EFFECTIVENESS OF UTILIZING FISH OIL AND PHOSPHATIDYLCHOLINE IN AMELIORATING THE PROGRESSION OF METABOLIC SYNDROME IN PIGS CONSUMING A WESTERN DIET

By

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

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July 14, 2022

St. John's, Newfoundland and Labrador

Abstract

The modern Western diet has been characterized as high in calories, saturated fat, omega-6: omega-3 ratio, added sugar, and sodium. Chronic consumption of a Western diet has been identified as an essential contributor to metabolic syndrome. Metabolic syndrome is characterized by the development of insulin resistance, dyslipidemia, obesity and hypertension. Omega-3 fatty acids have been identified as nutrients that may alleviate metabolic syndrome and recent studies found that phospholipids were effective in transporting omega-3 fatty acids even when included in a diet high in saturated fat and a high omega-6 to omega-3 fatty acid ratio. Our objectives were to assess the effects of fish oil and phospholipids on the expression of metabolic syndrome outcomes in Yucatan miniature pigs. Pigs were separated into three dietary treatment groups. To induce metabolic syndrome we utilized a Western diet (WD) and added either omega-3 fatty acids via fish oil (WFO) or fish oil and phosphatidylcholine via soy lecithin (WFOL) to identify the potential benefits of these compounds in preventing metabolic syndrome. The experimental diets were fed between 71-122 days. Body parameters obtained directly from the pigs and *in vivo* metabolic test data collected via laboratory analyses of tissue samples were subjected to ANCOVA statistical analysis. There was a significant difference in fasting blood glucose concentrations during intravenous glucose tolerance testing, and an increased trend was observed in fasting plasma cholesterol concentrations in WFO compared to WD and WFOL after being on the diet for six weeks. A definitive conclusion on the dietary effects of omega-3 fatty acids in ameliorating the factors of metabolic syndrome cannot be reached with our existing data as we currently have no data to correlate the metabolic outcomes with omega-3 fatty acid incorporation to measure the effectiveness of fish oil and phosphatidyl choline combination in ameliorating the Western diet induced metabolic syndrome.

Acknowledgments

I would like to thank my supervisor Dr. Robert Bertolo, my co-supervisor, Dr. Scott Harding, and my supervisory committee members Dr. Janet Brunton and Dr. Bruce Van Vliet for their support and guidance throughout the project.

I would also like to extend my appreciation to all Brunton and Bertolo lab members and lab members of Harding's lab. Special thanks to Bimal, Raniru, Khaled, Tiffany, Katie, Hannah, Keisha and Zack for lending their support. I would also like to thank Ocean Frontier Institute and NSERC for funding me throughout the project. Lastly, I would like to express my gratitude to my family and friends, for their continuous love and support, without which it would have been impossible to complete this project.

Thesis Impact Statement

The animal experiments began in August 2018 and were completed by May 2019. Prior to COVID-19, the primary objective of our research was to identify the effects of the experimental diets on the incorporation of omega-3 fatty acids into specific target tissues. The secondary objective was to identify the effects of the experimental diets on biomarkers for obesity, glucose intolerance, insulin resistance, dyslipidemia and hypertension. The outcomes for the secondary objective were mostly collected during the animal trial and various subsequent analyses were completed before March 2020 (the hypertension data were also not analyzed due to lack of time). The analyses for the primary objective were substantially affected by laboratory disruptions caused by shutdowns associated with COVID-19 after March 2020. For example, the sample preparations for gas chromatography began in January 2020, but were not completed, nor analyzed, before facility shutdown in March 2020. Due to limitations during partial re-openings, and backlog of samples to be analyzed, our primary analyses were not completed in time for this thesis. Therefore, the secondary objective on biomarkers of metabolic syndrome became the main objective of this thesis. This alteration impacted the methods, results and discussion of the overall project.

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List of abbreviations

AA	Arachidonic acid
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
BFBW	Back fat corrected for bodyweight
CM	Chylomicron
DAG	Diacylglycerol
DGAT	Diacylglycerol *O*-acyltransferase
DHA	Docosahexaenoic acid
EE	Ethyl ester
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FI	Feed intake
HDL	High-density lipoprotein
HSL	Hormone sensitive lipase
IL-6	Interleukin-6
IST	Insulin sensitivity test
IVGTT	Intravenous glucose tolerance test
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acid
LDL	Low density lipoprotein
LPCAT	Lysophosphatidylcholine acyltransferase
LPL	Lipoprotein lipase

LysoPC	Lysophosphatidylcholine
MAG	Monoacylglycerol
MetS	Metabolic syndrome
MGAT	Monoacylglycerol *O*-acyltransferase
MUFA	Monounsaturated fatty acids
OFTT	Oral fat tolerance test
NEFA	Non-esterified fatty acid
PC	Phosphatidylcholine
PI	Phosphatidylinositol
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
SA	Stearic acid
SCFA	Short-chain fatty acid
SDA	Stearidonic acid
SFA	Saturated fatty acid
TAG	Triacylglycerol
TMS	Trimethoprim and sulfadoxine
TNF-α	Tumor necrosis factor alpha
VLDL	Very low-density lipoprotein
WD	Western diet
WFO	Fish oil Western diet
WFOL	Fish oil and phosphatidylcholine Western diet

1. Literature Review

1.1 Metabolic syndrome

Metabolic syndrome (MetS) is a cluster of conditions, including insulin resistance, dyslipidemia, hypertension, and obesity (Eckel *et al*., 2005). A dramatic increase in the number of individuals with MetS has occurred over the past few decades. However, the condition's initiation and progression remain challenging to assign to a single point in risk factor development (Saklayen, 2018). Alteration of important lifestyle factors, such as food intake and physical activity, seems to be an effective way to control the factors that are involved in the pathogenesis of MetS (Aljohani, 2014; Alkerwi *et al*., 2009; Bergmann *et al*., 2014; Cena *et al*., 2011; Chiolero *et al*., 2008; Churilla & Zoeller, 2008; Edwardson *et al*., 2012; Ford & Li, 2008; He *et al*., 2014; Ngo *et al*., 2014; Park *et al*., 2004; Petersen *et al*., 2014; Sierra-Johnson *et al*., 2008; Slagter *et al*., 2015). When assessing the impact of diet on the development of MetS, conclusions from population-level or large cohort studies and smaller intervention trials do not always agree. It is accepted, regardless of study design, that dietary patterns are a key lifestyle factor in maintaining healthy body composition (Djousse *et al*., 2010). In general, epidemiological studies have shown a greater prevalence of MetS was found among consumers of “Western”-type diets. The “Western”-type diet is considered pro-inflammatory because it tends to supply a large proportion of calories from added sugar and saturated fats, and due to a high omega-6 fatty acid to omega-3 fatty acid ratio (Esmaillzadeh *et al*., 2007; Lutsey Pamela *et al*., 2008). MetS involves activation of chronic inflammatory pathways; Western dietary components play an important role in upregulating these pathways (Monteiro, 2009). Based on these observations it is reasonable to assume that if a dietary pattern is modified to address the pro-inflammatory aspects (e.g. high sugar, high saturated fats and high omega-6 to omega-3 fatty acid ratio the initiation or progression of MetS development could be slowed.

In vivo studies suggest omega-3 fatty acids are efficient in downregulating factors associated with chronic inflammation in MetS (DiNicolantonio & O'Keefe, 2018). Omega-3 fatty acids derived from vegetable sources in the Western diet are less efficiently converted to longer chain omega-3 fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that are involved in the synthesis of molecules with anti-inflammatory potential (Stark *et al*., 2008). Even though supplemental intake of EPA and DHA from refined fish oil among the Western diet consumers is present, research suggests that it is more beneficial to consume a diet that includes whole fish which increases the bioavailability of EPA and DHA by 10-fold compared to refined fish oil supplements (Visioli *et al*., 2003). The poorer bioavailability of EPA and DHA from refined fish oil suggests that including whole fish in the Western diet might be far more effective in elevating the omega-3 status and down-regulating chronic inflammation when compared to consuming omega-3 fatty acids from vegetable sources or supplements.

1.2 Components of Metabolic Syndrome

1.2.1 Obesity

Obesity is defined by the World Health Organization as an accumulation of excessive amounts of fat in the body. Obesity can be characterized as excessive peripheral and visceral adiposity (Björntorp & Rosmond, 2000). Peripheral adiposity is defined as the deposition of fat subcutaneously and generally is considered normal when the amount of subcutaneous fat is within the healthy ranges (Garg, 2004). Visceral adiposity is defined as the deposition of fat around the organs in the abdomen and it is associated with the development of metabolic risk factors and disease conditions, like MetS (Fairburn & Brownell, 2002). Visceral fat deposits surrounding organs can lead to the secretion of inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) and excess production of these cytokines may induce

higher levels of oxidative stress in the surrounding tissues and blood vessels (Hamdy *et al*., 2006). Increased secretion of pro-inflammatory cytokines into the circulation synthesized from excess visceral fat deposits has a strong correlation with risk factors associated with metabolic syndrome (Fairburn & Brownell, 2002). Omega-3 fatty acids are well known for their anti-inflammatory effects, in particular EPA and DHA, which are involved in the production of novel resolvins, protectins and maresins (Calder, 2017). *In vitro* studies suggest that supplementation of omega-3 fatty acids is especially effective in reducing hypertrophy and hyperplasia of adipose tissue compared to saturated fatty acids (SFA), which are present in higher amounts in the Western diet fat component (Albracht-Schulte *et al*., 2018). The reduction in hypertrophy and hyperplasia decreases the chances of a cell undergoing inflammation and functional impairment (Stenkula & Erlanson-Albertsson, 2018). The reduction in size and amount of adipose tissues improves its response to insulin (Stenkula & Erlanson-Albertsson, 2018). Decreased cytokine production and improved insulin response decreases the probability of developing MetS.

1.2.2 Insulin Resistance

Insulin is a polypeptide hormone secreted by pancreatic beta cells, that is involved in the regulation of glucose metabolism (Wilcox, 2005). Insulin regulates blood glucose concentrations by increasing glucose influx into target tissues such as the liver, which acts as the primary glycogen storage organ, followed by the secondary storage site, skeletal muscle (Reaven, 1995). Excess glucose in the circulation after glycogen storage is diverted towards fat synthesis in adipose tissue (Kahn & Flier, 2000). Insulin resistance is a state in which the target tissues have reduced their ability to respond to insulin. Insulin resistance in storage tissues leads to prolonged hyperglycemia, especially after a meal when the absorbed dietary glucose rapidly enters the circulation but the clearance of glucose via influx into target tissues is impaired (Rossetti *et al*., 1990). Impaired influx along with elevated fasting glucose and slow glucose

clearance over a prolonged period could excessively stimulate pancreatic beta cells and will lead to decompensation of insulin secretion (Dubois *et al*., 2007; LeRoith, 2002). Insulin resistance impairs glycogen storage in liver tissue and skeletal muscle tissues and also impairs glucose uptake in skeletal muscle, and increases lipoprotein lipase (LPL) activity leading to excess fatty acid uptake by skeletal muscles (Wang *et al*., 2009). The excess glucose in the circulation will be directed towards triacylglycerol (TAG) synthesis in liver along with fatty acids released from peripheral adipose tissue by lipolysis caused by hormone-sensitive lipase (HSL) will be deposited in fat depots surrounding visceral organs. Pro-inflammatory cytokines further exacerbate insulin resistance in target tissues responsible for glucose uptake, leading to a vicious pattern of repetition of hyperglycemia, visceral fat deposits and pro-inflammatory cytokine release (Borst, 2004; Kim *et al*., 2009). Omega-3 fatty acids might be beneficial in preventing insulin resistance via incorporation into beta-cell membrane layers which enhances the response of insulin secretion while incorporation into peripheral tissue membranes might improve insulin sensitivity leading to a better clearance by target tissues in humans (Lardinois, 1987). Omega-3 fatty acids also increased beta-oxidation of fatty acids in visceral fat of mice fed a high fat diet, through increased enzymatic activity of carnitine palmitoyl transferase-1 along with peroxisome proliferator-activated receptor-gamma coactivator 1 alpha and nuclear respiratory factor 1 (Flachs *et al.*, 2005). Improved beta-oxidation results in fewer adipose tissue deposits surrounding visceral tissues (Albracht-Schulte *et al.*, 2018). In addition to improved beta-oxidation, omega-3 fatty acids also inhibit the lipopolysaccharide-mediated release of inflammatory cytokines, including IL-6 and TNF-α, which are involved in the development and progression of insulin resistance (Albracht-Schulte *et al*., 2018; Eder *et al*., 2009; Rydén & Arner, 2007). Improved insulin sensitivity and decrement in visceral ectopic fat tissue deposition through beta-oxidation, followed by a reduction in inflammatory cytokine

synthesis, prevents or slows the progression of obesity and insulin resistance (Flachs *et al.*, 2005; Schenk *et al.*, 2008).

1.2.3 Dyslipidemia

An abnormal blood lipid concentration is known as dyslipidemia and includes elevated triacylglycerol and low-density lipoprotein (LDL)-cholesterol along with low high-density lipoprotein (HDL)-cholesterol levels (Rodriguez-Colon Sol *et al*., 2009). Blood lipids elevate because of the inability of peripheral adipose tissues to uptake triacylglycerols that are circulating in lipoproteins due to exceeded storage capacity and impaired LPL activity (Evans *et al*., 2002; Karpe *et al*., 2011). LPL is an extracellular enzyme in the capillaries that releases fatty acids at *sn*-1 and *sn*-2 position circulating triacylglycerols from lipoproteins for adipose tissue storage (Mead *et al*., 2002). In addition to obesity, insulin resistance elevates HSL activity in adipose tissue which increases intracellular lipolysis (Karpe *et al*., 2011). The increased activity of HSL elevates circulating non-esterified fatty acids (NEFA) even during fasting states (Watt *et al*., 2005). Excess glucose due to insulin resistance cannot flow into the liver for glycogen synthesis and will be directed towards lipid synthesis. Excess lipids accumulated in the adipose tissue will further undergo lipolysis, releasing NEFA. Insulin resistance also stimulates excess production and release of large VLDL particles from the liver which further adds lipoprotein load to the circulation for clearance (Gill & Sattar, 2011). Dyslipidemia ensues due to high the NEFA released by intracellular lipolysis and increased very low-density lipoprotein (VLDL) secretion from liver and an average clearance rate caused by reduced post-prandial uptake of lipid by adipose tissue (Fielding *et al*., 1996; Heimberg *et al*., 1974; McQuaid *et al*., 2011). Postprandial lipid clearance in the liver will be further downregulated due to congestion of excess lipoproteins synthesis in the hepatocytes due to insulin resistance. Chronic accumulation of lipids within the liver and surrounding visceral tissues will further contribute to the release of pro-inflammatory cytokines leading to chronic

inflammation interfering with efficient clearance of lipid and other metabolites (Stefan *et al*., 2011).

