unit was attached to a subcutaneous pouch on the left lateral side of the abdominal cavity.

#### 2.3.2.3 MEDICATIONS AND RECOVERY

The pigs were transferred to their individual pens after surgery. Buprenorphine (0.02 mg/kg) was administered as an analgesic 12 hours post-surgery. Trimethoprim and sulfadoxine (TMS) (Borgal™, Merck Animal Health) was administered immediately after surgery and once daily as an antibiotic intravenously at a dose of 16 mg/kg to minimize infection at the surgical site and to prevent sepsis. Meloxicam (Metacam, Boehringer 20 mg/mL) was administered as an analgesic intravenously once daily at a dose of 0.4 mg/kg. Meloxicam and TMS were administered consecutively for three days. The pigs were monitored for appetite, attitude (bright, alert, and responsive (BAR)), mobility, catheter, incision site infection, and signs of sepsis. The catheter patency was maintained by flushing the catheters daily with 5 mL of 1 IU/ml heparinized saline. Rectal temperature readings were obtained daily using a digital thermometer. If pigs had an elevated body temperature above 40℃, TMS was administered at a dose of 20 mg/kg once daily. The incision sites were monitored daily and treated with chlorhexidine ointment (DVL Inhibit™ 150 g 1% w/w) to prevent infection.

## 2.4 BIOCHEMICAL ANALYSIS

### 2.4.1 TOTAL FATTY ACID ANALYSIS

The total lipid analysis of various tissues was estimated using the Löfgren *et al.* (2016) method. Briefly, 300mg of the freeze-clamped tissue was weighed in a weighing boat, in an analytical balance, and transferred to a 5 mL glass test tube. The tissue was homogenized and transferred into a disposable test tube and 100 µL of the internal standard C17 (margaric acid

(370nmol/ml)). For the first phase of extraction, 600 µL of butanol/methanol (3:1, v/v) and the solution was vortex for 1min.

In contrast, 600 µL heptane/ethyl acetate (3:1, v/v) was added for the second extraction phase, and the solution was vortex for 1min. To induce the separation, 600 µL of 1% acetic acid was added and vortex for 1min; the solution was centrifuged at 1800 x g for 10 minutes. The upper layer was collected with a glass Pasteur pipette into a new test tube. An additional 1000 µL of heptane/ethyl acetate (3:1, v/v) was added to the bottom layer remaining in the first test tube, vortex, and centrifuge at 1800 x g for 10 minutes. The upper layer was also collected and combined with the solution in the new test tube (this step was repeated 3 times). Next, the combined organic upper layers were dried under $N_2$ gas, and the bottom layer was disposed. After drying, the lipids were resuspended in 1mL of hexane and vortex, and 1mL of fresh methylation reagent was added. The methylation reagent was prepared by adding 2%: 1mL $H_2SO_4$ and 48 mL methanol. The tubes were tightly capped, heated, and refluxed for 1 hour on a dry block heater at 70°C. After cooling, 3 mL of 5% $Na_2HCO_3$ in dd-water, 2 mL of hexane were added, and vortex, the solution was allowed to sit overnight in the fridge recapped to check for precipitate. The solution was centrifuged for 10minutes at 1800 rpm, and the hexane layer was collected and transferred into a new disposable test tube (this step was repeated twice). Samples were dried using a nitrogen evaporator and resuspended with 1ml of hexane and vortex for 1minute. 200 uL of resuspended lipid was transferred to a GC vial, and another 1.8 mL of hexane was added and stored for GC-FID analysis at -20 ℃.

### 2.4.2 PHOSPHOLIPID FRACTION (FAME) ANALYSIS

The phospholipid class separation procedure was carried out on the brain, retina, and liver using Wersto and Druyan, (1982) procedure. Briefly, 0.3 g silicic acid was activated at 100 ° C for

12 hours in a 15 mL conical glass centrifuge tube, 5 mL methanol was added, and vortex (it will form a slurry solution). Transfer the slurry to a disposable chromatography column in a Pasteur pipette pre-plugged with glass wool. Sequentially, the silicic acid columns were washed with 5 mL of chloroform, methanol, and chloroform, allowing the last chloroform wash to drain about 3 mm from the top of the column. The lipid was added to the column, and the neutral lipid and cholesterol lipid fraction was eluted by adding 6 mL of chloroform to the column, collecting the eluate into a 15 mL glass centrifuge tube. Phospholipids were eluted into another sample tube by adding 6 ml of methanol. The collected fractions were purged with nitrogen gas and stored at -20 ° C. The phospholipid samples were redissolved in 2mL of hexane and stored at -20 ° C under nitrogen gas.

#### 2.4.2.1 GAS CHROMATOGRAPHY

The FAME was analyzed on an Agilent 6890 GC FID equipped with a 7683 autosampler with an on-column injector and an Agilent 7890 equipped with a 7693 autosampler and a muli mode injector using split less mode. The GC column was a ZB wax+ (Phenomenex, U.S.A.). The column length was 30m with an internal diameter of 0.32mm. The column temperature began at 65$^{o}$C and held this temperature for 0.5 minutes. The temperature ramped to 195 $^{o}$C at a rate of 40 $^{o}$C/min, held for 15 minutes, then ramped to a final temperature of 220 $^{o}$C at a rate of 2 $^{o}$C/min. This final temperature was kept for 0.75 minutes. The carrier gas was hydrogen and flowed at 2 ml/minute. The injector temperature started at 150 $^{o}$C and ramped to a final temperature of 250 $^{o}$C at a rate of 120 $^{o}$C/minute. The detector temperature stayed constant at 260 $^{o}$C. Peaks were identified using retention times from standards purchased from Supelco, 37 component FAME mix (Product number 47885-U), Bacterial acid methyl ester mix (product number 47080-U), PUFA 1 (product number 47033), and PUFA 3 (product number 47085U). Chromatograms were

integrated using the Agilent Open LAB Data Analysis - Build 2.203.0.573. A quantitative standard purchased from Nu-Chek Prep, Inc (product number GLC490) was used to check the GC column about every 100 samples (or once a week) to ensure that the areas returned were as expected. The total and individual FA (mg/g sample dry weight) were calculated using the IS and the sample weight.

## 2.5 DETERMINATION OF SERUM CYTOKINES

Interleukin (IL-6), and tumor necrosis factor (TNFα) concentrations in the pig serum were measured by ELISA kit (Aviva systems biology, San Diego, USA) according to the producer's recommendation. Briefly, 100 µL of each sample was added to a 96-well plate. The plate was covered with the well plate sealer and incubated at room temperature for 2.5 hours with gentle shaking. After 4 washes with wash buffer, detection antibodies were added at a volume of 100 µL per well. The plates were sealed and incubated for 1 hour with gentle shaking. Following this incubation, plates were decanted, 100 µL of Streptavidin–horseradish peroxidase (HRP) conjugate was added to each well, and plates were incubated for 45 min, and then washed thrice. Finally, 100 µL of (3,3',5,5'-Tetramethylbenzidine) TMB substrate was added, and the plate was incubated at room temperature in the dark for 30 minutes. Finally, 50 µL of stop solution was added to each well. Cytokine concentrations were determined by comparing samples to standards of known concentration provided by the manufacturer.

## 2.6 STATISTICAL ANALYSES

All statistical analyses were performed using SPSS (version 19, SPSS Inc.). Data were expressed as means ± SEM. P values $<0.05$ were considered significant. Based on our study design,

a one-way analysis of variance (ANOVA) was used to with a Dunnett test was used for post hoc comparisons. Because the WD and FOPL groups were not compared against one another, the "control" groups in the Dunnett test were set as the FO group. This allowed for pairwise comparisons between the WD versus FO and FO versus FOPL separately.

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 DIETARY EFFECT ON BODY WEIGHT AND GIRTH.

We did not observe any differences in the final body weights of the pigs, regardless of dietary treatment (Table 3.1). Although, there were differences between WD and FO, pigs consuming the FOPL diet tended to have a larger girth than pigs consuming the FO diet. However, this difference in girth was less than 3% of the total average girth and not considered biologically significant (Table 3.1).

**TABLE 3.1.** DIETARY EFFECT ON BODY WEIGHT AND GIRTH.

| | WD | FO | FOPL |
|---|---|---|---|
| **Body weight (kg)** | 59.3±5.4 | 59.7±5.9 | 57.6±6.3 |
| **Girth (cm)** | 91.2±3.1 | 91.2±9.2 | 93.8±3.6 [#] |

Data are means with their standard errors of the mean, n= 8 pigs/group. [#] indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.2 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE LIVER.

Of the relevant results, I determined that there was a significant greater incorporation of ALA, EPA, and DHA in the FO group compared to the WD group, and there were no significant differences in the incorporation of ALA, EPA, and DHA in the FOPL group compared to FO group (Table 3.2). I also measured no significant increase in the incorporation of ∑ n-3PUFA in the FOPL compared to the FO group (Table 3.3).

**TABLE 3.2.** TOTAL FATTY ACID DISTRIBUTION IN THE LIVER

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 21.0±3.8 | 27.3±3.1 | 25.8±2.7 |
| **18:0** | **Stearic acid** | 25.1±1.6 * | 20.8±3.7 | 19.1±2.7 |
| **16:1w7** | **Palmitoleic acid** | 0.5±0.1 | 0.5±0.2 | 0.7±0.4 |
| **18:1w9** | **Oleic acid** | 19.8±4.5 * | 14.2±3.9 | 18.1±3.8 |
| **18:2w6** | **Linoleic acid** | 12.7±4.5 * | 5.2±1.0 | 4.6±2.4 |
| **18:3w6** | **γ-Linolenic acid** | 0.4±0.2 | 0.2±0.1 | 0.2±0.1 |
| **20:4w6** | **Arachidonic acid (AA)** | 12.3±2.9 * | 6.5±1.5 | 5.3±2.8 |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.4±0.0 * | 1.2±0.3 | 3.0±0.4 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 0.1±0.0 * | 2.5±0.8 | 3.9±0.6 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 2.6±0.5 * | 9.0±3.9 | 9.1±3.6 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.3.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE LIVER.

| Fatty acids % (w/w) | WD | FO | FOPL |
|---|---|---|---|
| ∑ **SFAs** | 46.0±5.5 | 47.3±7.1 | 45.3±5.5 |
| ∑ **MUFAs** | 22.4±5.2 * | 23.4±4.0 | 22.5±5.3 |
| ∑**PUFAs** | 28.5±8.1 | 24.6±7.6 | 25.9±9.9 |
| ∑**n 6 PUFAs** | 25.4±7.6 * | 11.9±2.6 | 9.9±5.3 |
| ∑**n 3 PUFAs** | 3.1±0.5 * | 12.7±5.0 | 16.0±4.6 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.3 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE HEART.

There was a significant increase of ALA, EPA and DHA in the FO group compared to the WD group and a significant increase of EPA and DHA in the FOPL group compared to the FO group (Table 3.4). I also measured a significant increase in the incorporation of ∑ n-3PUFA in the FO group compared to the WD, and a significant increase in the incorporation of ∑ n-3PUFA in the FOPL group compared to the FO. However, there was a significant decrease in the ∑ n-6 PUFA and ∑ PUFA in the FO compared to WD and in the FOPL group compared to the FO group (Table 3.5).

**TABLE 3.4.** TOTAL FATTY ACID DISTRIBUTION IN THE HEART.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 17.5±2.8 * | 28.0±0.9 | 14.2±1.4 |
| **18:0** | **Stearic acid** | 15.1±1.8 | 18.1±2.5 | 11.3±1.5 |
| **18:1w9** | **Oleic acid** | 17.5±4.3 | 19.5±5.4 | 32.9±5.1 |
| **18:2w6** | **Linoleic acid** | 20.4±5.4 * | 11.4±5.4 | 4.9±1.7 # |
| **20:4w6** | **Arachidonic acid (AA)** | 18.4±4.9 * | 12.0±4.2 | 8.1±1.5 # |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.2±0.0 * | 0.4±0.2 | 0.4±0.1 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 0.6±0.2 * | 3.4±2.8 | 7.5±4.5 # |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 1.4±1.2 * | 3.4±1.1 | 5.5±2.0 # |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.5.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE HEART.

| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| ∑ **SFAs** | 32.6±5.3 * | 37.1±7.0 | 25.5±2.9 # |
| ∑ **MUFAs** | 22.5±5.3 | 23.1±6.8 | 32.9±5.8# |
| ∑**PUFA** | 41.0±11.7* | 27.6±13.7 | 26.4±9.8 |
| ∑**n-6 PUFAs** | 38.8±10.3* | 23.4±9.6 | 13.0±3.2# |
| ∑ **n-3 PUFAs** | 2.2±1.4* | 4.2±4.1 | 13.4±6.6 # |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.4 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE BRAIN

Next, I assessed the fatty acid composition of brain tissue following the experimental diets. Both ALA and DHA content were significantly higher in FO fed pigs versus WD fed pigs (Table 3.6). This translated to a significantly higher ∑ n-3PUFA content in FO versus WD pigs, which appeared to be off-set by a lower ∑ n-6 PUFA in FO pigs compared to WD fed pigs (Table 3.7). I also observed a higher DHA and ∑ n-3PUFA content in the brain tissue of FOPL pigs compared to the FO group. There were no differences in individual or ∑ n-6 PUFA between the FO and FOPL groups. Finally, I did not observe any differences in the EPA content of brain tissue across any dietary comparison.

**TABLE 3.6.** TOTAL FATTY ACID DISTRIBUTION IN THE BRAIN.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 23.9±1.6 | 21.9±2.0 | 25.8±3.8 |
| **18:0** | **Stearic acid** | 25.0±7.4 * | 20.0±8.0 | 18.0±2.8 # |
| **18:1w9** | **Oleic acid** | 28.3±7.3 * | 22.2±2.1 | 23.4±2.1 |
| **24:1** | **Nervonic acid (NA)** | 1.7±0.3 | 3.4±0.8 | 3.5±0.5 |
| **18:2w6** | **Linoleic acid** | 0.8±0.1 | 0.6±0.1 | 0.2±0.0 |
| **20:4w6** | **Arachidonic acid (AA)** | 10.7±5.3 * | 5.2±0.2 | 4.3±0.7# |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.1±0.0 * | 5.3±0.7 | 5.1±1.7 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 0.1±0.0 | 0.3±0.1 | 0.3±0.1 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 3.1±0.9 * | 9.1±4.1 | 11.6±6.2 # |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.7.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE BRAIN.

| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| ∑ **SFAs** | 48.9±9.1 | 41.9±6.1 | 44.0±8.7. |
| ∑ **MUFAs** | 30.8±7.9* | 32.0±3.1 | 30.4±2.6 |
| ∑**PUFA** | 14.8±5.3 | 20.5±4.2 | 21.5±8.7 |
| ∑ **n-6 PUFAs** | 11.5±3.4* | 5.8±0.3 | 4.5±0.7 |
| ∑ **n-3 PUFAs** | 3.3±0.9* | 14.7±4.9 | 17.0±8.0 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.5 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE RETINA

For the retina, I measured a significant increase in the FO group compared to WD and no significant difference in the FOPL compared to FO, both for the incorporation of ALA, and DHA. However, there was no significant incorporation of EPA across all experimental groups (Table 3.8). It was also measured that there was a significant increase in the incorporation of ∑ n-3PUFA in the FO group compared to WD and no significant difference in the incorporation of ∑ n-3PUFA in FOPL compared to FO (Table 3.9).

**TABLE 3.8.** TOTAL FATTY ACID DISTRIBUTION IN THE RETINA

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 19.0±5.4 * | 17.9±6.1 | 15.7±2.9 # |
| **18:0** | **Stearic acid** | 22.8±6.5 * | 24.6±9.3 | 18.6±6.5# |
| **18:1w9** | **Oleic acid** | 11.3±5.1 * | 22.4±2.1 | 23.0±6.9 |
| **24:1** | **Nervonic acid (NA)** | 1.2±0.4 | 1.2±0.1 | 1.8±0.6 |
| **18:2w6** | **Linoleic acid** | 11.8±3.0 * | 3.7±2.4 | 4.3±1.1# |
| **20:4w6** | **Arachidonic acid (AA)** | 15.2±2.4 * | 9.8±2.3 | 5.6±1.8 # |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.2±0.0 * | 0.6±0.2 | 0.8±0.2 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 1.4±0.7* | 8.1±3.3 | 8.2±3.7 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 1.3±0.4 * | 6.2±2.1 | 6.4±2.3 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.9.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE RETINA

| | RETINA | | |
|---|---|---|---|
| **Fatty acids % WD (w/w)** | | **FO** | **FOPL** |
| ∑ **SFAs** | 42.1±6.0 | 42.7±15.4 | 34.6±9.5 # |
| ∑ **MUFAs** | 26.0±6.0 | 26.4±3.2 | 26.7±8.6 |
| ∑**PUFA** | 28.9±6.5 | 28.4±10.3 | 25.3±9.1 |
| ∑ **n-6 PUFAs** | 26.0±5.4 * | 13.5±4.7 | 9.9±2.9 # |
| ∑ **n-3 PUFAs** | 2.9±1.1 * | 14.9±5.6 | 15.4±6.2 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.6 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE PANCREAS

For the incorporation of ALA, DHA, and EPA in the pancreas, I measured a significant increase in the FO compared to the WD group, and no significant difference was observed in the FOPL compared to the FO group (Table 3.10). Also, for ∑ n-3PUFA, it was measured that there was a significant increase in the FO compared to WD, and no significant difference in the FOPL compared to FO (Table 3.11).