Dietary lipid composition plays an essential role in regulating blood lipid profile (Calder, 2002). Consumption of omega-3 fatty acids increases the beta-oxidation of fat through an increase in carnitine palmitoyl transferase-1 synthesis in heart, skeletal muscles and especially in liver converting fat into energy which reduces fat deposition in visceral tissues (Flachs *et al.*, 2005; Power & Newsholme, 1997). Omega-3 fatty acids modify the response of HSL to adipose tissue macrophage infiltration and cytokines released by reducing lipolysis (Shearer *et al.*, 2012). In addition, an omega-3 fatty acid intake of 4 g/day will effectively reduce triacylglycerol levels and reduce low-density lipoprotein particle size while affecting HDL cholesterol metabolism through Apolipoprotein-E upregulation, which will correct dyslipidemia (Goldberg & Sabharwal, 2008; Poudyal *et al*., 2011).

1.2.4 Hypertension

Hypertension is defined as elevated blood pressure of systemic arteries beyond the normal range in a particular population over regular periods (Giles *et al*., 2009). Obesity, insulin resistance and dyslipidemia collectively contribute to the development of hypertension (Sowers, 1992). Increased cardiac output is commonly observed in obese individuals to meet the blood flow demanded by excess body weight beyond the healthy range (Ashraf & Baweja, 2013). The increased cardiac output contributes to hypertension in obese individuals. In addition, insulin resistance has been identified in elevating endothelin-1 secretion in endothelial cells both *in vivo* and *in vitro* models, causing vasoconstriction that elevates blood pressure (Sarafidis & Bakris, 2007). Individuals with chronically elevated blood pressure possess a higher cardiovascular risk (Kannel, 1996; Kikuya *et al*., 2007; Klag *et al*., 1996; Vasan *et al*., 2001). The chronically

elevated blood pressure could also lead to microvasculature dysfunction within various tissues (Giles *et al*., 2005). Omega-3 fatty acids could significantly reduce blood pressure by indirectly regulating the factors associated with MetS such as dyslipidemia. In addition, omega-3 fatty acids may directly alleviate hypertension by inhibition of renin secretion, angiotensin-converting enzyme inhibition, reduction of thromboxane A_2 secretion, structural modification of blood vessels and inhibition of Na^+/K^+ ATPase and Na^+/Ca^{2+} exchange (Poudyal *et al*., 2011).

1.3 Western Diet

1.3.1 Composition of Western diet

A Western diet can be characterized as a diet high in calories derived from saturated fat, with a high omega-6 fatty acid: omega-3 fatty acid ratio, high in added sugars, low in fiber, and high in sodium (Manzel *et al*., 2013; Simopoulos, 2002). The calories supplied by saturated fat with a higher omega-6 fatty acid: omega-3 fatty acid ratio and high added sugar are associated with the initiation and progression of chronic inflammation. The most common form of omega-3 fatty acid consumed in a Western diet is alpha-linolenic acid (ALA) derived from plant sources (Kris-Etherton *et al*., 2000). Omega-3 fatty acid consumed as ALA is an inefficient method of acquiring EPA and DHA as the conversion efficiency is low; the rate of ALA to EPA conversion was reported as 8%-20% and to DHA was 0.5%-9% (Stark *et al*., 2008). Regular consumption of pro-inflammatory caloric content with a high omega-6: omega-3 ratio will prime our system towards chronic inflammation contributing to the development of MetS.

1.3.2 Lipid composition and Western Diet

Saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) can be synthesized through *de novo* lipogenesis by desaturases and elongases, but mammals are less efficient in synthesizing longer chain PUFA from oleic acid (18:1n-9) due to a lack of a delta-12 desaturase

and delta-15 desaturase enzymes. However, delta-5 desaturase and delta-6 desaturase are involved in the *de novo* lipogenesis of PUFA in mammals, which results in unconventional fatty acids. The interference in further synthesis of long-chain polyunsaturated fatty acid (LCPUFA) requires the dietary intake of omega-6 LA (18:2n-6) and omega-3 ALA (18:3n-3) as precursors for the synthesis of EPA and DHA, which are long-chain PUFA and essential fatty acids (EFA). Vegetable sources of fat common in the Western diet are important for dietary EFA intake and is composed of a high LA content (e.g. corn oil, safflower oil) and lower ALA content (e.g. flaxseed oil, high oleic/high ALA canola oil). Both omega-3 fatty acids and omega-6 fatty acids compete for the same enzyme pathways responsible for the synthesis of long chain PUFAs. In a Western diet, low ALA intake means less potential to compete with LA for the delta-6-desaturase enzyme. Less ALA is converted to stearidonic acid (SDA), which is further converted into EPA and DHA. The low efficiency conversion rates of ALA to EPA and DHA lead to even less EPA and DHA assimilation when dietary LA content is high (Innes & Calder, 2018).

Dietary fat may regularly contribute up to 40% of energy in the typical Western diet and saturated fat could account for 10%-15% of the total (Weisburger, 1997). The content of saturated fat in the Western diet is higher than the recommended level, which ideally should be less than 10%. Every 1% increase of calories through saturated fat elevates LDL cholesterol by 0.031-0.049 mM (Denke, 2006). Energy contributed through MUFA and PUFA has been identified as beneficial compared to SFA (Joris & Mensink, 2016). In particular, the ratio of the daily intakes of omega-6 to omega-3 fatty acids plays a crucial role in regulating the progression of chronic low-grade inflammation. In a Western diet, the omega-6 to omega-3 fatty acid intake ratio is 40:1 ratio (Cordain *et al*., 2005; Simopoulos, 2006), whereas a healthy ratio is considered as 1:1 (Molendi-Coste *et al*., 2011; Simopoulos, 2016). Compared to vegetable

sources supplying ALA, the inclusion of whole fish as a dietary source of omega-3 fatty acids in the Western diet will help to directly improve the omega-3 fatty acid status especially, EPA and DHA, which are directly involved in the anti-inflammatory cytokine synthesis, and which could slow down the progression of chronic inflammation associated with MetS.

1.3.3 Dietary Lipids and Structure and Digestion

1.3.3.1 Triacylglycerols

TAG (triacylglycerol) is the predominant form of lipid found in our diets (Lichtenstein, 2013). TAG comprises a glycerol backbone and three fatty acyl CoA connected to the glycerol backbone's stereospecific positions *sn*-1, *sn*-2 and *sn*-3 (Lichtenstein, 2013). TAG is minimally digested by lingual and gastric lipase before reaching the small intestine (Carriere *et al*., 1993; Hamosh *et al*., 1989; Roman *et al*., 2007). The gastric and lingual lipases cleave some fatty acids from TAG producing diacylglycerol (DAG) (Chavda & Shah, 2017). TAG and DAG reach the small intestine where bile emulsifies the lipids, and pancreatic triacylglycerol lipase digests the TAG (Lichtenstein, 2013). The pancreatic lipase, an alkaline lipase, and bile secretion work to digest DAG and TAG into monoacylglycerol (2-MAG) and FFA (Brobst, 1980). Pancreatic lipase cleaves the *sn*-1 and *sn*-3 positions of TAG and DAG while the *sn*-2 position remains intact. The 2-MAG and FFA are absorbed into the intestinal cells (Buhman *et al*., 2002).

1.3.3.2 Phospholipids

Phospholipids are amphipathic molecules with a hydrophilic polar head and hydrophobic non-polar tails composed of fatty acyl CoA. Phospholipids make-up 1%-10% of the lipids in our diet (Cohn *et al*., 2010; Phan & Tso, 2001). They are also commonly found as

structural lipids in cellular membranes (Tanford, 1980). Like triacylglycerols, phospholipids have a glycerol 3-carbon backbone with *sn*-1 and *sn*-2 available for FFA and *sn*-3 position for the phosphate group (Lichtenstein, 2013). The organic compound attached to the phosphate molecule of the polar head determines the type of phospholipids. There are various types of phospholipids, depending on the organic compound attached to the phosphate molecule of the polar head. These include phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidylcholine (PC) and phosphatidylglycerol. Phosphatidylcholine is the predominant form of phospholipid in most dietary sources. Phosphatidylcholine has a choline molecule at the *sn*-3 position of the glycerol backbone. During the digestion of phospholipids in the small intestine, the *sn*-2 position acyl link is cleaved by group 1B phospholipase A_2 leading to the formation of lysophosphatidylcholine (lysoPC) and FFA. The acyl link at the *sn*-1 position of the lysoPC is cleaved by phospholipase A_1 generating 2-acyl-lysophospholipid and a fatty acid. The activity of intestinal phospholipase A_1 is not as efficient as intestinal phospholipase A_2 (Cohn *et al*., 2010). Ninety percent of the phospholipids are digested in the intestine as lysophospholipid and an unsaturated fatty acid at *sn*-2 (Zierenberg & Grundy, 1982). The 10% undigested phospholipids are assumed to be absorbed intact by the small intestine (Zierenberg & Grundy, 1982). Group 1B phospholipase in the intestine exhibits a higher preference for dietary PC. This preference increases the digestion of PC compared to other phospholipids and results in cleaving the existing fatty acid in the *sn*-2 position. The free-floating lysoPC with a hydroxyl group in the *sn*-2 position in the intestinal pool will increase the probability of absorbed omega-3 free fatty acids being re-attached in the intestinal cells to make PC enriched with omega-3 fatty acid. This process is thought to be more efficient at absorbing omega-3 fatty acids than when omega-3 fatty acids are bound to MAG.

1.3.3.3 Emulsification and transportation of fat

Lipids are non-polar and cannot directly undergo digestion or absorption in an aqueous environment. The unique process that lipids undergo at the intestinal lumen level before digestion and absorption is known as emulsification. Emulsification creates an amphipathic molecule within the intestinal lumen (Hussain, 2014). Bile salts are involved in the process of emulsification and the resulting amphipathic molecules are known as micelles (Porter *et al*., 1971). Micelles reduce the surface area and increase the water solubility of lipids, increasing the efficiency of digestion by lipases (Brobst, 1980) (Hussain, 2014). After digestion, the resulting micelle is composed of PL, cholesterol, TAG, MAG, cholesteryl esters, DAG, other lysophospholipids, fat-soluble vitamins and FFA. The digested components within the micelles such as MAG, FA, lysoPC are absorbed through the intestinal cells intact via receptor-mediated passive absorption.

1.3.4 Intestinal re-esterification and transportation of lipids

1.3.4.1 Re-esterification of TAG within the intestinal cells

The dietary TAG is 95% digested and absorbed into the intestinal cells (Pan & Hussain, 2012). After being absorbed into the intestinal cells, MAG and two FFA are used to synthesize TAG (Hiramine *et al*., 2010). The enzymes involved in the re-esterification of acyl CoA to the monoacylglycerol back bone are mainly monoacylglycerol *O*-acyltransferases (MGAT) and in the absence of MAG, fatty acids can be resynthesized through the G3P pathway which involves G3P acyltransferase, 1-acylglycerol-3-phosphate acyltransferase and phosphatidic acid phosphatase (Yen & Farese, 2003). However, the pancreatic lipase can efficiently digest triacylglycerols to form MAG and fatty acyl CoA for monoacylglycerol pathway synthesis. There are two isoforms of MGAT in the intestines, MGAT-2 and MGAT-3. MGAT -2 has a higher preference in selecting 2-MAG containing either LA (18:2n-6) or ALA(18.3n-3) at the

sn-2 position for esterification of fatty acyl CoA. MGAT-3 has a preference to conjugate either palmitic or oleic fatty acyl chain to MAG *sn*-1 or *sn*-3 position to form DAG (Yen *et al*., 2002). The DGAT enzyme involved in DAG synthesis is DGAT- 2 and it has a higher preference to conjugate free-floating LA and ALA into TAG (Zammit *et al*., 2008). The preference of these enzymes will increase the linoleic acid, alpha-linolenic acid and oleic acid to be linked to TAG, minimizing the competition for LCPUFA such as EPA and DHA to link with PC that will be readily available for efficient incorporation into tissues.

1.3.4.2 Re-esterification of phospholipids within the intestinal cells

Phospholipids are digested by group 1B pancreatic phospholipase A_2 (Murakami & Kudo, 2002). The predominant form of phospholipid in our diet is PC. Phospholipase A_2 catalyzes phosphatidylcholine forming lysoPC and a free fatty acid cleaved from the *sn*-2 position of the molecule, which usually is an unsaturated fatty acid (Murakami & Kudo, 2002). The FFAs will be conjugated to lysophospholipid in the intestinal cells by lysophospholipid acyltransferases to form phospholipids (Zhang *et al*., 2015). The LPCAT3 is the predominant enzyme in the intestine and is involved in synthesizing PC from lysoPC and has a higher preference for PUFA (Li *et al*., 2015; Zhao *et al*., 2008) . Preference for omega-3 PUFA by LPCAT3 enzyme indicates that naturally, omega-3 PUFA tends to exist and be transported within phospholipids in the biological systems for efficient incorporation. Omega-3 fatty acids, especially EPA and DHA consumed as TAG, which are cleaved by pancreatic lipase are lost in relinking within the intestinal cells due to MGAT and DGAT enzyme preferences. In the presence of lysoPC within the intestinal cells, omega-3 fatty acids, especially EPA and DHA will be efficiently linked with lysoPC. The resulting EPA- and DHA- containing phospholipids are transported in chylomicrons and will be readily available for rapid incorporation into tissues. Increased incorporation of omega-3 fatty acids in cellular membranes improves the

overall fluidity of the membranes and improves cellular responses (Wassall & Stillwell, 2008). Increasing EPA and DHA dietary content and improving overall transportation in the presence of phospholipid would be more beneficial than consuming them in TAG forms.

1.3.4.3 Intestinal clearance of FFA

Free fatty acids, 14 carbons or less are directly transferred into the portal vein (Bernard & Carlier, 1991; Hyun *et al*., 1967). The FFA of 14 carbons or less binds to albumin, enters the portal vein, directly reaches the liver (van der Vusse, 2009). The fatty acids of larger than 14 carbons need to be relinked with 2-MAG, DAG and lysoPC before incorporation and transportation via chylomicrons (Chon *et al*., 2007). Chylomicrons primarily contain TAG from the dietary origin and will be transported into lacteals and eventually transported into the circulation (Engelking, 2015). TAG and a small portions of phospholipids within the circulating chylomicrons undergo lipolysis by LPL in adipose tissue or skeletal muscles or cardiac muscles, followed by FFA uptake for storage in adipose and oxidation in muscular tissues (Pirahanchi *et al*., 2021). FFA uptake from chylomicron by LPL results in a chylomicron remnant with lower TAG levels and higher cholesterol levels and the chylomicron remnant accepts apo-E as a ligand and induces the chylomicron remnant uptake by liver. Chylomicron remnants undergo clearance in the liver by hepatic lipase (Eleftheriadou *et al*., 2008).