**TABLE 3.10.** TOTAL FATTY ACID DISTRIBUTION IN THE PANCREAS.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 33.9±5.1 | 33.9±5.9 | 34.3±11.3 |
| **18:0** | **Stearic acid** | 21.8±9.4* | 12.9±5.6 | 18.8±9.3# |
| **18:1w9** | **Oleic acid** | 18.4±0.8 * | 22.2±6.5 | 14.5±2.1# |
| **18:2w6** | **Linoleic acid** | 8.4±2.3 * | 4.2±0.7 | 3.6±0.7 |
| **20:4w6** | **Arachidonic acid (AA)** | 0.8±0.2 | 0.4±0.1 | 0.2±0.0 # |
| **18:3w3** | **α Linoleic acid (ALA)** | 5.0±2.0 * | 12.7±3.9 | 13.8±3.9 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 1.3±0.7 * | 5.7±2.6 | 6.9±2.6 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 0.3±0.0 * | 1.5±0.6 | 1.9±0.6 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.11.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE RETINA

| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| ∑ SFAs | 63.5±16.6 * | 51.4±11.4 | 49.5±8.2# |
| ∑ MUFAs | 18.8±1.0 * | 22.5±6.6 | 18.6±4.1# |
| ∑ PUFA | 15.9±5.2 | 26.9±8.6 | 27.7±8.9 |
| ∑n-6 PUFAs | 9.2±2.5 * | 4.6±0.8 | 3.8±0.7 |
| ∑ n-3 PUFAs | 6.7±2.7* | 22.3±7.8 | 23.9±8.2 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.7 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE DORSAL SUBCUTANEOUS FAT.

I also analyzed the total fatty acids in the dorsal subcutaneous fat**,** there were no changes in the FO compared to the WD group, and no significant difference was measured in the FOPL compared to the FO group for ALA, DHA, and EPA distribution (Table 3.12). Also, in palmitic acid there was a significant incorporation in the FOPL group compared to FO group and a significant decrease in the FO group compared to the WD group. While for ∑ n-3PUFA, it was measured that there was no significant increase in the FO compared to WD, and no changes in the FOPL compared to FO. Also, there was no significant difference in the incorporation of ∑ MUFA across all experimental groups (Table 3.13). (These are the relevant results mentioned).

**TABLE 3.12.** TOTAL FATTY ACID DISTRIBUTION IN THE DORSAL SUBCUTANEOUS FAT.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 20.9±1.4* | 18.2±8.7 | 23.2±1.8# |
| **18:0** | **Stearic acid** | 18.1±12.6 | 19.0±8.7 | 14.6±8.3# |
| **18:1w9** | **Oleic acid** | 49.7±13.5 | 50.1±19.4 | 51.7±16.0 |
| **18:2w6** | **Linoleic acid** | 7.5±2.3 * | 9.1±3.3 | 7.5±3.7 |
| **20:4w6** | **Arachidonic acid (AA)** | 0.2±0.1 | 0.2±0.0 | 0.1±0.0 |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.2±0.2 | 0.8±0.1 | 0.7±0.3 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 0.1±0.0 | 0.2±0.1 | 0.4±0.1 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 0.1±0.0 | 0.3±0.2 | 0.4±0.1 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.13.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE DORSAL SUBCUTANEOUS FAT.

| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| ∑ **SFAs** | 40.6±14.3 | 39.1±17.9 | 37.8±10.4[#] |
| ∑ **MUFAs** | 50.1±20.6 | 50.3±21.5 | 52.3±13.0 |
| ∑ **PUFA** | 7.9±2.8[*] | 10.6±3.8 | 9.1±4.2 |
| ∑ **n-6 PUFAs** | 7.5±2.4 [*] | 9.3±3.3 | 7.6±3.7 |
| ∑ **n-3 PUFAs** | 0.4±0.2 | 1.3±0.5 | 1.5±0.5 |

Data are means with their standard errors of the mean, n= 8 pigs/group. [*] indicates FO is significantly different from WD, [#] indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

## 3.8 DISTRIBUTION OF THE TOTAL FATTY ACIDS IN THE VISCERAL FAT

I further assessed the fatty acid composition of visceral fat following the experimental diets. For ALA, DHA, and EPA distribution, there were no changes in the FO compared to the WD group, and no significant difference was measured in the FOPL compared to the FO group (Table 3.14). While for ∑ n-3PUFA distribution, it was measured that there was no significant difference in the FO compared to WD, and no significant difference in the FOPL compared to FO. However, there was a significant decrease in the incorporation of ∑ n-6 PUFA in the FOPL compared to the FO group (Table 3.15).

**TABLE 3.14.** TOTAL FATTY ACID DISTRIBUTION IN THE VISCERAL FAT

| Fatty acids % (w/w) | Common Name | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 26.3±11.4* | 20.8±12.2 | 13.4±5.4# |
| **18:0** | **Stearic acid** | 11.3±3.2 * | 17.3±5.2 | 15.0±5.3 |
| **18:1w9** | **Oleic acid** | 56.0±12.6* | 40.9±6.7 | 46.2±22.4# |
| **18:2w6** | **Linoleic acid** | 7.6±2.4 | 7.8±2.2 | 2.3±0.4 # |
| **20:4w6** | **Arachidonic acid (AA)** | 0.2±0.1 | 0.2±0.0 | 0.1±0.0 |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.2±0.1 | 0.2±0.1 | 0.2±0.1 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | 0.2±0.0 | 0.4±0.2 | 0.4±0.1 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 0.1±0.1 | 0.1±0.0 | 0.1±0.0 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.15.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE VISCERAL FAT.

| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
|---|---|---|---|
| ∑ **SFAs** | 27.3±12.6* | 35.3±16.7 | 19.3±6.9# |
| ∑ **MUFAs** | 56.4±12.7* | 40.9±6.7 | 46.6±22.4# |
| ∑ **PUFA** | 8.3±2.7 | 8.7±2.5 | 3.1±0.6# |
| ∑ **n-6 PUFAs** | 7.8±2.5 | 8.0±2.2 | 2.4±0.4 # |
| ∑ **n-3 PUFAs** | 0.5±0.2 | 0.7±0.3 | 0.7±0.2 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

### 3.9 DISTRIBUTION OF THE FATTY ACIDS IN THE BRAIN PHOSPHOLIPID.

Next, I assessed the fatty acid composition in brain phospholipid following the experimental diets. There was a significant increase in the FO compared to the WD group for both ALA and DHA content and no EPA was detected in the brain (Table 3.16). Also, for ∑ n-3PUFA distribution, there was a significant increase in the FO compared to WD, and a significant increase in the FOPL compared to FO (Table 3.17).

**TABLE 3.16.** DISTRIBUTION OF THE FATTY ACIDS IN THE BRAIN PHOSPHOLIPID.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 29.7±3.5 | 25.7±3.5 | 26.3±4.3 |
| **18:0** | **Stearic acid** | 5.0±2.9* | 25.1±7.8 | 22.5±8.5 # |
| **24:1** | **Nervonic acid (NA)** | 2.0±0.4 | 1.3±0.5 | 6.0±1.7# |
| **18:1w9** | **Oleic acid** | 38.5±10.6 * | 49.3±10.1 | 27.0±7.1# |
| **18:2w6** | **Linoleic acid** | 12.2±3.4* | 8.3±1.6 | 3.5±0.9 |
| **20:4w6** | **Arachidonic acid (AA)** | 2.7±0.7 * | 0.3±0.1 | 0.1±0.0 |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.3±0.1 * | 2.2±0.4 | 4.4±0.8# |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | ND | ND | ND |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 0.1±0.0 * | 0.5±0.2 | 5.3±3.3# |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.17.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN THE BRAIN PHOSPHOLIPID

| Fatty acids % (w/w) | WD | D FO | FOPL |
|---|---|---|---|
| ∑ **SFAs** | 34.7±12.3 | 50.8±12.1 | 53.1±14.9[#] |
| ∑ **MUFAs** | 38.5±12.8 | 37.8±11.4 | 33.8±7.7[#] |
| ∑ **PUFA** | 15.3±4.2 [*] | 12.2±2.6 | 13.6±4.1 |
| ∑ **n-6 PUFAs** | 14.9±4.1 [*] | 8.3±1.7 | 3.6±0.9[#] |
| ∑ **n-3 PUFAs** | 0.4±0.1 [*] | 2.7±0.6 | 9.7±4.1[#] |

Data are means with their standard errors of the mean, n= 8 pigs/group. [*] indicates FO is significantly different from WD, [#] indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

### 3.10 DISTRIBUTION OF THE FATTY ACIDS IN THE RETINA PHOSPHOLIPID.

No changes were measured in the incorporation of ALA in the FO group compared to the WD group, and no significant difference in the FOPL compared to the FO group in the retina phospholipid. EPA was not detected in the FO and WD experimental groups (Table 3.18). However, there was a significant increase in DHA incorporation in the FOPL compared to FO. For the distribution of ∑ n3-PUFA, there was a significant increase in the FO group compared to WD and a significant increase in the FOPL group compared to the FO group (Table 3.19).

**TABLE 3.18.** DISTRIBUTION OF THE FATTY ACIDS IN THE RETINA PHOSPHOLIPID.

| Fatty acids % (w/w) | Common Names | WD | FO | FOPL |
|---|---|---|---|---|
| **16:0** | **Palmitic acid** | 22.9±2.2 * | 27.1±11.4 | 24.2±0.7# |
| **18:0** | **Stearic acid** | 22.9±1.5 | 21.1±1.9 | 21.2±4.9 |
| **24:1** | **Nervonic acid (NA)** | 1.1±0.5 | 1.4±0.1 | 2.3±0.9 |
| **18:1w9** | **Oleic acid** | 19.0±1.4 * | 24.7±1.5 | 17.6±1.1 |
| **18:2w6** | **Linoleic acid** | 11.2±1.3 | 6.1±0.5 | 2.0±0.4 |
| **20:4w6** | **Arachidonic acid (AA)** | 3.8±0.7 * | 0.3±0.1 | 0.1±0.0 |
| **18:3w3** | **α Linoleic acid (ALA)** | 0.3±0.1 | 2.3±0.5 | 2.2±0.5 |
| **20:5w3** | **Eicosapentaenoic acid (EPA)** | ND | ND | 1.8±0.4 |
| **22:6w3** | **Docosahexaenoic acid (DHA)** | 0.1±0.0 | 0.5±0.1 | 5.9±2.2# |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.19.** SUMMARY OF THE DISTRIBUTION OF THE FATTY ACIDS IN THE RETINA PHOSPHOLIPID.

| | OLIPIL | | |
|---|---|---|---|
| **Fatty acids % (w/w)** | **WD** | **FO** | **FOPL** |
| ∑ **SFAs** | 59.6±6.3 | 57.3±13.4 | 60.7±7.5 |
| ∑ **MUFAs** | 20.1±4.6 * | 25.6±3.1 | 22.3±2.0# |
| ∑ **PUFA** | 17.1±2.1 * | 10.0±1.2 | 12.5±3.4 |
| ∑ **n-6 PUFAs** | 15.0±2.0 * | 6.4±0.6 | 2.1±0.4# |
| ∑ **n-3 PUFAs** | 0.4±0.1 * | 2.8±0.6 | 9.9±3.1# |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

### 3.11 DISTRIBUTION OF THE FATTY ACIDS IN THE LIVER PHOSPHOLIPID.

I measured no significant difference in the incorporation of ALA in the FO group compared to the WD group, and no significant difference in the FOPL compared to the FO group in the liver phospholipid. EPA was also not detected in the three experimental groups. However, there was no significant difference in DHA incorporation across all experimental groups (Table 3.20). While for the distribution of ∑ n3-PUFA, there was a significant increase in the FO group compared to WD, and there was no significant increase in the FOPL group compared to the FO group. The WD had the highest level of ∑ MUFA, compared to all other groups (Table 3.21).

**TABLE 3.20.** TOTAL FATTY ACID DISTRIBUTION IN THE LIVER PHOSPHOLIPID.

| Fatty acids % (w/w) | Common Name | WD | FO | FOPL |
|---|---|---|---|---|
| 16:0 | Palmitic acid | 31.2±4.1 * | 38.5±14.3 | 36.6±6.4 |
| 18:0 | Stearic acid | 21.5±2.2 * | 30.9±11.0 | 25.4±9.8 |
| 18:1w9 | Oleic acid | 29.6±6.3 * | 21.0±2.4 | 25.4±5.7# |
| 18:2w6 | Linoleic acid | 10.2±2.3 * | 5.63±2.6 | 4.27±1.1# |
| 20:4w6 | Arachidonic acid (AA) | 3.70±0.6 * | 0.40±0.0 | 0.33±0.1 |
| 18:3w3 | α Linoleic acid (ALA) | 0.63±0.1 | 2.37±0.4 | 2.68±0.3 |
| 20:5w3 | Eicosapentaenoic acid (EPA) | ND | ND | ND |
| 22:6w3 | Docosahexaenoic acid (DHA) | 0.16±0.1 | 2.59±1.0 | 3.34±1.4 |

Data means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

**TABLE 3.21.** SUMMARY OF THE TOTAL FATTY ACID DISTRIBUTION IN LIVER PHOSPHOLIPID.

| Fatty acids % (w/w) | WD | FO | FOPL |
|---|---|---|---|
| ∑ **SFAs** | 52.7±6.3 * | 68.6±25.3 | 62.0±17.2 |
| ∑ **MUFAs** | 29.6±3.3 * | 21.0±2.4 | 25.4±3.7# |
| ∑ **PUFA** | 14.7±3.6 | 11.0±3.3 | 10.6±2.6 |
| ∑ **n-6 PUFAs** | 13.9±2.9 | 6.03±2.6 | 4.6±1.2# |
| ∑ **n-3 PUFAs** | 0.7±0.2 * | 4.96±1.4 | 6.0±1.4 |

Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from WD, # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

### 3.12 THE EFFECT OF ADDING PL TO FISH OIL OMEGA-3 FATTY ACID ON TNFα.

There was a significant increase in TNFα concentrations in the FOPL group compared to the other dietary group.