1.3.4.4 Plant-based omega-3 fatty acids versus fish omega-3 fatty acids

Delta-6-desaturase more efficiently acts on LA than ALA when elevated levels of LA are present (Ferreri, 2013). The PUFA content in the Western diet is mainly high in LA derived from plants and lower in ALA. ALA conversion to EPA (6%) and DHA (3.8%) is not efficient and a diet rich in omega-6 PUFA will further reduce this conversion by 40%-50% percent (Gerster, 1998). A higher concentration of omega-6 fatty acids competing with omega-3 fatty acids during the conversion will favor the synthesis of pro-inflammatory cytokines. Omega-6

cytokine derivatives are pro-inflammatory and can induce thrombosis and inflammation compared to omega-3 derivatives (Kang, 2003; Simopoulos, 2001; Simopoulos, 2008). The inclusion of whole fish in the Western diet will be beneficial in improving EPA and DHA bioavailability which will bypass the synthesis of EPA and DHA from ALA. Increased bioavailability and incorporation of potential omega-3 fatty acids EPA and DHA into tissues may slow down chronic inflammation through anti-inflammatory cytokine synthesis.

1.3.4.5 Fish oil supplements versus whole fish

Consumption of whole fish, which is typically low in the Western diet, is far more efficient in increasing the bioavailability of EPA and DHA compared to purified supplements (Visioli *et al*., 2003). The standard supplement form that occupies most of the market share is ethyl ester (EE) (Rubio-Rodríguez *et al*., 2010). Omega-3 fatty acids within EE cannot be efficiently digested by pancreatic lipase for efficient absorption (Lawson & Hughes, 1988). Provision of omega-3 fatty acids as triacylglycerol or phospholipids leads to a higher bioavailability than EE forms as they can be naturally digested and absorbed (Rubio-Rodríguez *et al*., 2010). Compared to these purified single forms of omega-3 fatty acids, consumption of whole fish increases the bioavailability of omega-3 fatty acids by 9-10 fold (Visioli *et al*., 2003). The process of refining fish oil supplements to separate omega-3 fatty acids also leads to oxidation and degradation and formation of radicals in these supplements, which can be harmful (Albert *et al*., 2013). Compared to purified fish oil supplements, which are purified to either synthetic EE or TAG or PL, whole fish includes multiple lipid components that could have a synergistic effect in increasing the bioavailability of omega-3 fatty acids (Visioli *et al*., 2003). The whole fish diet includes a combination of triacylglycerols, phospholipids and cholesterol. A recent study has shown that the addition of PC increased the bioavailability of omega-3 fatty acids even though omega-6 fatty acids were present at a higher percentage by weight in the diet

(van Wijk *et al*., 2016). This suggests that adding PC to an omega-3 fatty acid rich diet could improve the overall bioavailability of omega-3 fatty acids in Western diet consumers (van Wijk *et al*., 2016). The involvement of synergistic digestion of TAG and PL coupled with digestive and resynthesizing enzyme preferences for MAG, DAG, LysoPC and FFAs formed at the intestinal level plays a vital role in transporting the essential omega-3 fatty acids for an overall better bioavailability, compared to digestion of only triacylglycerols. Inclusion of whole fish in the diet would lead to synergistic digestion that will improve the overall omega-3 status and could ameliorate the factors associated with MetS compared to refined omega-3 fatty acid supplements.

1.4 Rationale

The Western diet is typically high in fat, sugar and salt. In addition to calories supplied via added sugar content, the fat content is predominantly composed of saturated fat and high omega-6 fatty acid content and lower omega-3 fatty acid content. The common omega-3 fatty acid consumed in the Western diet is ALA which results in less efficient conversion to EPA and DHA. Even though omega-3 fatty acids are included in the Western diet their ability to compete with SFA, MUFA and omega-6 fatty acids for absorption and transportation from the intestines is limited. Although the supplemental intake of purified fish oil provides EPA and DHA, the efficiency of both the absorption and incorporation of these omega-3 fatty acids are much higher when consumed as a whole fish meal. One component of whole fish meal is phosphatidylcholine which has been recently identified to efficiently transport and incorporate omega-3 fatty acids, especially EPA and DHA from fish oil, even when included in a diet rich in omega-6 fatty acids. The efficient absorption, transportation and incorporation of omega-3 fatty acids depend on the type of lipids, preferential positioning of these fatty acids and enzymes involved in digestion and re-esterification to the glycerol. The preferential positioning of fatty

acids at the *sn*-1 and *sn*-3 in TAG is targeted explicitly by pancreatic lipase and fatty acids at *sn*-2 positioning of PL, specifically cleaved by pancreatic phospholipase A_2. The digestive enzymes pancreatic lipase cleaves fatty acids at both the *sn*-1 and *sn*-3 of TAG and DAG, leading to 2-MAG. Pancreatic phospholipase A_2 cleaves fatty acid at the *sn*-2 position. LysoPC, 2-MAG and FFA molecules are then transported into the intestinal cells creating a pool of fatty acids and backbones for fatty acid synthesis. MGAT-2 prefers re-esterification of fatty acids to 2-MAG with LA or ALA at the *sn*-2 position. MGAT-3 prefers re-esterification of palmitoyl acyl-CoA and oleoyl acyl-CoA to the *sn*-1 and *sn*-3 positions of MAG. In addition to MGAT, DGAT-2 specifically prefers free LA, an active enzyme in converting DAG to TAG. LPCATs specifically prefer re-esterification of PUFA to LysoPC *sn*-2 position, leading to a higher probability for the omega-3 fatty acids, especially EPA and DHA, to be transported as phospholipids in the circulation, resulting in rapid incorporation. Altering the dietary fat composition of a Western diet by especially including dietary omega-3 fatty acids via fish oil and utilizing phosphatidylcholine to efficiently transport omega-3 fatty acids in Western diet might improve the overall omega-3 status of Western diet consumers and might prevent or slowdown the development of metabolic syndrome.

1.5 Hypotheses

The primary hypothesis was that providing phosphatidylcholine (i.e. soy lecithin) and fish oil in the Western diet would increase the bioavailability of omega-3 fatty acids through the body's normal digestion and re-esterification processes. The increase in the availability of omega-3 fatty acids were further hypothesized to improve MetS risk factors induced by the Western diet, when fish oil is included in the Western diet as an added fat. A secondary hypothesis was that adding fish oil and phosphatidylcholine (as soy lecithin) to the Western diet would increase omega-3 fatty acid bioavailability as phosphatidylcholine will increase the

bioavailability of omega-3 fatty acids, compared to fish oil alone. This secondary hypothesis could not be addressed due to research restrictions imposed by COVID-19.

1.7 Objective

The primary objective of this study was to determine the effects of dietary fish oil and PC on regulating each component of MetS in Yucatan Miniature pigs that were group fed over a period of 71-122 days with three experimental diets (Western diet, fish oil with the Western diet and fish oil and PC with the Western diet). Dietary omega-3 fatty acids and omega-3 fatty acids and PC in combination were expected to alleviate the conditions below.

1. Insulin resistance: Fasting plasma glucose concentration, post-prandial glucose clearance via endogenous insulin (IVGTT) and post-prandial glucose clearance via exogenous insulin (IST)
2. Dyslipidemia: Fasting plasma samples and postprandial triacylglycerol clearance (OFTT)
3. Obesity: Bodyweight and back fat thickness
4. Hypertension: Telemetry implant

The secondary objective of this study was to determine the effects of dietary fish oil and phosphatidylcholine on fatty acid assimilation in target tissues including red blood cells, liver, adipose tissue, visceral fat, brain and retina. These analyses could not be completed due to the COVID restrictions.

CHAPTER 2. Materials and Methods

2.1 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 obtained from the swine stock at the Vivarium were included in our study (Memorial University of Newfoundland). The piglets were obtained following a natural farrowing process (114 ± 3 days). The piglets were housed with the sows and were allowed to suckle colostrum and sow milk following birth for 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.

2.2 Diets and Housing

2.2.1 Grower diet

The grower diet (CO-OP Hog Grower Pellets, Atlantic Farm Services Inc, Moncton, NB) (Table 1) was introduced during the last week of suckling to slowly transition weaning. Piglets also had access to the sow's feed pellets throughout suckling, although typically had showed little interest until > 3 weeks of age. The pigs were group-housed by size, regardless of age and sex as part of normal Vivarium herd management. They were group fed restricted amounts of grower diet appropriate for growth phase (~3% of total body weights) by scattering pellets in the pen; they had free access to water. Pigs were fed grower diet in their respective groups until the start of experimental study.

Table 1. Guaranteed analysis of nutrients in grower diet by weight percentage

Nutrients	Guaranteed nutrient levels
Crude Protein (minimum)	16%
Crude fat (minimum)	3.4%
Crude Fibre (maximum)	7%
Calcium (actual)	0.7%
Phosphorous (actual)	0.68%
Sodium (actual)	0.2%
Zinc (actual)	130 mg/kg
Copper (actual)	20 mg/kg
Vitamin A (minimum)	8800 IU/kg
Vitamin D_3	1040 IU/kg
Vitamin E (minimum)	40 IU/kg

2.2.2 Experimental Diet

Individual housing for pigs was not available 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).

Table 2. Experimental dietary grouping of pigs and their total number of days on experimental diets

Batch number	WD (Pig number)	WFO (Pig number)	WFOL (Pig 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	816	101
2	820	821	819	122
3	*823	824	822	80
3	826	827	825	106

*(n=106 days on experimental diet)
WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

All pigs were females and were at least 6 months of age when they were assigned to their respective experimental group. Previous studies conducted in our laboratory pointed out that female pigs are highly susceptible to metabolic syndrome compared to males (McKnight *et al*., 2012; Myrie *et al*., 2012). Experimental diet groups (n=8 per group) were comprised of 3 of these batches (n=2 or 3 pigs per each group). Age, size and litter were considered as the primary factors when selecting individual pigs for a grouping. A maximum of 3 pigs was housed in each experimental pen to provide sufficient space for mobility and to allow socialization. These batch pens were used in sequence, so a new set of pigs was introduced after the complete removal of the previous set. This approach avoided introduction of new pigs to batches, which necessitates a stressful re-establishment of social hierarchy each time.

Individual pigs were removed from each batch to individual cages to undergo surgery, surgical recovery and *in vivo* tests, which took 2 weeks (see below). 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. Previous studies conducted by our research group used adult Yucatan minipigs which were fed a Western diet for a minimum of 4 weeks (Myrie *et al*., 2012) . We assumed that a minimum of 2.5 months was adequate to adapt to a Western diet, so that was the minimum time each grouping was left intact on experimental diet before the first set of pigs were removed. As a result, the mean total number of days (97±17 days) the pigs were fed on the experimental diets did not differ between experimental groups.

2.3 Study protocols

2.3.1 Experimental Western diets

Experimental 'Western' diets were formulated using a grower diet and adding sugar, salt and fat (Table 3). The ingredients used to formulate the base grower diet was requested from the 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).

Table 3. Weight composition of ingredients in each experimental diet

Feed Ingredient	**WD**	**WFO**	**WFOL**
Grower Diet (g/kg)	660	660	660
Sugar (g/kg)	100	100	100
Salt (g/kg)	40	40	40
Shortening (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

WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

The caloric composition of each diet is summarized in Table 4. The three experimental Western diets (WD) were formulated by adding 10% sugar (Red Path™ 20 kg/bag), 4% salt (Windsor™ hi-grade, granulated sodium-containing sodium salt and sodium ferrocyanide decahydrate and yellow prussiate soda as anti-caking; 20 kg/bag) and 20% fat (see below) by weight. 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 shortening oil (Tenderfry™, 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™), fish oil (crude fish oil, Barry Group, Inc, Corner

Brook, NL) and soy lecithin (Bulk Barn, Product code: 000943). The experimental Western diets were isocaloric (Table 4). The diet was mixed using a horizontal ribbon mixer at the Marine Bioprocessing Plant (Memorial University of Newfoundland). The processed diet was immediately stored in a -20°C freezer until used for feeding.

Table 4. Caloric composition of ingredients in each experimental diet

Components	WD	WFO	WFOL
Grower diet	1918	1918	1918
Sugar	379	379	379
Salt	0	0	0
Shortening	1080	1080	1080
Safflower oil	720	270	225
Fish oil	-	450	450
Soy lecithin	-	-	38
Total kcal/kg	**4097**	**4097**	**4091**

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) WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

The 20% of added fat by weight in the W diet was composed of shortening oil (12%) and safflower oil (8%). The 20% of the added fat by weight in the WFO diet was composed of shortening oil (12%), safflower oil (3%) and fish oil (5%). The 20% added fat by weight in the WFOL diet was composed of shortening oil (12%), 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.

Table 5. Percent composition of fatty acids by weight (%, w/w) via added fat content

	WD	WFO	WFOL
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

Percentage (%) = (fat component by weight/ total fat content) ×100
WD=Western diet (shortening oil + safflower oil), WFO= Fish oil Western diet shortening oil + safflower oil + fish oil)., WFOL=Fish oil and phosphatidylcholine Western diet (shortening oil + safflower oil + fish oil + soy lecithin).

2.3.2 Body weight measurements

The pigs were weighed once every month using a small scale capable of measuring a maximum of 15 kg (Cole-Palmer® IS30) until two months of age. After two months of age, pigs were weighed every 6 weeks on a digital scale (Weigh South Medical VS-2501) capable of weighing a maximum weight of 300 kg. After being placed on the experimental diets, pigs were weighed once every 6 weeks.

2.3.3 Fasting blood sample collection

Initial fasting blood samples (baseline) were collected before placing the pigs on their respective experimental diet. Fasting blood samples were then collected once every 6 weeks until necropsy. The pigs were fasted for 14 hours before the collection of blood samples. Pigs were flipped and restrained by two individuals in the supine position using a V-trough. The right external jugular vein was punctured using an 18 gauge needle with evacuated blood tube (BD Vacutainer®). Two 10 ml samples of blood were collected from each pig in a dipotassium ethylenediaminetetraacetic acid (K_2EDTA) (BD® 7.2 mg) tube and a serum tube (BD®). The blood samples were immediately centrifuged at 4000x*g* at 4°C and the resulting plasma and serum were pipetted using a borosilicate glass pasture pipette. The plasma and serum samples were transferred to microcentrifuge tubes (Eppendorf®) and were stored at -80°C.

2.3.4 Surgical procedures

2.3.4.1 Transportation and housing

Our *in vivo* metabolic testing facility allowed individual housing for three pigs at a time. After being on the diet for a minimum of 56 days one pig from each experimental diet group was transported to the Animal Care Unit at the Health Sciences Centre (Memorial University

of Newfoundland) for surgery, *in vivo* metabolic testing and telemetry data collection. The pigs were kept in these individual cages before and after the surgery and for all testing. After testing was completed, the pigs were transported back to the Vivarium for necropsy.

2.3.4.2 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 5-10 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.

2.3.4.3 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 was 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

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.

2.3.4.4 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, 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°C, 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.

2.3.5 *In vivo* metabolic tests

2.3.5.1 Intravenous glucose tolerance test (IVGTT)

The pigs were considered recovered from surgery after returning to their pre-surgery daily feed intake levels. The pigs were then fasted 14 hours before conducting an IVGTT. In the morning, three fasting blood samples were collected at five-minute intervals. The pigs were then infused with 50% w/v D-glucose (Sigma-Aldrich G8270) solution intravenously at a dose of 0.5 g/kg body weight through one of the venous catheters. 10 ml of heparinized saline (1 IU/ml) was used to flush the glucose that remained within the catheter. From the time the glucose was administered, samples were collected at five-minute intervals for 50 minutes. After 50 minutes, samples were then collected at 10 minute intervals until the pigs returned to their fasting blood glucose concentrations. Blood samples were collected through a catheter that was not used to administer glucose. Blood glucose concentrations were immediately measured using a digital glucometer (Contour® Next) and test strips (Contour®). The blood samples were collected in a 4 ml K_2EDTA (BD®) tube and immediately centrifuged at 4000x*g* at 4°C. The separated plasma was pipetted into microcentrifuge tubes and were stored at -80°C for glucose assay analysis.

2.3.5.2 Insulin sensitivity test (IST)

The day after the IVGTT, pigs underwent the IST. Three fasting blood samples were collected at 5 minute intervals before the administration of a glucose bolus. A bolus of dose of somatostatin (as octreotide acetate omega 500 µg/ml) was administered intravenously at a dose of 4 µg/kg through the catheter after collecting the second fasting blood sample to inhibit endogenous insulin secretion. The third fasting blood glucose sample was measured to ensure that there were no abrupt changes in the blood glucose concentration due to the administration of somatostatin. After confirming the absence of abrupt fluctuation in the blood glucose concentration a bolus dose of 50% w/v D-glucose solution was administered at a dose of 0.5 g/kg of body weight through one of the intravenous catheters. A maintenance dose of

somatostatin at a dose of 2 µg/kg of body weight was administered following the glucose bolus administration through the same catheter. A blood sample was collected through the other intravenous catheter at 3 minute intervals for 15 minutes and blood glucose concentrations were measured to identify stabilization. Somatostatin was injected repeatedly after each sample was collected. The stable blood glucose concentrations indicated that glucose clearance was inhibited by somatostatin. The exogenous insulin (Humulin® R 100 IU/ml) was administered at a dose of 5 IU/kg immediately after the blood glucose concentration stabilized. Following the administration of exogenous insulin, blood samples were collected at every 5 minute intervals. The maintenance dose of somatostatin was administered at 5 minute intervals after each blood sample was collected. The blood glucose concentrations were immediately measured using a glucometer (Contour® Next) and strips (Contour®). The blood samples were collected in 4 ml K_2EDTA tubes. The samples were centrifuged at 4000 g at 4°C and were pipetted into microcentrifuge tubes. The separated plasma was pipetted into microcentrifuge tubes and was stored at -80°C until measurement of plasma glucose concentrations and insulin concentrations.

2.3.5.3 Oral fat tolerance test (OFTT)

Two days after the IST, an oral fat tolerance test was conducted in order to measure the experimental dietary effects on postprandial triacylglycerol clearance. The pigs were fasted for 14 hours and the blood samples were collected. An oral fat bolus was provided following the fasting blood sample collection. The fat bolus for each experimental group was formulated at a dose of 1.5 g/kg of body weight. The grower diet provided 5% of the total fat content by weight across all groups. The remainder (95%) of the total fat in the fat boluses were determined by the added fat content of their respective experimental diets. The percentages of each component's contribution to the total fat in the fat boluses are listed below (Table 6).

Table 6. Percentages of each component's contribution by weight to the total fat in the fat boluses

	W	WFO	WFOL
Grower diet	5%	5%	5%
Shortening	57%	57%	57%
Safflower oil	38%	14.2%	11.8%
Fish oil	-	23.8%	23.8%
Soy lecithin	-		2.4%

WD=Western diet, WFO=Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

After collecting the fasting blood sample, the fat boluses were provided and an hour was given to consume at least 80% of the bolus. The pigs were given an additional hour if they had not consumed the minimum requirement. The OFTT was only performed on pigs which consumed 80% of the bolus within either one or two hours. A 10 ml blood sample was collected following the consumption of 80% of the fat bolus. Deuterium oxide (D_2O) was then administered at a dose of 1 ml/kg to determine fatty acid synthesis rates (analyses not

completed). Blood samples were collected at hourly intervals for a total of 11 hours. Blood samples were collected in K_2EDTA (BD® 7.2 mg) tubes. Immediately after collection the samples were centrifuged at 4000xg for two minutes and the plasma was pipetted and was transferred into microcentrifuge tubes and were stored at -80°C.

2.3.6 Cardiovascular and activity monitoring

The telemetry system monitored the cardiovascular parameters along with an index of locomotor activity over two circadian cycles (48 hours). The three pigs each belonging to an experimental group (WD, WFO and WFOL) were simultaneously monitored by receivers in the room. The signals emitted by each wireless transmitter was simultaneously picked up by the four receivers (DSI PhysioTel™ Digital Transceiver TRX-1 series, Data Sciences International, St. Paul, MN) which were placed around the cages for optimal signal reception. The received signals passed through the communication link controller (CLC) to E2S-1 (Data Sciences International, St. Paul, MN). The signals were then passed to an ambient pressure reference (APR-1, Data Sciences International, St. Paul, MN) which assisted the system to calculate the arterial blood pressure as the absolute pressure recorded by the telemeter. In addition to the blood pressure, heart rate (HR), systolic arterial pressure (SAP), diastolic arterial pressure (DAP) and mean arterial pressure (MAP), locomotor activity level and body temperature were monitored. The data collected were processed by DSI software (Ponemah® version 6.33 Data Sciences International, St. Paul, MN). The wireless transmitters were calibrated prior to implantation and after removal. Due to time constraints, the acquired data were not processed in time for this thesis but will be analyzed by other students.

2.3.7 Necropsy

Following the completion of metabolic tests and telemetry data collection, the pigs were transferred back to the Vivarium (Memorial University of Newfoundland). The pigs were placed on the experimental diet for three to four days until necropsy. They were fasted for 14 hours prior to necropsy. After fasting, pigs were anesthetized by administering sodium pentobarbital (Euthanyl, Biomeda-MTC 240 mg/ml) intravenously at a dose of 50 mg/kg of body weight. Anesthetized pigs were intubated and ventilated during the necropsy. Blood samples were collected using vaccutainer needles in K_2EDTA tubes and serum tubes via cardiac puncture. Tissues including the pancreas, liver, spleen, stomach, visceral fat, subcutaneous fat, small and large intestines, muscle (longissimus dorsi, gastrocnemius), heart, aorta, brain and retina were removed, weighed and samples collected in freezer bags (Fisherbrand™). The freezer bags were immediately stored in a liquid nitrogen container and later transferred to a -80°C freezer. A sample of the pancreas, liver, duodenum, heart and aorta were stored in a solution of 10% neutral-buffered formalin (Vavantor™, 89370-094). The telemeters and catheters were removed from the pigs at the end of necropsy.

2.3.8 Biochemical analyses

The samples were stored at -80°C and were thawed on ice before transferring to the assay plate wells for biochemical analysis. The plasma glucose concentrations were measured before the introduction of the experimental diets, after 6 weeks on the experimental diet, at necropsy, at IVGTT and IST. The plasma glucose concentrations were measured using an assay kit and 96-well microplates (Non-Treated, Polystyrene, Clear, Flat-Bottom, Non-Sterile, Greiner Bio-One 655101). The reagent was prepared using glucose oxidase peroxidase reagent (Sigma-Aldrich® G3660-1CAP) and O-dianisidine dihydrochloride (Sigma-Aldrich D3252-5G) as instructed by the manufacturer. The reagent was kept on ice along with the samples and

standards. The reagent was added to the samples and standards in the microplate and was incubated at 37°C for 30 minutes. Readings were obtained at 450 nm wavelength. The plasma triacylglycerol concentrations were measured using an assay kit (Sekisui Diagnostics™ 236-60). A series of dilutions was prepared using the triacylglycerol standard (250 mg/dL, Sigma-Aldrich® G7793). The reagent was added to the samples and standards and were incubated at 37°C and shaken for 10 minutes at 70 RPM. Readings were obtained at 500 nm wavelength. The total plasma cholesterol concentrations were also measured using an assay kit (Sekisui Diagnostics™ 234-60). A series of dilutions was prepared using the cholesterol standard (200 mg/dL) included in the kit. The reagent was added to the samples and standards and were incubated/shaken at 37°C at 70 RPM. Readings were obtained at 505 nm wavelength.

All samples in the three assays were measured in duplicate. Readings from the incubated samples and standards were obtained using a microplate reader (Biotek's SynergyTM Mx Fluorescence, UV/Vis Plate Reader). Duplicated readings that exceeded the covariance limit of 10% were repeated. The plasma glucose concentrations, plasma triacylglycerol concentrations and plasma cholesterol concentrations were calculated using the equations derived from the standard graphs, which were unique for each microplate.

2.3.8 Statistical analyses

The data were analyzed using SPSS (19.0) software. A one-way analysis of covariance (ANCOVA) and one-way analysis of variance (ANOVA) were performed on a general linear model after accounting for variance caused by covariates and nominal factors (Table 7). The area under the curve (AUC) was calculated using the GraphPad PRISM (7.0). Even though certain covariates were not significantly correlated, they were included in the analysis due to their significance in previous studies. Body weight was given priority over age since litter was

already included as a nominal factor. Litter is interchangeable with age when both these factors are considered as a nominal factor. The P-value of significance was determined as 0.05 for both correlations and ANCOVA. A P-value of 0.1 was considered a trend in ANCOVA. The analyzed data were expressed as mean ± standard error (SE).

Table 7. Nominal factors and covariates included in ANCOVA analysis

Analysis	Nominal Factor	Covariates	Interactions
Growth rate (Baseline to 6 weeks) (6 weeks to necropsy)	Diet Litter	-	-
Abdominal circumference	Diet Litter	Bodyweight Days on diet	Diet × Days on diet
BFBW	Diet Litter	Days on diet	Diet × Days on diet
Baseline Fasting plasma concentration (glucose, triacylglycerols and cholesterol)	Diet Litter	Body weight	-
6 weeks Fasting plasma concentration (glucose, triacylglycerols and cholesterol)	Diet Litter	Body weight	-
Necropsy Fasting plasma concentration (glucose, triacylglycerols and cholesterol)	Diet Litter	Days on diet BFBW	Diet × Days on diet
IVGTT IST	Diet Litter	Bodyweight Days on diet	Diet × Days on diet
OFTT	Diet Litter	Bodyweight Days on diet	Diet × Days on diet

BFBW (backfat thickness corrected per kg of body weight), IVGTT (intravenous glucose tolerance test), IST (insulin sensitivity test), OFTT (oral fat tolerance test). Diet (WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet)

Chapter 3: Results

3.1 Dietary effects on body composition and growth

There were no significant differences in the body composition and growth among the groups at any of the three timepoints (Table 8). The growth rates were measured between baseline and six weeks and between six weeks and necropsy. The growth rates at these two intervals were compared across the groups using non-repeated measures one-way ANOVA which also includes the litter effect (Table 7).

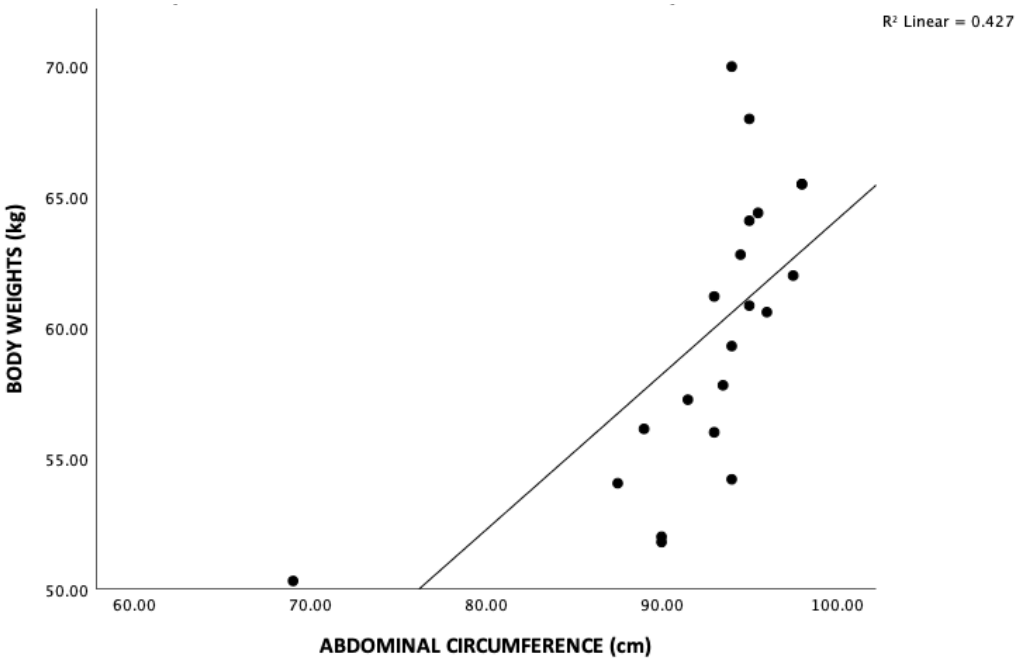
Table 8. Body parameters analysis of experimental groups

Growth Data	WD	WFO	WFOL	P-value	**Covariates**
Growth rate (Baseline to 6 weeks) (kg/day)	0.30 ± 0.035	0.30 ± 0.019	0.30 ± 0.030	0.899	-
Growth rate (6 weeks to necropsy (kg/day)	0.12 ± 0.028	0.105 ± 0.0453	0.10 ± 0.035	0.992	-
Abdominal circumference at necropsy (cm)	91.31 ± 1.085	91.19 ± 3.438	92.1 ± 1.196	0.14	Body weights
BFBW (mm/kg)	1.05 ± 0.0291	1.05 ± 0.0651	1.02 ± 0.0394	0.99	-

Note: data are means ± SE, n=8 pigs per group
BFBW (back fat thickness corrected for body weights)
WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

There were no significant differences in mean growth rates among the groups at both intervals. In addition to the litter effect, the non-repeated measures one-way ANCOVA analysis of abdominal circumference at necropsy across groups, accounted for the effects caused by bodyweights, days on diet and an interaction factor (diet × days on diet) (Table 7). Body weights measured at necropsy were positively correlated with the abdominal circumference (R^2=0.427, P=0.001) (Fig. 1).