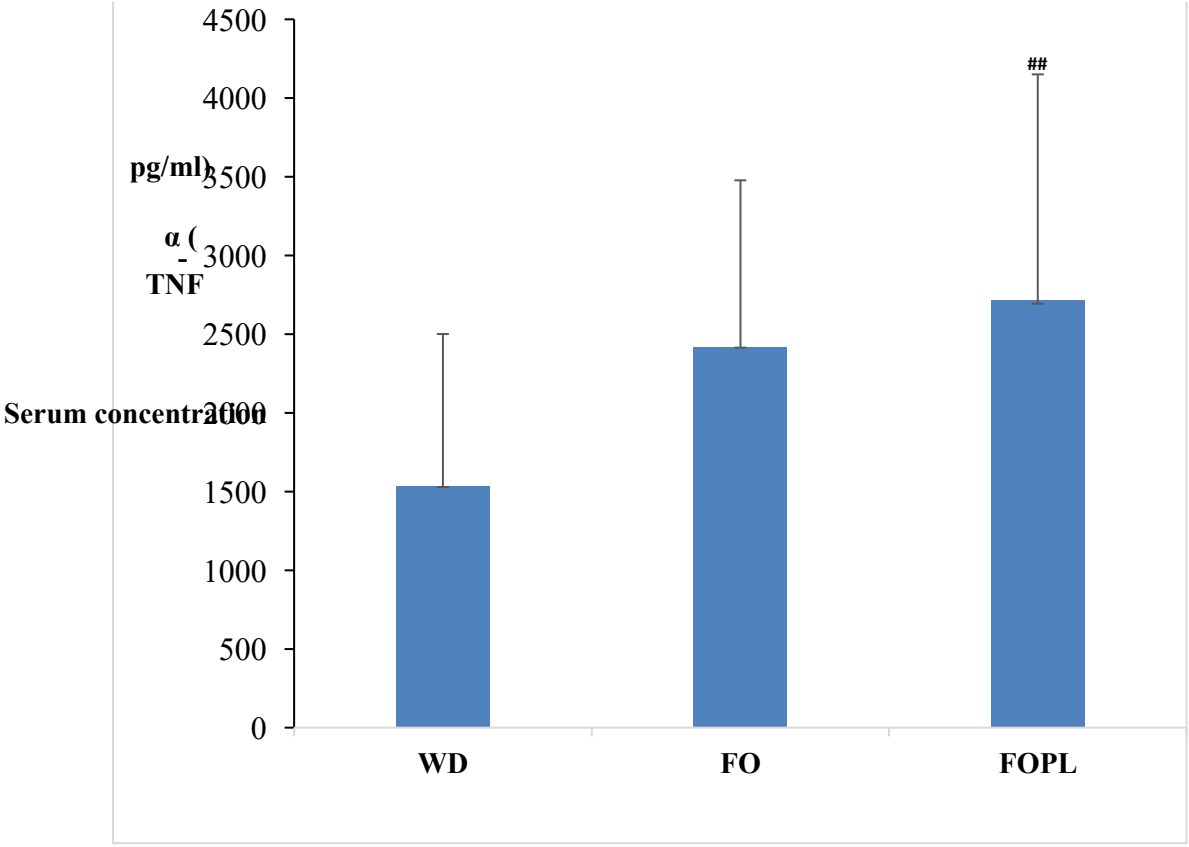


**Figure 3.1.** Effect of adding PL on fish oil on serum TNFα. Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from control (WD), # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

### 3.13: THE EFFECT OF ADDING PL TO FISH OIL OMEGA-3 FATTY ACID ON IL-6.

There were no significant differences across all dietary groups.

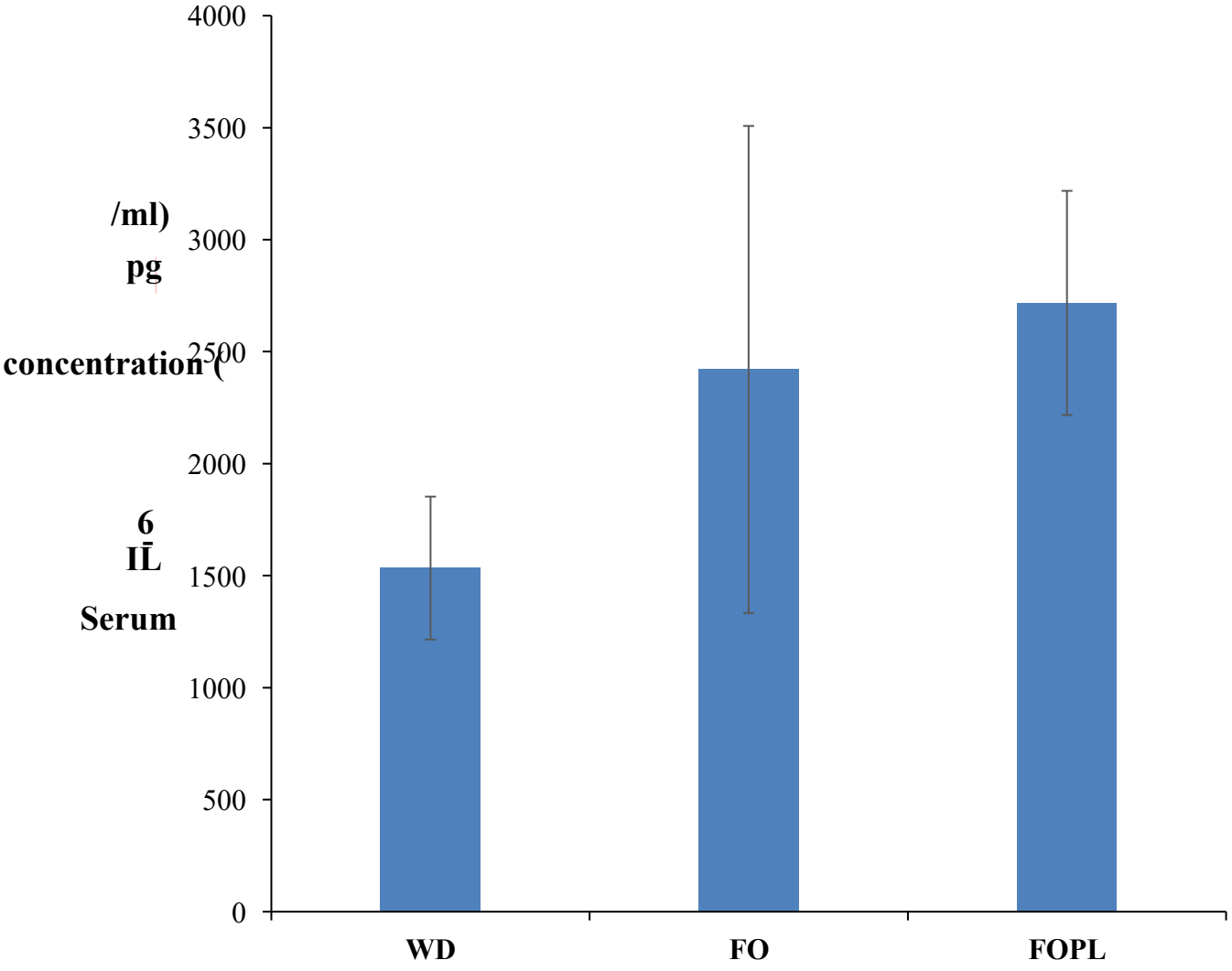


**Figure 3.2.** Effect of adding PL on fish oil omega-3 fatty acid on serum interleukin (IL-6). Data are means with their standard errors of the mean, n= 8 pigs/group. * indicates FO is significantly different from control (WD), # indicates FOPL is significantly different from FO (FO vs. FOPL). "One-way ANOVA with Dunnett post hoc test for pairwise comparisons, $p < 0.05$."

# CHAPTER FOUR

## 4.0 DISCUSSION

### 4.1 TOTAL FATTY ACID DISTRIBUTION AND CONCENTRATION IN THE BRAIN AND RETINA.

Omega-3 fatty acids are important for central nervous system development (DiNicolantonio and O'Keefe, 2018). The brain and retina are particularly enriched with n-3 fatty acids, mostly DHA. My data showed that the addition of PL to fish oil (FOPL) resulted in a significant increase in the incorporation of DHA compared to the FO group. However, no differences were observed in EPA incorporation across all experimental groups in the brain. These findings agree with previous studies, which showed that PL n-3 fatty acids, predominantly DHA, are preferentially incorporated into the brain (Sugasini *et al*., 2017). The isotope study by Kitson *et al*. (2016) showed that [U-$^{3}$H] PL-DHA significantly increased the level of DHA in the cerebellum, cortex, hippocampus, and other brain sections. The reason could be the brain relies on PL as a molecular carrier for the accretion of n-3 fatty acids. Recent studies showed an expression of a particular protein in the blood-brain barrier called major facilitator superfamily domain 2a (Mfsd2a), specific for the transport of PL n-3 fatty acid, which may explain the preferential uptake of DHA when esterified in PL (Ben-Zvi *et al*., 2014). It may also imply that LPCAT specifically prefers the re-esterification of DHA in the brain compared to other n-3 fatty acids. Furthermore, it may be that the digestive capacity of pancreatic lipase was higher in the FO group (usually in TAG form) than PLA-1 on PL and may cleave off the DHA in either the sn-1 or 2 position, which may not be re-esterified into the glycerol backbone, thereby decreasing the accretion of FO-DHA in the brain.

Additionally, dietary soy lecithin is an active source of choline, and studies have revealed that PC increases plasma and brain choline levels, and PL, in particular PC (DHA), is taken up by

the brain more readily because of the choline. In thus study, humans and mice were fed 2.3g of lecithin (free form) and fish oil mixed with choline, respectively (Wurtman *et al*., 1977; Li *et al*., 2023). Choline is a precursor for acetylcholine synthesis, a neurotransmitter in the brain; therefore, supplemental PC provides a synergic benefit in delivering n- 3 fatty acids and choline to the brain. For example, for each molecule of DHA entering the brain, a molecule of choline is provided through the specific high-affinity Mfsd2a transporter (Nguyen *et al*.,2014). Furthermore, my data showed that the addition of PL to fish oil caused a significant increase in the ∑ n3 PUFA compared to the FO in the brain. Therefore, increasing the level of n-3 fatty acids via PL in the plasma is necessary to efficiently concentrate DHA in the brain. Also, my data showed the FO diet significantly increased the incorporation of DHA in the brain and retina compared to WD.