Fig 1: Correlations between measurements of bodyweights and abdominal circumference at necropsy



However, there was no significant difference in mean abdominal circumference among the groups at necropsy. The non-repeated measures one-way ANCOVA analysis of BFBW across groups accounted for the effects caused by litter, days on diet and an interaction factor

(diet × days on diet) and there was no significant difference among the groups at necropsy. The expected and observed results of body parameters analysis are summarized below (Table 9).

Table 9. Summary of expected and observed results from growth data measurements

	Expected Results	Observed Results
Growth rate (Baseline to 6 weeks)	Significantly higher growth rate after 6 weeks on the diet (WD>WFO>WFOL)	There was no significant difference among the groups.
Growth rate (6 weeks to necropsy)	Significantly higher growth rate (WD>WFO>WFOL)	There was no significant difference among the groups.
Abdominal circumference	Significantly higher abdominal circumference (WD>WFO>WFOL)	There was no significant difference among the groups.
BFBW (back fat thickness corrected for body weight)	Significantly higher BFBW (WD>WFO>WFOL)	There was no significant difference among the groups.

3.2 Dietary effects on fasting plasma glucose

The fasting plasma glucose concentrations across the groups within a time point were compared using one-way ANCOVA. The mean plasma glucose concentrations of each group at all three time points are illustrated (Fig.2). There were no significant differences among the groups at all three time points (Table 10).

Table 10. Fasting plasma glucose concentration (mM) of experimental groups at baseline, 6 weeks and necropsy

Plasma glucose (mM)	WD	WFO	WFOL	P-value	Significant covariates
Baseline (mM)	9.23 ± 1.02	9.54 ± 0.701	11.3 ± 1.63	0.789	
6 weeks (mM)	9.90 ± 0.609	9.46 ± 0.774	11.7 ± 1.31	0.131	
Necropsy (mM)	18.9 ± 4.19	16.9 ± 2.59	14.9 ± 4.14	0.899	BFBW

Note: data are means ± SE, n=8 pigs, necropsy n=7 pigs
BFBW (backfat thickness corrected for bodyweights)
WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

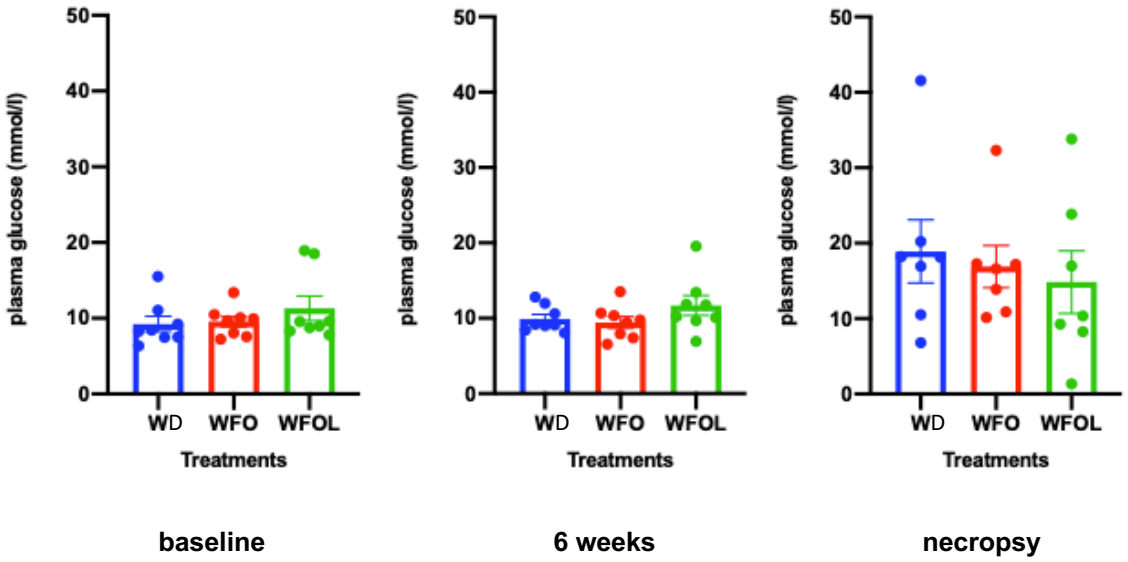
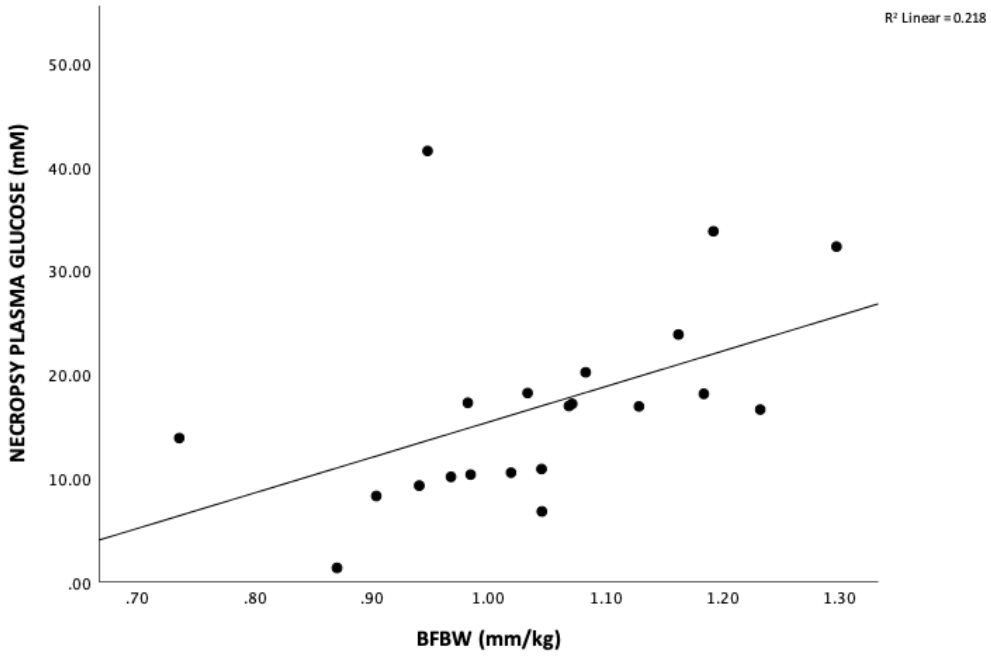


Fig 2: Mean plasma glucose concentrations in each experimental group during baseline, 6 weeks and necropsy. Experimental groups: WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet. Note: Each bar represents the mean ± SE in n=8 pigs, except in necropsy n=7 pigs.

In addition to the litter effect, the one-way ANCOVA analysis of fasting plasma glucose concentrations at necropsy accounted for effects from days on diet and the interaction factor (diet × days on diet) (Table 7). The back fat thickness corrected for body weight provided an approximate distribution of fat per kilogram of total body weight in pigs which is a more accurate representation of obesity rather than depending on body weight. BFBW replaced body weight as a covariate in the one-way ANCOVA analysis of necropsy plasma glucose concentrations across the groups as it could only be measured during necropsy. The BFBW, which is an accurate indicator of total body fat distribution and obesity in Yucatan miniature pigs, was considered over body weight and it was positively correlated with plasma glucose concentrations at necropsy (R^2=0.218, P=0.033) (Fig. 3). The expected and observed results of fasting plasma glucose analysis are summarized below (Table 9).

Fig 3: Correlations between measurements of necropsy plasma glucose concentrations and backfat thickness corrected per kg of body weight during necropsy



3.3 Dietary effects on IVGTT and IST outcomes

Body weight was negatively correlated with fasting blood glucose concentrations during IVGTT (R^2=0.440, P=0.000) (Fig. 5). The IVGTT outcomes and the slopes of clearance during IST were compared across the groups using one-way ANCOVA and there were no significant differences between the groups, with the exception of fasting blood glucose concentrations (Table 11). The mean blood glucose concentrations of all three groups are illustrated below (Fig.4). The expected and observed results of IVGTT and IST analyses are summarized below (Table 9).

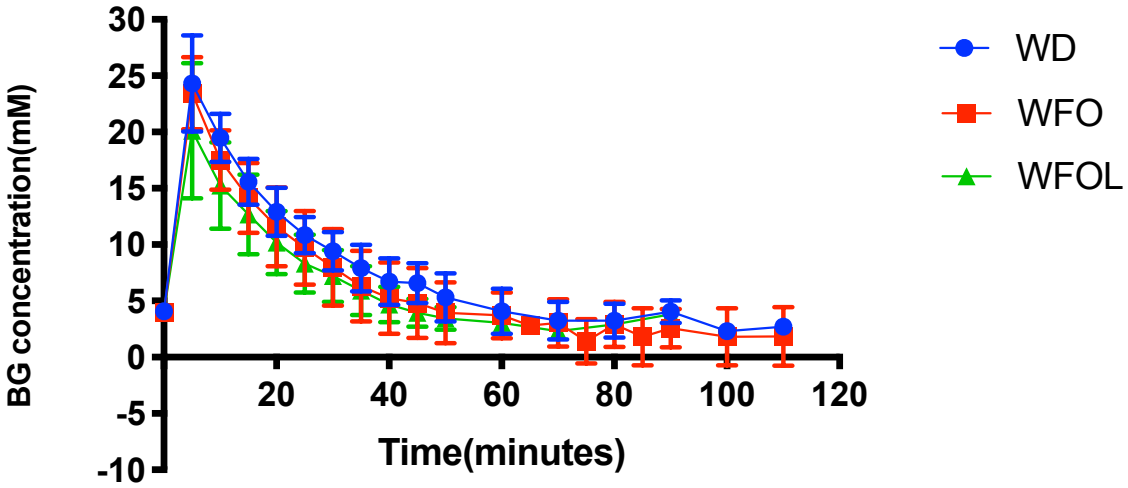


Fig 4: Mean blood glucose concentrations in each experimental group during intravenous glucose tolerance test (IVGTT). Experimental groups: WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet. Note: Each symbol represents the mean ± SE (n=8)

Fig 5: Correlations between measurements of baseline blood glucose concentrations and body weights during intravenous glucose tolerance test (IVGTT)

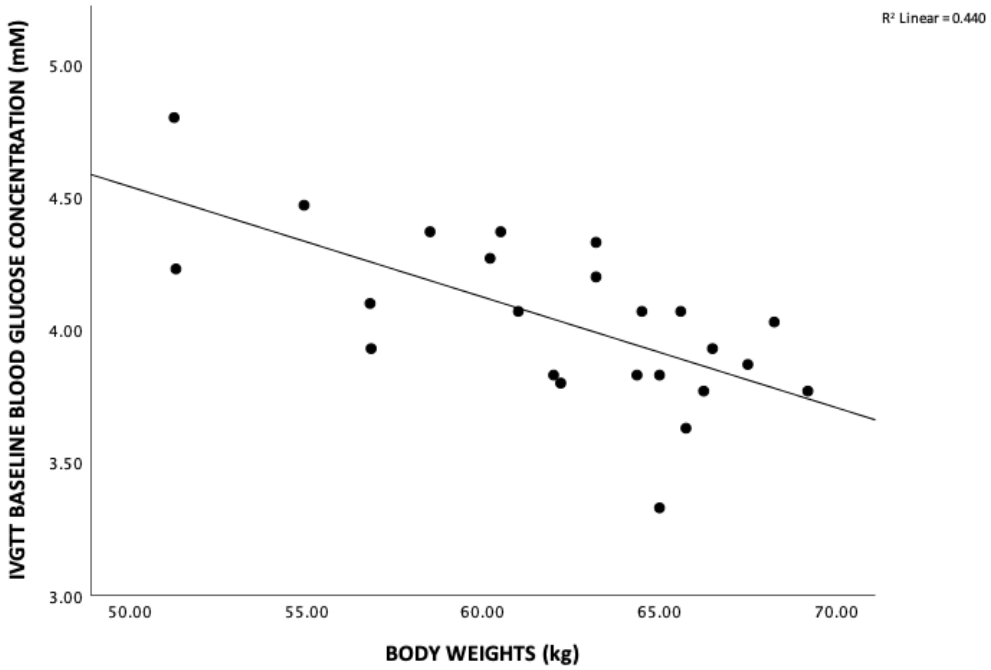


Table 11. Measurements of glucose tolerance derived from intravenous glucose tolerance tests (IVGTT) and insulin sensitivity test (IST)

IVGTT	WD	WFO	WFOL	P-value	Significant covariates
Fasting plasma glucose (mM)	6.39 ± 0.580	5.92 ± 0.466	6.84 ± 0.626	0.429	-
Fasting blood glucose (mM)	4.08 ± 0.137	3.95 ± 0.0594	4.09 ± 0.125	0.035*	Body weights
Peak blood glucose (mM)	24.3 ± 1.51	20.1 ± 2.13	23.5 ± 1.13	0.758	-
AUC (mM **x** minutes)	402.6 ± 34.58	269.1 ± 39.43	340.9 ± 56.92	0.807	-
Time to return to fasting blood glucose (minutes)	58.6 ± 5.26	44.5 ± 2.98	47.3 ± 7.25	0.648	-
Slope of glucose clearance (ln(glucose)× $minutes^{-1}$)	-0.031 ± 0.003	-0.040 ± 0.0052	-0.045 ± 0.0052	0.485	-
IST					
Slope of glucose clearance (ln(glucose)× $minutes^{-1}$)	0.009 ± 0.003	0.011 ± 0.002	0.009 ± 0.001	0.284	-

Note: data are means ± SE, n=8 pig
*Data are different than W pigs, P≤ 0.05 as assessed by ANCOVA
AUC (area under curve), IST (insulin sensitivity test)

Table 12. Summary of expected and observed results from fasting plasma glucose analysis, intravenous glucose tolerance test (IVGTT), insulin sensitivity test (IST)

	Expected Results	Observed Results
Fasting plasma glucose analysis	Significantly elevated plasma glucose concentration after 6 weeks and at necropsy (WD>WFO>WFOL)	There was no significant difference among the groups.
Intravenous glucose tolerance test (IVGTT)	Significantly elevated fasting baseline blood glucose concentration (WD>WFO>WFOL)	A significant difference in fasting baseline blood glucose (P=0.035) (WFOL>WD>WFO)
	Significantly larger AUC (WD>WFO>WFOL)	There was no significant difference among the groups.
	Significantly faster clearance rates (WFOL>WFO>W)	There was no significant difference among the groups.
	Significantly shorter time to return to baseline (WFOL>WFO>W)	There was no significant difference among the groups.
Insulin sensitivity test (IST)	Significantly faster blood glucose clearance rates (WFOL>WFO>W)	There was no significant difference among the groups.

3.4 Dietary effects on fasting plasma triacylglycerols

The fasting plasma triacylglycerol concentrations at each time point were compared across the groups using one-way ANCOVA. The mean plasma triacylglycerol concentrations of each group at all three time points are illustrated (Fig.6). There were no significant differences among the groups at any of the three time points (Table 13).). The expected and observed results of fasting plasma triacylglycerol are summarized below (Table 15).

Table 13. Fasting plasma triacylglycerol concentration (mM) of experimental groups at baseline, 6 weeks and necropsy

Plasma TG (mM)	W	WFO	WFOL	P-value
Baseline (mM)	0.39 ± 0.056	0.53 ± 0.11	0.41 ± 0.052	0.228
6 weeks (mM)	0.51 ± 0.065	0.50 ± 0.060	0.52 ± 0.070	0.908
Necropsy (mM)	0.54 ± 0.079	0.41 ± 0.032	0.52 ± 0.12	0.256

Note: data are means ± SE, n=8 pigs, except necropsy n=7 pigs
WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

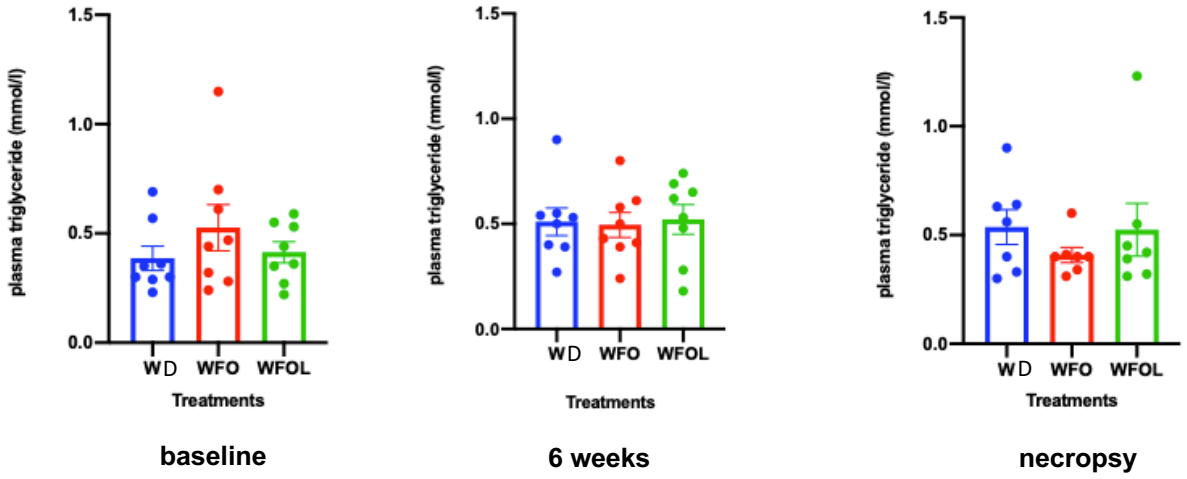


Fig 6: Mean plasma triacylglycerol concentrations in each experimental group during baseline, 6 weeks and necropsy. Experimental groups: WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet. Note: Each bar represents the mean ± SE in n=8 pigs, except in necropsy n=7 pigs.

In addition to litter effect, the one-way ANCOVA analysis of fasting plasma triacylglycerol concentrations at necropsy accounted for effects from days on diet and an interaction factor (diet × days on diet) (Table 7). Because BFBW is an indicator of total body fat and obesity in Yucatan miniature pigs, BFBW replaced bodyweights as a covariate in the one-way ANCOVA analysis of necropsy plasma triacylglycerol concentrations across groups (Table 7).

3.5 Dietary effects on OFTT outcomes

The mean plasma triacylglycerol concentrations of each group at all timepoints is illustrated below (Fig 7). The OFTT outcomes were compared across the groups using one-way ANCOVA and there were no significant differences among the groups (Table 14). In addition to the effects caused by litter and bodyweights, the one-way ANCOVA analyses of OFTT outcomes accounted for the effects from days on diet and an interaction factor (diet × days on diet) (Table 7). The expected and observed results of OFTT analysis are summarized below (Table 15).

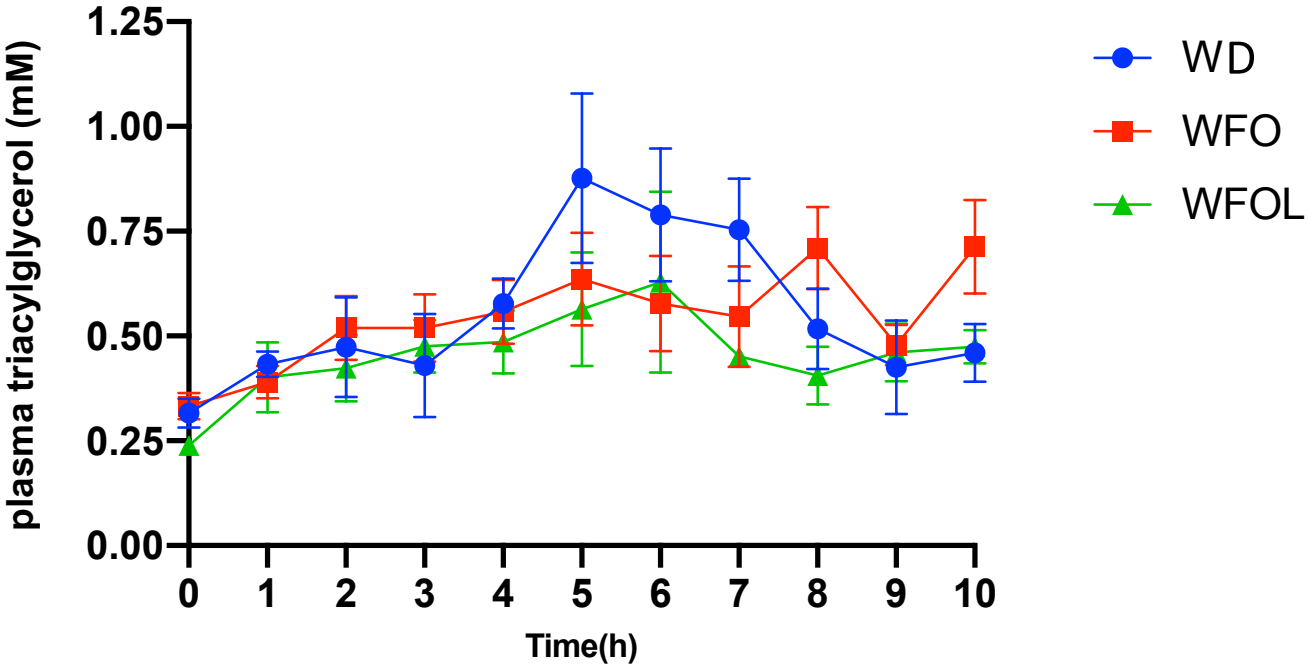


Fig 7: Mean plasma triacylglycerol concentrations in each experimental group during oral fat tolerance test (OFTT). Experimental groups: WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet. Note: Each symbol represents the mean ± SE (WD=5, WFO=7, WFOL=6).

Table 14. Measurements of fat tolerance derived from fat tolerance test (OFTT)

Parameter	W(n=5)	WFO(n= 7)	WFOL(n=6)	P-value
Fasting plasma triacylglycerol (mM)	0.31 ± 0.034	0.31 ± 0.033	0.24 ± 0.012	0.777
Peak plasma triacylglycerol (mM)	5.2 ± 1.5	4.8 ± 0.91	5.8 ± 1.5	0.110
Adjusted Peak (mM)	0.55 ± 0.11	0.50 ± 0.11	0.49 ± 0.18	0.440
Adjusted AUC (mM **x** h)	2.2 ± 0.44	2.4 ± 0.49	1.9 ± 0.66	0.958

Note: data are means ± SE
AUC (area under curve)
WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

Table 15. Summary of expected and observed results from fasting plasma triacylglycerol analysis and oral fat tolerance test (OFTT)

	Expected Results	Observed Results
Fasting plasma triacylglycerol analysis	Significantly elevated fasting plasma triacylglycerol concentration at 6 weeks and necropsy (WD>WFO>WFOL)	There was no significant difference among the groups
Oral fat tolerance test (OFTT)	Significantly elevated fasting plasma triacylglycerol concentration at baseline (WD>WFO>WFOL) Significantly large adjusted AUC (WD>WFO>WFOL) Significantly adjusted peak (WD>WFO>WFOL)	There was no significant difference among the groups There was no significant difference among the groups There was no significant difference among the groups

3.6 Dietary effects on fasting plasma cholesterol

The fasting plasma cholesterol concentrations at each time point were compared across the groups using one-way ANCOVA. The mean plasma triacylglycerol concentrations of all groups at all three time points are illustrated below (Fig.8). There were no significant differences among the groups at any of the three time points (Table 16). The expected and observed results of fasting plasma cholesterol analyses are summarized below (Table 17).

Table 16. Fasting plasma cholesterol concentration (mM) of experimental groups at baseline, 6 weeks and necropsy

Plasma cholesterol	W	WFO	WFOL	P-value	Covariates
Baseline (mM)	2.01 ± 0.277	2.37 ± 0.366	2.26 ± 0.363	0.243	
6 weeks (mM)	2.35 ± 0.346	3.36 ± 0.557	2.32 ± 0.236	0.081	
Necropsy (mM)	2.62 ± 0.121	3.10 ± 0.270	2.80 ± 0.180	0.255	BFBW

Note: data are means ± SE, n=8 pigs, except necropsy n=7 pig WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet

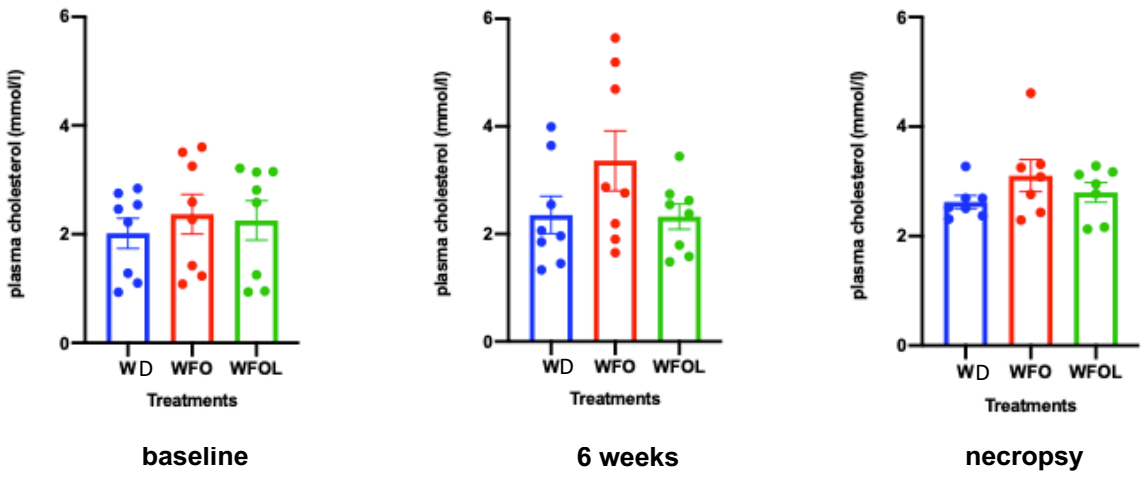


Fig 8: Mean plasma cholesterol concentrations in each experimental group during baseline, 6 weeks and necropsy. Experimental groups: WD= Western diet, WFO= Fish oil Western diet, WFOL=Fish oil and phosphatidylcholine Western diet. Note: Each bar represents the mean ± SE in n=8 pigs, except in necropsy n=7 pigs.

In addition to litter effect, the one-way ANCOVA analysis of fasting plasma cholesterol concentrations at necropsy also accounted for effects from days on diet and an interaction factor (diet × days on diet) (Table 7). The BFBW, which is an indicator of total body fat distribution and obesity in Yucatan miniature pigs, replaced bodyweights as a covariate in the one-way ANCOVA analysis of necropsy plasma cholesterol concentrations across groups. The BFBW was negatively correlated with plasma cholesterol concentrations at necropsy (R^2=0.242, P=0.024) (Fig. 9).

Fig 9: Correlations between measurements of plasma cholesterol concentrations and BFBW (backfat thickness corrected per kg of body weight) during necropsy

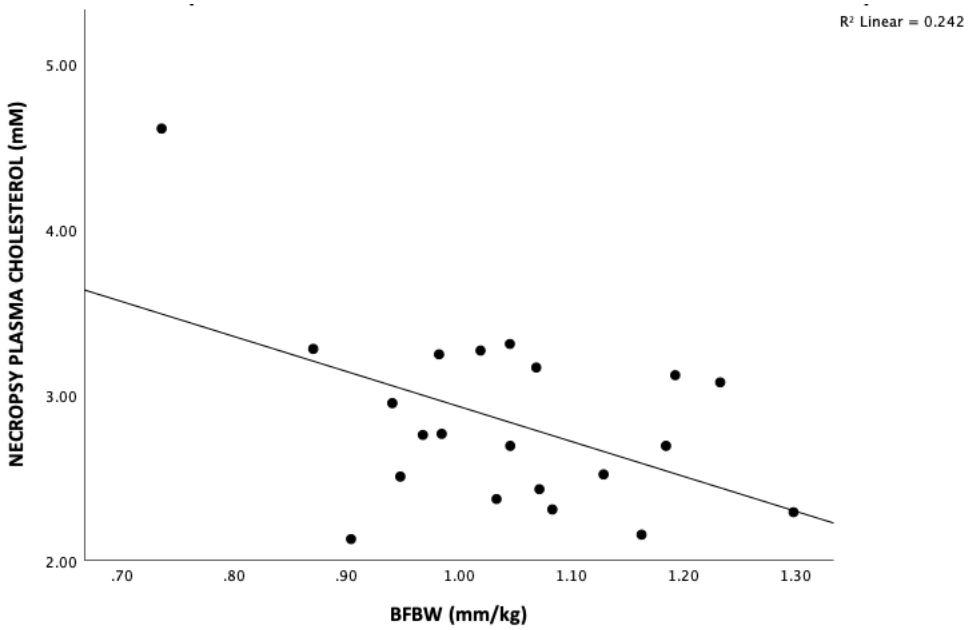


Table 17. Summary of expected and observed results from fasting plasma cholesterol analysis

	Expected Results	Observed Results
Fasting plasma cholesterol analysis	Significantly elevated fasting plasma cholesterol levels at 6 weeks and necropsy (WD>WFO>WFOL)	There was no significant difference among the groups but a trend of increased mean plasma cholesterol concentration was observed after 6 weeks on the WFO diet

Chapter 4: Discussion

The chronic consumption of a Western diet is detrimental to health. The initiation and progression of chronic inflammatory mechanisms induced by obesity may lead to further development of insulin resistance, dyslipidemia and hypertension. *In vivo* studies have identified that omega-3 fatty acids are beneficial in alleviating these outcomes both individually and collectively (de Camargo Talon *et al*., 2015). However, ALA derived from plant sources is the common form of omega-3 fatty acid consumed in the Western diet. Alpha-linolenic acid needs to compete with the higher omega-6 content for the same enzyme, in order to be converted to potent omega-3 fatty acids such as EPA and DHA. The lower levels of ALA in the Western di*et al*ong with a lower conversion efficiency from ALA to EPA and DHA results in decreased assimilation of EPA and DHA, which are precursors for anti-inflammatory cytokine synthesis. Although the supplemental intake of purified fish oil provides EPA and DHA, the efficiency of both the absorption and incorporation of these omega-3 fatty acids are much higher when consumed as a whole fish meal (Visioli *et al*., 2003). One component of the whole fish meal is phosphatidylcholine, which has been recently identified to efficiently transport and incorporate omega-3 fatty acids, especially EPA and DHA from fish oil, even when included in a diet rich in omega-6 fatty acids (van Wijk *et al*., 2016). Based on these previous studies, we hypothesized that the effect of combining fish oil and phosphatidylcholine in a Western diet may alleviate the factors of metabolic syndrome compared to only including fish oil in a Western diet or to a standard Western di*et al*one. The secondary hypothesis of our research was that the experimental dietary effects on the factors of metabolic syndrome may depend on the systemic distribution and incorporation of omega-3 fatty acids, especially EPA and DHA.