Also, the FO group significantly increased ALA compared to WD in the brain; however, the addition of PL to fish oil did not result in a significant increase in the incorporation of ALA compared to the FO group. Nervonic acid (NA) regulates the function of brain cell membranes and has a neuroprotective property. It functions as an intermediate of myelin biosynthesis, formed from the differentiation of plasma membrane Schwann cells (Bolino, 2021). The main characteristics differentiating myelin from other membranes are fatty acid composition and the high lipid: protein ratio (Lewkowicz *et al*., 2019). During myelinogenesis, NA is the most abundant fatty acid incorporated into sphingomyelin (Martinez and Mougan, 1998; Lewkowicz *et al*., 2019). However, my data showed no significant increase in NA content in the brain in the FO group compared to the WD group.

Similarly, there was no significant increase in NA content in the brain when PL was added to fish oil compared to the FO group. Omega-3 fatty acid deficiency in the brain may be related to numerous neurodegenerative conditions, and my study showed that there was a significant

decrease in brain AA in the FO group compared to WD and a significant decrease in the FOPL compared to the FO group. Although there was a higher ratio of n-6/n-3 fatty acids in the WD, it may also imply that the more the n-3 fatty acids in the diet, the less re-esterification of n-6 fatty acids, resulting in a lesser uptake into the tissues. Wainwright *et al.,* 1997 study showed that the increased levels of n-3 fatty acids in the diet increased brain DHA and decreased ARA, in pregnant and lactating B6D2F1 mice fed with a low and high ratio of n-6/n-3 fatty acids (fish oil).

For the incorporation of ∑ n-3 PUFA in the retina, the addition of PL to fish oil, did not result in a significant increase in the incorporation of ∑ n-3 PUFA compared to the FO group in the retina. It could be an indication of a suppressed LPCAT activity in the retina, however, the level of DHA was high in both the FOPL and FO, which may imply that there was an increased desaturase and elongase activity in the retina (Kulkarni *et al*., 2022). This result agreed with Vidal *et al*. (2020), who reported that n-3 fatty acids containing PL (krill oil) significantly increased the concentration of DHA in the retina in aged rats (Vidal *et al*. 2020). It could be that the oil that was enriched with PL may effectively increase the uptake of DHA in the retina. However, the FO group had higher ∑ n-3 PUFA incorporation compared to the WD, and this outcome was expected because the WD group did not contain DHA and EPA. The uptake of ALA in the retina was lower than the uptake of DHA and EPA in the FO group compared to the WD group, which agreed with the findings from Prokopiou *et al*. (2019), who reported that there was a low ratio of ALA/EPA in the plasma and retina in aged C57BL/6J mice (Prokopiou *et al*., 2019), these findings may be linked to increased desaturase and elongase enzyme activity in the retina.

Although there is scarce data showing PL's importance in the retina, some studies in mice and guinea pigs show that reducing retinal DHA levels caused retinal dysfunction (Weisinger *et*

*al*., 1998). The current result indicates that DHA may be integrated into the retina of the pig, which may be essential for healthy visual acuity.

## 4.2 TOTAL FATTY ACID DISTRIBUTION AND CONCENTRATION IN THE HEART.

My data showed that the intervention of PL caused a significant increase in the incorporation of EPA and DHA in the hearts of the FOPL group compared to the FO group. These data may explain that the ALA in the heart was highly utilized for beta-oxidation or was involved in a rapid conversion into its longer-chain derivatives (EPA, DPA, and DHA). Also, there may be an increased LPCAT activity in the heart, which caused an increased level of DHA and EPA in the FOPL compared to other experimental groups. However, these findings do not agree with other data that show low elongase-2 activity in the rat heart and hence can not effectively convert ALA to DHA (Kulkarni *et al*., 2022). The suppressed elongase-2 activity in the heart could be species-specific. One of the limitations of this study was that the desaturase and elongase activities were not measured in any of the tissues; hence, I cannot categorically say that the heart had increased elongase-2 activity. There was a significant increase in the incorporation of ∑ n-3 PUFA in the FO group compared to the WD group, and a significant increase in the incorporation of ∑ n-3 PUFA in the FOPL group compared to the FO group. The increased level of ∑ n-3 PUFA in the FO group in the heart may suggest 1) the fatty acids from circulating lipoproteins could enter the heart to a measurable extent via the VLDL receptor, or 2) there could be an enzyme substrate preference (i.e., the competition between n-6 and n-3 fatty acids), the enzyme DGAT may have a preference for the incorporation of n-3 fatty acid into the heart lipids and enzymatic pathways
(Cunnane *et al*., 1990). My data also showed a significant increase in the uptake of ALA in the heart in the FO group compared to the WD group, and there was no significant increase in the incorporation of ALA in the FOPL compared to the FO group.

The fish oil group did not result in any change in ∑ SFA compared to the WD group. However, the addition of PL to FO resulted in a significant decrease in ∑ SFA compared to the FO group. This could be because PL preferred to increase the accretion of n-3 fatty acids in the heart compared to saturated fatty acids. Also, there was a significant decrease in the incorporation of LA in the FO group compared to WD and a significant decrease in the FOPL group compared to the FO group, and this was expected as the WD group had a high ratio of n-6/n-3 fatty acid compared to the other dietary group, and there is a preferential re-esterification of n-3 fatty acid into PL than n-6 fatty acids. Therefore, giving preference to n-3 bioavailability in the heart, where they can perform their biological functions. Studies have shown that n-3 fatty acid is a promising nutrient in preventing CVD (Yang *et al.,* 2017). However, previous work from our lab showed that when all the pigs were hypertensive, the addition of FO and PL did not have any impact on 24hour blood pressure (Manoharan, 2022).