However, we found no significant differences in most metabolic syndrome outcomes in the current study. The mean fasting blood glucose concentrations among the groups only differed significantly during IVGTT, while the other outcomes related to glucose tolerance or insulin sensitivity were not significantly different among the groups. In addition, there were no significant differences among the groups in terms of both the fasting plasma triacylglycerol concentrations at all three time points as well as the OFTT outcomes. Although the mean plasma triacylglycerol concentration of the fish oil group (WFO) declined throughout the study, this decline was not observed in the other two groups. However, the plasma triacylglycerol concentrations were not analyzed within the groups due to the difference in total number of days on diet. The mean plasma cholesterol concentrations of the fish oil group (WFO) indicated an elevated trend after being on the diet for 6 weeks.

4.1 Body parameters

The consumption of a Western diet over a chronic period leads to the development of obesity (Rakhra *et al*., 2020). Each of our experimental groups was consuming a modified version of the Western diet. A combination of fish oil and phosphatidylcholine, when included in the Western diet, was expected to slow down the development of obesity, as it was expected to efficiently transport omega-3 fatty acids compared to only including fish oil in a Western diet or the standard Western diet. However, the combination of fish oil and phosphatidylcholine did not significantly affect the body weight growth rates that were measured after being on the diet for 6 weeks and between 6 weeks and necropsy. The absence of significant differences in growth rates may be due to the equal dilution of the nutrients by the added fat content in our experimental diets. In other words, all groups may have gained weight due to the high fat content of the Western diet, masking any beneficial effects from fish oil and/or phosphatidylcholine. However, the shortened duration on the experimental diet, as well as the

Western diet composition, may not have allowed the preventive effects of the fish oil in alleviating the factors of MetS, with or without phosphatidylcholine. In addition, diet intake plays a primary role in the development of obesity. Although individual diet intakes were periodically measured, available resources made it necessary to group house the pigs and daily individual intake in each group likely varied depending on the established social hierarchy in the group, which cannot be measured. In addition, the pigs were healthy young adults and may be resistant to the development of obesity within the short time period we used, or all three of our groups were obese regardless of the inclusion of fish oil or a combination of fish oil and phosphatidylcholine in a Western diet. Because we did not use a chow-fed control, we cannot establish whether all pigs were obese or not. A previous study, which investigated the effects of a Western diet on obesity, was conducted in Yucatan pigs over a span of 11 months (Myrie *et al*., 2012). Visceral fat plays a key role in the outcomes of metabolic syndrome. Although we did not measure visceral fat content directly, there was no significant difference in the abdominal circumference among our experimental groups at necropsy. The Myrie *et al*. study did demonstrate that there is a significant correlation between visceral obesity and abdominal circumference (Myrie *et al*., 2012). The BFBW, which is an indicator of subcutaneous or peripheral obesity, was measured at necropsy and did not significantly differ among the groups. However, the absence of a control group due to limiting housing resources was a limiting factor in our study. However, large pigs used in the previous studies conducted in our laboratory cannot be directly compared with our pigs. Omega-3 fatty acids, especially EPA and DHA, are involved in the reduction of both peripheral and visceral adipose tissue accumulation. However, both fish oil and a combination of fish oil and phosphatidylcholine were ineffective in counteracting the effects of Western dietary components. If the total duration on the experimental diet is increased, the dosing effect may improve the assimilation of EPA and

DHA, and can help identify the long-term beneficial effects of including fish oil or a combination of fish oil and phosphatidylcholine in a Western diet

4.2 Fasting plasma glucose concentrations

Previous studies that used Yucatan miniature pigs as a model to investigate diabetes observed that the blood glucose concentrations of non-diabetic pigs ranged between 3.0-5.0 mM (Otis *et al*., 2003). However, at baseline before introducing the Western diet, the fasting mean blood glucose concentrations of our experimental groups were calculated by dividing the mean plasma glucose concentrations by an average hematocrit factor (1.5, obtained from IVGTT baseline blood glucose concentrations and baseline plasma glucose concentration). The calculated values before the introduction of the Western diet were 2.5 times higher than the non-diabetic reference range (6.5-7.5 mM). However, these readings were not as elevated as the diabetic group (19.6-20.0 mM) in the previous study (Otis *et al*., 2003). There can be various factors associated with the elevation of plasma glucose concentrations at baseline. Acute stress caused by restraining the pigs during jugular venipuncture can elevate fasting plasma glucose concentrations, which can lead to an overestimation of plasma glucose concentrations due to elevated cortisol levels (Nowotny *et al*., 2010). Therefore, a long-term intravenous catheterization similar to IVGTT or training the pigs at an early age, can minimize the stress during blood collection which would enable us to measure fasting glucose concentrations more accurately. After 6 weeks on the diet, the mean plasma glucose concentrations were unchanged and the mean calculated blood glucose values were above the non-diabetic reference range (6.3-7.8 mM). A flipping method of restraint was used for blood collection, both at baseline and at 6 weeks on the experimental diets. The flipping stress elevates glucose concentration in the circulation and might have masked the effects of both fish oil and a combination of fish oil and phosphatidylcholine on fasting plasma glucose concentrations even after being on the diet for 6 weeks. The final blood sample collection was performed under anesthesia at necropsy via

direct cardiac puncture. Pentobarbital was administered as the anesthetic agent, which has also been identified as a factor in increasing both blood glucose concentrations and plasma insulin concentrations (Windeløv *et al*., 2016). As a result, mean plasma glucose concentrations at necropsy approximately doubled, compared to baseline and at 6 weeks and the calculated mean blood glucose concentrations were above the non-diabetic reference range (9.9-12.6 mM). The hematocrit value for these samples were unknown; however, it should also be noted that these mean blood glucose concentrations from these three time points are elevated beyond the non-diabetic reference range for minipigs (3.0-5.0 mM). Our three experimental groups consumed a modified version of a Western diet. The combination of fish oil and phosphatidylcholine (WFOL) was expected to improve the utilization of EPA and DHA in the form of phospholipids from the fish oil, resulting in a better glucose tolerance during fasting states compared to both the Western diets which included only fish oil (WFO) and the standard Western diet (W), but the outcomes were not significantly different. However, the duration that our young adult Yucatan miniature pigs were on the diet ranged between 71-122 days. This may not have been sufficient enough for the assimilation of EPA and DHA from either fish oil or a combination of fish oil and phosphatidylcholine to counteract the effects of the Western dietary components; alternatively, all three Western diets were equally detrimental regardless of the addition of fish oil and phosphatidylcholine.

4.3 Intravenous glucose tolerance test (IVGTT) outcomes

The absence of significant differences in the fasting plasma glucose concentrations among our groups and elevated mean fasting blood concentrations beyond the non-diabetic reference at all three time points may have been influenced by flipping stress and anesthesia (during necropsy). This influence on both the plasma and blood glucose concentrations might have masked the effects of our dietary components. However, the fasting blood glucose

concentrations (3.95-4.09 mM) measured in IVGTT samples which were collected via catheters before the administration of the glucose bolus remained closer to the upper limit of a non-diabetic reference range for blood glucose concentrations (3.0-5.0 mM). Bodyweights were negatively correlated with fasting blood glucose concentrations during IVGTT (R^2=0.440, P=0.000) (Fig.5). In addition, the mean blood glucose concentration was significantly different among our experimental groups (P=0.035). However, no significant differences were observed between groups during analysis of fasting plasma glucose concentrations. In addition to fasting plasma glucose concentration, IVGTT also measured peak blood glucose concentration, time taken for peak blood glucose concentrations to return to fasting levels, area under the curve (AUC) and slope of blood glucose clearance as outcomes.

Obesity is associated with the development of glucose intolerance and insulin resistance. Previous studies that used Yucatan miniature pigs have successfully validated this pig model to study the outcomes of glucose tolerance (McKnight *et al*., 2012). The Western diet can induce obesity in pigs and can accelerate growth rates primarily due to deposition of fat. Obesity, in particular visceral obesity, is associated with the development of glucose intolerance (Borst, 2004). Glucose intolerance will affect the glucose curve by slowing down the clearance, which will also affect the AUC. Omega-3 fatty acids have been identified as a preventive measure in reducing fat deposition by improving beta-oxidation (Ukropec *et al*., 2003). Our three experimental groups consumed a modified version of a Western diet. The combination of fish oil and phosphatidylcholine (WFOL) was expected to improve the utilization of EPA and DHA in the form of phospholipids from the fish oil, resulting in a better glucose tolerance during both the fasting and fed states. However, none of our growth data, including abdominal circumference and BFBW, were significantly different among the groups; furthermore, none were correlated with the slope of clearance or AUC during IVGTT.

The glucose bolus was administered through IV catheters which led to an immediate elevation of glucose concentration in the circulation. The peak blood glucose concentrations were measured in all three groups within five minutes following administration. As expected, there were no significant differences in either the time taken to reach peak value or peak blood glucose concentration, so the test outcomes were primarily focused on detecting the effectiveness of the dietary interventions. Intravenous glucose tolerance tests (IVGTT) bypass the incretin hormone mechanism that is stimulated by oral intake of glucose and this leads to a cascade of events that stimulates gradual release of insulin from the pancreas. However, IVGTT leads to an instant hyperglycemic state which allows us to accurately detect the sensitivity of beta cells in pancreas for glucose stimuli during the first phase of insulin secretion that affects AUC and clearance.

AUC is composed of two components: the absorption and clearance. However, as mentioned previously, the absorption aspect of the AUC may not play an important role due to the direct infusion of glucose into the blood stream. As a result, any significant differences will solely depend upon the effectiveness of the endogenous glucose clearance mechanism. The AUC was expected to be the highest in the group which was consuming a standard Western diet (W) and the lowest in the group which was consuming a combination of fish oil and phosphatidylcholine in a Western diet (WFOL). However, there were no significant differences among the groups which indicates that the addition of neither fish oil nor a combination of fish oil and phosphatidylcholine to a Western diet significantly affected the blood glucose regulation in the pigs. Even though the glucose was cleared by endogenous insulin, the plasma insulin concentrations were not measured for these time points. It may have been valuable to measure

insulin to determine insulin sensitivity, which may lead to a more accurate interpretation of the results.

Slope of clearance was calculated by plotting the natural logarithm of blood glucose concentrations against their respective time points. The slope of clearance, similar to AUC, primarily depends on the effectiveness of endogenous insulin secretion and the sensitivity of the peripheral tissues for uptake. However, there were no significant differences in the clearance rate among the experimental groups, suggesting neither fish oil nor a combination of fish oil and phosphatidylcholine included in the diet were effective enough to alter glucose clearance in pigs compared to the pigs consuming a standard Western diet. The effects of omega-3 fatty acids on insulin sensitivity might have been masked if the pigs were at their maximum insulin sensitivity, regardless of the diets. Omega-3 fatty acids have been identified in the modification of adipokines and cellular response, which may lead to both improved endogenous insulin secretion and sensitivity (Bhaswant *et al*., 2015). Omega-3 fatty acids in the pancreatic beta cell membrane have been known to prevent cell death induced by cytokines, as well as improve the overall response to glucose stimuli (Baynes *et al*., 2018). EPA and DHA were found to be effective in improving overall glucose regulation compared to ALA, even in obese individuals (Liu *et al*., 2013; Mori *et al*., 1999; Muramatsu *et al*., 2010; Sarbolouki *et al*., 2013). In addition, *in-vivo* studies found that the incorporation of omega-fatty acids into tissues was much higher when absorbed as phospholipids (Batetta *et al*., 2009). The combination of fish oil and phosphatidylcholine (WFOL) was expected to improve the utilization of EPA and DHA in the form of phospholipids from the fish oil, resulting in a better glucose tolerance, compared to both the Western diet which included only fish oil (WFO) and the standard Western diet (W), but the outcomes were not significantly different. The AUC and slope of clearance were not significantly different between groups which suggests that the glucose tolerance of these pigs

were neither altered by the addition of fish oil nor by the addition of fish oil and phosphatidylcholine to the Western diet. The absence of significant difference in AUC or slope of clearance suggests that the Western dietary content which occupied a higher percentage of the experimental diets might have influenced these outcomes or the fish oil or the combination of fish oil and phosphatidylcholine in the Western diet was less effective. The absence of significant difference between groups, especially AUC and slope of clearance suggest that the influence of the Western dietary components on glucose tolerance might be higher than the effects elicited by fish oil or the combination of fish oil and phosphatidylcholine. However, measuring of plasma insulin concentrations and omega-3 fatty acid distribution in tissues associated with glucose clearance, such as pancreas, skeletal muscles and liver, may improve the accuracy of our conclusions.