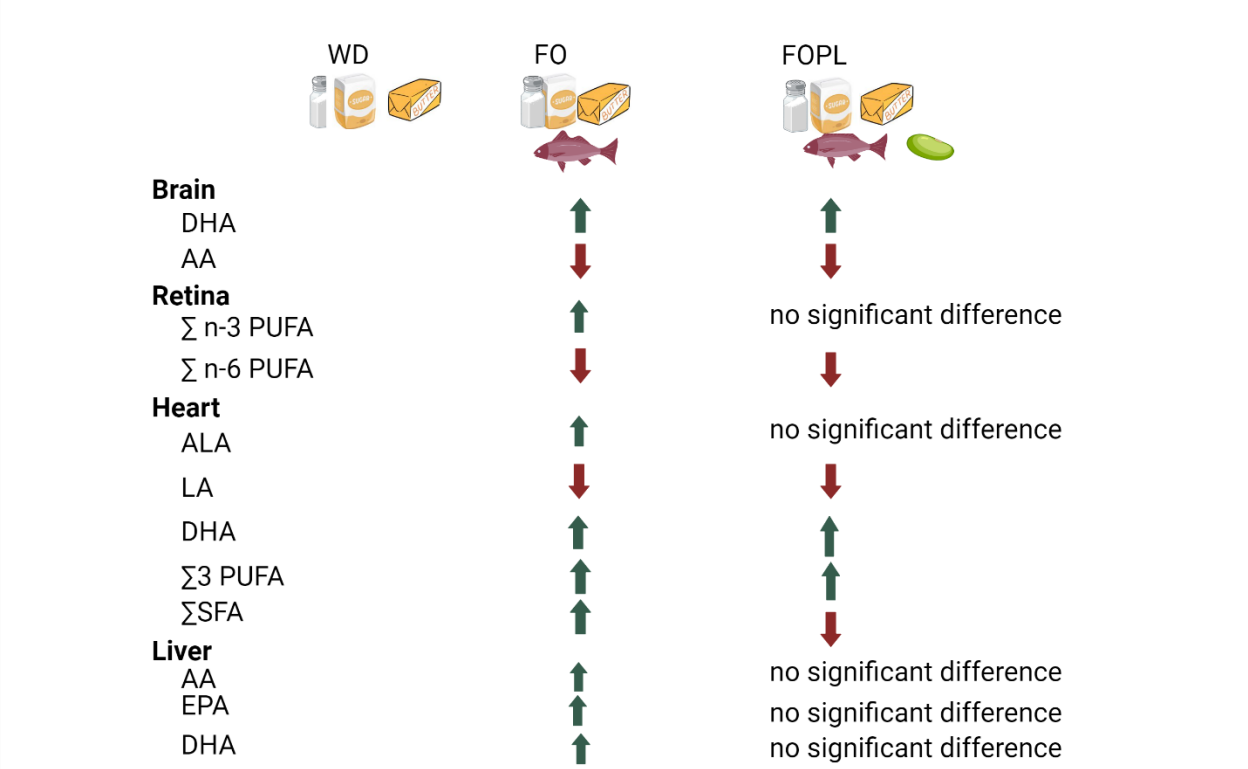


**Figure 4.1:** A summary result of the distribution of fatty acid in some organs .

## 4.3 TOTAL FATTY ACID DISTRIBUTION AND CONCENTRATION IN THE LIVER.

There was no significant increase in the incorporation of n-3 fatty acids (ALA, EPA, and DHA) in the FOPL group compared to the FO group. My result differed from Hosomi *et al*. (2019), which demonstrated a significantly higher level of n-3 fatty acid in the liver when DHA-lys phosphatidylcholine (LPC) oil was fed to rats compared to soybean oil (Hosomi *et al*., 2019). This could be because the uptake of n-3 fatty acid in the liver differs among species. Also, the level of n-3 fatty in the PL group present in the experimental diet may be higher than what I used in this current study. Studies have shown that the n-3 fatty acid bound to TAG, primarily EPA, is a poor substrate for carnitine palmitoyl transferase I (CPT I), making them less available for the betaoxidation pathway (Calder *et al*., 2009) and therefore retained for tissue accretion. Also, there was a significant increase in the incorporation of n-3 fatty acids in the liver of the FO group compared to the WD group. Since the WD group had a high ratio of n-6: n-3 fatty acid, the lower incorporation of n-3 fatty was expected.

Furthermore, my data showed a significant higher level in the uptake of DHA in the liver in the FO group compared to the WD group. I also observed that DHA had the highest uptake compared to other n-3 fatty acids in the FO group compared to the WD group, however no difference was observed when PL was added to FO in the uptake of DHA in the liver compared to the FO group. It could indicate that the liver had high desaturase and elongase activities, which may have resulted in higher DHA uptake in the liver because ALA may be easily converted into DHA in the liver.

## 4.4 DISTRIBUTION OF THE FATTY ACIDS IN THE BRAIN, RETINA, AND LIVER PHOSPHOLIPID.

Phospholipids are a structural and functional component of the cell membrane, and they maintain the structure fluidity and promote enzyme activity in the membrane (Dyall, 2015; Kuschner *et al*., 2018; and Kim *et al*., 2013(a)). They also play a significant role in lipid metabolism (Chanted *et al*., 2021). The brain and the retina have distinct fatty acid compositions, and PL is the most abundant lipid in the brain compared to other organs (Kushner *et al*., 2018). The level of PL present in the membrane is a function of the dietary lipid composition (Kim *et al*., 2013()).

My data showed a significant increase in the uptake of ∑ n-3 PUFAs (notably DHA) when PL was added to FO compared to the FO group. The significant increase in DHA in the brain agrees with previous studies (Lim *et al.,* 2005; Cardoso et al., 2013; Eldho *et al.,* 2003; Dyall *et al*., 2015). Although the mechanism has not been fully elucidated on how DHA is specifically enriched in brain phospholipids, it may be that DHA is one of the most abundant unsaturated fatty acids that maintain membrane fluidity (by reducing the packing between membrane PL) compared to other n-3 fatty acids. In contrast, SFA increases the fatty acid packing (i.e., they are closely bonded) between membrane PL, thus reducing membrane fluidity (Dyall *et al*., 2015). Additionally, since the FOPL experimental diet was enriched with supplemental PL, it is expected that ALA, and LA competition for an enzyme will be in favor of n-3 fatty acids, as PL give preference to n-3 fatty acid over n-6 fatty acids (Able *et al*., 2014), and therefore inhibit the synthesis of n-6 fatty acids in the brain.

Several studies have shown low incorporation of EPA in the brain PL (Chen *et al*., 2015; Kaur *et al*., 2013). The decreased uptake could be due to increased beta-oxidation, rapid elongation to DPA, or poor phospholipid recycling through the Land's cycle (de-acylation–reacylation reactions process) (Cummings et al., 2014; Lacombe *et al*., 2018). However, in this current study,

the level of EPA was not detected in all experimental groups. Nervonic acid (NA) is the most important fatty acid needed for myelin synthesis in the brain (Martínez, and Mougan, 1998). It bonds to sphingosine, forming sphingomyelin, a necessary component of myelination, which has been considered to improve brain development, function, and cognition (Song *et al*., 2022). Although I did not observe a significant synthesis of NA in the FO group compared to WD, my data showed a significant increase in the synthesis of NA in FOPL compared to the FO groups.

Previous investigation on fatty acid uptake in retina PL is limited. I observed no significant changes in the FO group compared to the WD group in the uptake of DHA in the retina, however, there was a significant increase in the distribution of DHA in retina PL in the FOPL compared to the FO group. The increase in FOPL DHA incorporation in the retina may be due to its degree of unsaturation or preferential membrane uptake. Phospholipid may have greater permeability into the retina PL than TAG, as the dietary fatty acid composition may also affect the tissue phospholipid accretion (Herndon et al., 2020), this agrees with other findings, showing DHA is the most abundant LCPUFA in the retina (Fliesler *et al*., 1983; Bazen et al.,1982). Also, the significant increase in the FOPL ∑ n-3 PUFAs and the significant decrease in FOPL ∑ n-6 PUFAs retina phospholipid may be due to the enzyme/substrate specificity (Kitson et al., 2016), as PL would prefer the re-esterification of n-3 fatty acid compared to n-6 fatty acid in retina PL. Studies have shown that the outer part of the photoreceptor cells has the highest level of n-3 fatty acids, which may play important biophysical and biochemical functions in visual acuity and inflammation (Jacobson and Cideciyan, 2010).

The liver is the central organ for fatty acid metabolism, and dysregulation of fatty acid composition in liver phospholipids may affect its fluidity and functionality (Ranković et al., 2017). My study showed no significant difference in the distribution of DHA in the FO group compared

to the WD group, and there were no significant changes in the distribution of DHA in the FOPL compared to the FO group, EPA was not detected in all experimental groups. It could be that EPA in the FOPL liver phospholipid has decreased expression enzymes involved in the Land's cycle, and they are channeled into fatty acid metabolism and other metabolic processes. There was no significant change in the incorporation of ∑n-6 PUFAs in the FO group compared to the WD group, while the addition of PL to FO showed a significant decrease in ∑n-6 PUFAs incorporation compared to the FO group. Perhaps the FO n-6 fatty acid in the liver may augment the n-6 fatty acid uptake rate, mostly LA, to fulfill the n-6 fatty acid PL requirement, maintain membrane integrity, and preserve the pool of eicosanoid fatty acids precursor.

Apart from the superior functionality of PL over TAG, such as the antioxidant activities, suppressing inflammation, and higher absorbability, when PL n-3 fatty acid is absorbed, it is also incorporated into HDL (Sugasini *et al*., 2017). There was an increase in the amount of DHA recovered in lymph HDL after PL-DHA absorption, and HDL has a longer half-life of about 1224 hours, and this would lead to greater tissue exposure and therapeutic advantage (Sugasini *et al.,* 2017), in contrast, TAG is incorporated into CM, which has a half-life of about 30mins. Therefore, the greater tissue exposure, the more bioavailability of n-3 fatty acids.

## 4.5 TOTAL FATTY ACID DISTRIBUTION AND CONCENTRATION IN THE PANCREAS, DORSAL SUBCUTANEOUS FAT, AND VISCERAL FAT.

. There is a paucity of data showing n-3 fatty acids accretion in the pancreas, dorsal subcutaneous fat, and visceral fat. For the pancreas, in the presence of ALA, there was a lower accretion of the LCPUFA (EPA and DHA) in both the FOPL and FO group, suggesting that the pancreas may efficiently take up ALA but does not efficiently convert ALA to EPA and DHA due to low desaturase and elongase activities in the pancreas (Kulkarni *et al*.,2022). Although there are

scarce data showing the distribution of n-3 fatty acid in the pancreas in animal studies (Waylandt *et al*., 2008), the importance of n-3 fatty acid in humans is well documented (Ding *et al*., 2018; Heller *et al*., 2004). In a randomized control trial study (n=229), the administration of n-3 fatty acids reduces the risk of new-organ failure in patients with acute pancreatitis (AP) (Wolbrink *et al*., 2020), which could be due to the anti-inflammatory properties of n-3 fatty acids. However, my data showed more ALA was incorporated into the pancreas in the FO group compared to the WD group, however, there was no significant increase in the incorporation of ALA when PL was added to FO compared to the FO group.

In the subcutaneous and visceral fat, my data showed no significant difference in the uptake of n-3 fatty acids in the FO group compared to the WD group nor between the FO and FOPL groups. It could be that these organs are not prioritized for n-3 fatty acid storage. However, in Kulkarni *et al.* (2022) study, there was a significant uptake of dietary ALA in the visceral fat of a rat (3g of soybeans for 33 days). My data also showed a higher incorporation of dietary ALA in the visceral and subcutaneous fat in the FO and FOPL groups. In these organs, the higher uptake of ALA and lower uptake of EPA and DHA suggest that both organs efficiently take up ALA but do not effectively convert ALA to its longer chain metabolites, either due to low desaturase and elongase enzyme activities (Kang *et al*., 2017) or, because these higher metabolites are not prioritized for energy storage both in the visceral and subcutaneous fat perhaps because they are easily oxidized (Kulkarni *et al*., 2019). These results agreed with a previous study, which showed no significant accumulation of n-3 fatty acids in the visceral fat and body composition when supplemented with fish oil compared to the control in overweight men consuming 4 × 500 mg capsules of fish oil for 12 weeks (Parker *et al*., 2019). However, my findings differ from Hames *et al*. (2017), who found a significant increase in EPA and DHA in the plasma and subcutaneous

adipose tissue in the group supplemented with n-3 fatty acid in a randomized, placebo-controlled, double-blind study in insulin-resistant adult (3.9g of EPA and DHA) (Hames *et al*. 2017).

However, no effect was seen in the inflammation status in the subcutaneous adipose tissue (Hames *et al*., 2017), the difference in the results may be due to the fatty acid composition in the experimental diets or differences between humans and pigs.

Myrie *et al*., (2012) showed that WD caused metabolic syndrome in pigs. Interestingly, in my current study, although there were no changes in stearic acid in the dorsal subcutaneous fat in the FO group compared to WD, there was a significant decrease in the FOPL group compared to the FO group. Studies have shown high SFA intake is associated with obesity, insulin resistance, and hepatic steatosis (Zacek *et al*., 2019; Zhou *et al*., 2020). My study agreed with Kroupova *et al*., (2020), which showed that PL- EPA and DHA had an anti-steatosis effect in male C57BL/6N mice. Also, in the visceral fat, I observed a significant decrease in the ∑ MUFAs in the FO group compared to WD, and a significant increase in the FOPL group compared to the FO group. Although no studies show that n-3 fatty acids decreased MUFA in humans and animals, TAGMUFA may be less preferentially incorporated in the visceral fat. However, the addition of PL to FO increased MUFA in the visceral fat, which may have an anti-inflammatory effect (Ravaut *et al*., 2020).

**4.6 EFFECT OF OMEGA-3 FATTY ACID ON PRO-INFLAMMATORY CYTOKINES.**

I further tested the hypothesis that the increased tissue PL n-3 fatty acid would alleviate the level of pro-inflammatory cytokines compared to the WD and FO alone. The anti-inflammatory effects of n-3 fatty acid are based on the competition with n-6 fatty acid ARA in eicosanoid synthesis (Schmitz and Ecker, 2008). The conversion of ALA and LA to their respective

eicosanoids results in competition for metabolism by the same enzymes Δ6 desaturase, elongase, and Δ5 desaturase. Therefore, increasing the n-3 fatty acid in the diet would reduce inflammatory responses in a competitive manner compared with diets containing high n-6 fatty acids. There are few studies showing the effect of PL n-3 fatty acid on pro-inflammatory cytokines *in vivo*. However, PC significantly inhibited TNF-α-induced pro-inflammatory signaling in Caco-2w cells (Treede *et al*., 2009).

According to Ramirez-Ramirez *et al.* (2013), the intake of fish oil (4g of fish oil for 12 months) decreased the concentration of pro-inflammatory cytokines (IL-6, IL-1*β,* and TNF*α*). Similarly, Upadhaya *et al*. (2015) work showed that supplementation of n-3 fatty acid reduced serum TNF-*α* in LPS-challenged pigs, and there was a decrease in pro-inflammatory cytokines concentration (IL-6, TNF-*α*) when EPA/DHA was ingested (Oliver *et al.,* 2010). However, these findings were contrary to my results, which showed a significant increase in TNF*α* concentrations in the FO group compared to WD groups (both baseline and necropsy) and a significant increase in TNF*α* concentrations in the FOPL group compared to the FO groups (both baseline and necropsy). Also, no changes were observed in the concentration of IL-6 in the FO group compared to WD groups (both baseline and necropsy), and no difference in IL-6 concentrations in the FOPL group compared to the FO groups (both baseline and necropsy). The higher concentration of TNF*α* in my result agrees with Vaisman *et al.* (2005), who reported a significant increase in the concentration of pro-inflammatory cytokines (TNF-*α*, IL-6) and anti-inflammatory cytokines and in LPS- stimulated peripheral blood mononuclear cells in n-3 fatty treated group compared with controls. Also, Petursdottir and Hardardottir (2007) found that dietary fish oil increased LPSinduced TNF-*α* secretion by murine splenocytes. Similarly, EPA and DHA increased the release of TNF-α in non-stimulated cells (Paschoal *et al*., 2013). Cormier *et al*. (2016) showed

slightly over-expressed TNFα and IL6 genes in human subjects supplemented with fish oil (5 g/day) for six weeks. However, my study looked at the baseline state of inflammation versus an LPS-stimulated (or other stimulus) experiment that looked for responses to an external inflammatory agent.

I did not see a difference in any groups, so we do not know if the background Western diet is raising the inflammatory cytokines and the n-3 fatty acids do not have any effect or if all pigs are at a low level of inflammation and the n-3 fatty acids would not have an effect anyway. The unequivocally positive effects of n-3 fatty acid were mainly obtained from *in-vitro* studies, which uses a higher concentration of n-3 fatty acid than physiological concentrations (Komprda *et al*., 2018). The anti-inflammatory potential of n-3 fatty acids may be specie specific, as the signaling pathway of PPAR*γ* ligation by n-3 fatty acid in rodents is not similar to humans and pigs (Luci *et al.,* 2007). Studies have shown that ligand-induced activation of PPAR*γ* does not decrease proinflammatory cytokines but enhances their production in weaned pigs subjected to LPS challenge (Liu *et al.,* 2009).

**LIMITATIONS AND FUTURE DIRECTIONS.**

The limitations of my study are that I only used female pigs for this study, hence, I cannot extend my conclusions to male pigs, to ascertain if the bioavailability of fish oil n-3 fatty acid with or without PL is sex-specific. Also, there was no positive control (healthy control group) in the experimental design to correlate the significant differences from the other dietary groups. This group would have helped us to achieve a better comparison between the dietary group in the inflammatory study and the bioavailability of n-3 fatty acids in most tissues evaluated. The study duration may also have impacted the data, as this was a short-term study that extended for a

maximum of 3 months, which may not have been long enough to elicit an effect in the proinflammatory outcomes and accretion of n-3 fatty acids in some tissues.

The overall goal of this research was to determine the effects of FO or a combination of FOPL in a Western diet in the accretion of n-3 fatty acid into various tissues and to evaluate the effect of PL n-3 fatty acid in decreasing serum pro-inflammatory cytokines in Yucatan miniature pigs. Although the combination of fish oil and PL to a Western diet significantly increased the distribution of n-3 fatty acids in most tissues, however, the combination of fish oil and PL to a Western diet had a negligible effect on serum cytokine concentrations. A long-term feeding trial or higher doses of fish oil and phosphatidylcholine given with a Western diet may identify whether fish oil or a combination of phosphatidylcholine with fish oil has an anti-inflammatory effect.

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