4.4 Insulin sensitivity test (IST) outcomes

The outcomes of glycemic state in our animals was inconclusive, as both fasting plasma glucose analysis and IVGTT were not different. The goal of IST was to assess the rate of glucose clearance from the circulation under a pre-determined dose of exogenous insulin, while endogenous secretion was expected to be completely inhibited by somatostatin. WFO and WFOL groups consumed modified versions of a Western diet. However, the group that consumed a combination of fish oil and phosphatidylcholine (WFOL) was expected to effectively utilize omega-3 fatty acids, especially compared to the groups consuming a Western diet with or without fish oil. The exogenous insulin dose of 0.05 IU/kg was determined from previous research and was shown to induce a hypoglycemic state in healthy Yucatan miniature pigs (Otis *et al*., 2003). Although we expected our pigs to become hypoglycemic, a dose of 0.5 g/kg of bodyweight of glucose was administered, which is the same dose administered for non-diabetic pigs to prevent any negative outcomes due to the exogenous insulin dose (Otis *et al*.,

2003). The blood glucose concentrations were measured following administration of a glucose bolus along with the maintenance doses of somatostatin at 3 minute intervals, to detect the inhibition of glucose clearance by endogenous insulin secretion. However, measuring plasma C-peptide concentrations in these samples would provide more accurate data on whether endogenous insulin secretion was completely inhibited (Leighton *et al*., 2017) (McKnight *et al*., 2012). The absence of significant difference in the slope of clearance in IST indicated that neither fish oil nor a combination of fish oil and phosphatidylcholine in the Western diet were effective in improving insulin sensitivity. *In vivo* studies conducted in rats and mice using purified omega-3 fatty acids suggested that insulin resistance was counteracted by both EPA and DHA compared to ALA (Andersen *et al*., 2008; Shimura *et al*., 1997). In addition, supplementing omega-3 fatty acids as phospholipids appears to be efficient in alleviating insulin resistance, and an inverse correlation was observed between insulin resistance and the ratio of omega-3 fatty acids to omega-6 fatty acids incorporation in skeletal muscle membranes (Liu *et al*., 2013; Storlien *et al*., 1996). We predicted that the group consuming a combination of both fish oil and phosphatidylcholine (WFOL) would respond significantly different than the fish oil group (WFO) and the standard Western diet (WD). The lack of effect in both WFO and WFOL may be due to the Western dietary components, which were higher by content compared to omega-3 fatty acid content which might have masked the beneficial effects of omega-3 fatty acids on improving insulin sensitivity. However, our pigs were young adults and the Western dietary components might have failed to induce insulin resistance in these pigs as it is a chronic inflammatory process; 71-122 days on the diet might not be sufficient to induce an effect in this model. In addition, the Yucatan miniature pig might be resistant to the development of diabetes and might not be a suitable model for the study.

4.5 Fasting plasma triacylglycerol concentration

Compared to humans, pigs have lower fasting plasma triacylglycerol concentrations, yet their lipid metabolism is similar. The mean plasma triacylglycerol concentrations of our groups remained within the normal range (1.0-1.64 mM) prior to switching the pigs to the experimental diets (Ye *et al*., 1994). However, after placing the pigs on the experimental diet for 6 weeks and at necropsy, the mean fasting plasma triacylglycerol concentrations did not significantly differ among our experimental groups. In previous research in adult Yucatan miniature pigs, fasting mean plasma triacylglycerol concentrations gradually declined by 0.1 mM between the ages of 3-6 months and plateaued after 6 months. The reduction in the fasting plasma triacylglycerol concentrations in these pigs was assumed to be due to the fluctuation and stabilization in reproductive changes during sexual maturity (Myrie *et al*., 2009). The mean fasting plasma triacylglycerol concentration of the fish oil group declined by 0.12 mM but there was no significant difference within the WFO (P=0.6 paired t-test) group between baseline and necropsy. The ability to detecting a significant change within WFO group might have been masked by outliers in the data (Fig. 3). Because our pigs were above 6 months of age and female Yucatan miniature pigs normally reach sexual maturity between the age of 3.5-4 months and stabilizes around 6 months, the impact due to hormonal changes on fasting plasma triacylglycerol concentrations in our study was expected to be minimal. The impact of fish oil in reducing VLDL synthesis in the liver and lipolysis in adipose tissues and leading to reduced NEFA load in circulation has been observed in humans with both normal and elevated triacylglycerol levels after being treated with fish oil (Shearer *et al*., 2012). We predicted that this effect would be prominent in the WFOL group; however, the mean plasma triacylglycerol concentrations were elevated at 6 weeks and remained similar at necropsy (Table 13). Fish oil has been reported to reduce VLDL synthesis in the liver which contributes to the reduction of fasting plasma triacylglycerols (Shearer *et al*., 2012). Omega-3 PUFA increases LPL activity

especially in peripheral adipose tissues and the excess influx of fatty acids into the adipose tissue might inhibit LPL activity. The reduction in LPL activity inhibits the production of chylomicron remnants followed by VLDL. However, VLDL triacylglycerol in fasting plasma samples need to be separated by ultracentrifugation and need to be analyzed to identify whether there is any significant difference among the groups. This will enable us to detect how the total plasma triacylglycerol concentrations were declining only in the WFO group but not in the WFOL group, which was expected to effectively absorb and incorporate omega-3 fatty acids. Epidemiological studies also suggest that fasting plasma triacylglycerol concentration appears to be an independent risk factor in the prediction of cardiovascular disease (CVD) in patients with metabolic syndrome (Hokanson & Austin, 1996). In addition, *in vivo* studies that utilized omega-3 phospholipid identified a significant reduction in body weight gain, adipose tissue accumulation and reduced fasting NEFA levels in C57BL/6J mice (Liu *et al*., 2013). However, we found no correlations between back fat corrected for body weights and fasting plasma triacylglycerol concentrations. In addition, plasma triacylglycerol concentrations did not elevate abruptly due to flipping or anesthesia and instead remained within normal levels in contrast to fasting plasma glucose concentrations, which suggests that blood glucose concentrations are more sensitive to cortisol induced by flipping, as opposed to triacylglycerol.

4.6 Oral fat tolerance test (OFTT) outcomes

Compared to the values measured from a single time point after 14 hours of fasting, the post-prandial clearance of plasma triacylglycerols measures data over a 12 hour period at hourly intervals. OFTT includes multiple aspects of fat regulation such as digestion, absorption and clearance. The digestion and absorption effect is mainly measured by peak triacylglycerol concentration that enabled us to detect the effects of both fish oil and a combination of fish oil and phosphatidylcholine in effectiveness in the absorption of dietary fat. The AUC might

provide data on how effectively the triacylglycerols were absorbed and cleared from the plasma. The AUC data could help to identify treatment effects in improving overall handling of triacylglycerols when included in a Western diet. Fasting plasma triacylglycerol concentrations, peak plasma triacylglycerol concentrations, adjusted peak and adjusted AUC were analyzed and there were no significant differences between the groups. All pigs, that consumed 80% of the fat bolus, reached peak plasma triacylglycerols after 5 hours but there was no significant difference among the groups. In addition, the postprandial plasma triacylglycerol concentration at each time point during OFTT was adjusted by the respective fasting plasma triacylglycerol concentration of the pig in order to negate the influence of circulating plasma NEFA concentrations. Yet, there were no significant differences in either the peak or the adjusted peak plasma triacylglycerol concentrations among the groups. Measuring chylomicron triacylglycerol concentrations may be more accurate due to synthesis being limited to the gut and induced by the presence of dietary fat (Jialal *et al*., 2010). Studies conducted in humans often use plasma triacylglycerol concentrations to detect dyslipidemia and the effectiveness of post-prandial triacylglycerol absorption and clearance (Hansen *et al*., 1998; Rambjør *et al*., 1996; Rössner *et al*., 1974; van Barlingen *et al*., 1996). In the absence of chylomicron triacylglycerol concentrations, the adjusted plasma triacylglycerol concentrations are considered as an alternative. Adjusted AUC provided data on absorption and clearance, but no significant difference among groups was found. However, 6 pigs (WD=2, WFO=1, WFOL=3) included in the analysis did not return to fasting plasma triacylglycerol concentrations and only 18 pigs completed the test. Excluding the pigs from the analysis reduces the degree of freedom for error with all considered factors, which results in an inconclusive result. Therefore, the adjusted AUC in these 6 pigs (WD=2, WFO=1, WFOL=3) were likely underestimated. If OFTT was completed including these pigs and AUC data were included in the analysis, the outcome might trend towards the effect of the diet which might have the larger AUC curves. There were

6 pigs (WD=3, WFO=1, WFOL=2) completely excluded from all analysis of OFTT outcomes because they failed to consume the fat bolus within the first two hours, which may have underpowered this outcome. Overall, the results from the incomplete OFTT data suggested that the dietary interventions may not have been effective, and we do not know if complete data may have depicted a different outcome. Our experimental groups consumed a Western diet treatment for a period ranging between 71-122 days. However, in a previous study, Yucatan miniature pigs were fed a Western diet for 11 months in and identified significant differences between groups in lipid profiles (Myrie *et al*., 2009). As such, our null results might be due to an adequate feeding duration. In addition, Western dietary components might be detrimental to overall health, even though omega-3 fatty acids were included in the diets via fish oil or a combination of fish oil and phosphatidylcholine and was expected to alleviate dyslipidemia.

4.7 Fasting plasma cholesterol concentrations

At the beginning of the study, mean fasting total plasma cholesterol concentrations did not differ among groups and tended to be lower than the normal reference range. The reference range for total plasma cholesterol concentrations for adult Yucatan miniature pigs is 1.5-3.0 mM (Myrie *et al*., 2012; Myrie *et al*., 2017; Ye *et al*., 1994). Previous studies used female Yucatan miniature pigs as a model which reach sexual maturity between the age of 3.5-4 months (McKnight *et al*., 2012). The effects of a Western diet on plasma cholesterol levels detected in the previous study indicated there was an abrupt reduction between 5-6 months of age that might be due to ongoing hormonal changes and after 6 months of age the plasma cholesterol concentrations plateaued (Myrie *et al*., 2009). However, this change could not be measured in our study because the initial fasting blood sample was collected at 6 months of age. Pigs in the previous study were fed a similar Western diet to ours, and they reported that the total plasma cholesterol concentrations gradually declined until 9 months of age (Myrie *et*

al., 2009). However, the mean total fasting plasma cholesterol concentrations in our pigs continued to elevate in all three groups throughout the study. After 6 weeks on the experimental diets, the fish oil group's mean plasma cholesterol concentration ($P < 0.1$) revealed an elevated trend compared to the other two groups, but it was not significantly different. Fish oil has been identified in reducing total plasma cholesterol concentrations, and increasing HDL-cholesterol concentrations and lowering LDL-cholesterol concentrations due to a reduction in VLDL secretion in humans (Regnström *et al*., 1992). The trend towards elevated total cholesterol may be due to either LDL-cholesterol or HDL-cholesterol. In fact, LDL-cholesterol may be elevated as a result of the Western diet, the fish oil or a combination of both (Escolà-Gil *et al*., 2011) . On the other hand, fish oil can also increase HDL-cholesterol. Further investigation is required to confirm which of these the fractions were elevated as a result of dietary intervention in fish oil group and why this elevation was absent in the group that consumed a combination of fish oil and phosphatidylcholine. High LDL-cholesterol is considered an atherogenic factor even if it is the result of fish oil consumption. If LDL-cholesterol concentrations increase, consuming fish oil with a Western diet may lead to detrimental effects because omega-3 fatty acids associated with LDL particles have a short life and are highly susceptible to oxidation leading to plaque formation. However, if the fish oil increases total plasma cholesterol concentrations due to higher HDL-cholesterol, the omega-3 fatty acids are transported in HDL with less susceptibility to oxidation due to its longer half-life and atheroprotective nature. As a result, this may impact cardiovascular health. However, if the total plasma cholesterol concentration is elevated due to higher HDL-fractions, this may confer a benefit of consuming fish oil along with a Western diet. If both fractions are elevated, the HDL/LDL ratio should be measured to confirm whether adding fish oil to a Western diet is beneficial or detrimental. Previous studies in *in vitro and in vivo* models that used purified supplements reported outcomes that were in contrast to *in vivo models* that used marine omega-3 sources (Harris, 1989; Nestel, 2000). In

addition, adult Yucatan miniature pigs fed a high-fat di*et al*ong with fish oil had decreased incidence of atherosclerosis compared to pigs placed solely on an atherogenic diet (Barbeau *et al*., 1997). In our study, it may have been useful to collect a cross section of the aorta for staining with Sudan IV to identify plaque formation in addition to measuring HDL-cholesterol and LDL-cholesterol concentrations. As all three of our groups were consuming a Western diet, the inclusion of these factors in the analysis would further confirm whether the increments observed in the total plasma cholesterol concentrations were either beneficial or detrimental due to fish oil or a combination of fish oil and phosphatidylcholine supplementation.

Chapter 5: Conclusion

The overall goal of this research was to determine the effects of fish oil or a combination of fish oil and phosphatidylcholine in a Western diet in ameliorating the progression of metabolic syndrome in Yucatan miniature pigs. The omega-3 content an adult human weighing approximately around 75 kg requires 1400-1600mg of omega-3 fatty acids which translates to an average dose of 20mg/kg per day. The body weights of our pigs were expected between 45-60kg and their daily feed intake was expected between 2%-4% of their bodyweights. The minimum consumption was expected to provide an approximate of 4260g of EPA and DHA for a 45kg pig which is 95.5mg/kg of bodyweight which is higher than the average dietary requirement for humans. Even though this high dose of fish oil and phosphatidylcholine in a Western diet was expected to ameliorate the factors associated with the progression of metabolic syndrome, no significant differences were found except for fasting blood glucose concentrations during IVGTT (Table 11). An elevated trend in total fasting plasma cholesterol concentrations in the fish oil group after 6 weeks on the experimental diet was also found (Table 16). The significant difference in blood glucose concentrations between groups during IVGTT

and a trend with cholesterol concentrations in WFO group is not sufficient to conclude that omega-3 fatty acids had an effect on the factors of metabolic syndrome. Based on our existing data, we reject the primary hypothesis. The secondary hypothesis was not analyzed and therefore no conclusions cannot be made. However, necessary accommodations to our experimental protocol may have altered the outcomes of the study. The limitation of available resources led to group housing of pigs that affected the total number of days on the experimental diet and measuring individual feed intake. For example, individually housing the pigs throughout the study could allow us to measure the individual feed intake of these pigs on a daily basis and also would have allowed us to add a control group on a low caloric grower diet which would have permitted us with a better assessment when comparing the three Western diets (WD, WFO, WFOL). Individual housing would have eliminated the variability in the total number of days each animal was fed the test diets. The measurement of individual feed intake data at multiple time points may be of more significance compared to only considering the total number of days on feed. Another factor that might improve the study design would be the use of a stress free method to collect blood, such as a long term catherization; this may result in more accurate measurements similar to fasting blood glucose concentrations during IVGTT. The expected experimental dietary outcomes in the development and prevention of metabolic syndrome between our pigs were not significantly different. The three experimental diets were mainly composed of Western dietary components and fish oil only contributed to 5% of the diet by weight in both WFO and WFOL. A previous study that utilized a similar Western diet with the objective to identify the effects of Western diet in Yucatan miniature model fed the pigs for a maximum duration of 11 months (Myrie *et al*., 2012). However, these experimental diets were consumed by young adult Yucatan miniature pigs and this was a short-term study which extended for a maximum of 122 days which may not have been long enough to elicit an effect in metabolic syndrome outcomes. In addition, it is evident that the fish oil and

phosphatidylcholine content combined with the Western diets did not counteract the effects of the Western dietary components. A long-term feeding trial or higher doses of fish oil and phosphatidylcholine given with a Western diet may serve to identify whether there is the potential for a dietary treatment to avoid the onset of adverse metabolic outcomes related to a Western diet.

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