LIPID METABOLISIM AND THE RISK FACTORS OF CARDIOVASCULAR DISEASE: IMPLICATION OF DIETARY OMEGA-3 POLYUNSATURATED FATTY ACIDS

By

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ABSTRACT

Cardiovascular disease (CVD) is a complicated and multifarious disease, and is the number cause of mortality worldwide. The pathogenesis of CVD is attributed to the interaction between genetics and environment. There are numerous data that support the cardioprotective properties of omega (*n*)-3 polyunsaturated fatty acids (PUFA); however, there are also controversial reports. Considering the reported sex and age differences in the pathophysiology of CVD and the metabolism of *n*-3 PUFA, it is imperative to consider these factors in the cardioprotective effects of *n*-3 PUFA. The main objective of the current thesis was to investigate the effects of *n*-3 PUFA on the risk factors of CVD such as dyslipidaemia and obesity, with particular focus on how sex, age and dose of *n*-3 PUFA affect lipid and lipoprotein metabolism. The set of experiments presented in this thesis was investigated using the C57BL/6 mouse strain which has been established as a mouse model of choice for the study of diet-induced pathological conditions of lipid and lipoprotein metabolism. Plasma concentrations of lipids and lipoproteins of mice offspring at weaning and 16 weeks postweaning were chosen as study outcomes to assess the sex, age and dose-specific effects of *n*-3 PUFA on markers of dyslipidaemia, a well-known risk factor of CVD. It was observed that a longer exposure to a postnatal diet high in *n*-3 PUFA increased plasma concentration of low-density lipoprotein cholesterol (LDL-c) in the offspring in a sex-specific manner; however, the profile of this increase was less atherogenic , as the high *n*-3 PUFA group had a lower plasma concentration of very small LDL-particles in both males and females. There was no effect of high *n*-3 PUFA diet observed on plasma concentration of high-density lipoprotein cholesterol (HDL-c); however, the high *n*-3 PUFA group had a higher cholesterol efflux in the male offspring but not in female offspring, further demonstrating the effect of sex on cholesterol metabolism. Lipidomic analyses revealed that high *n*-3 PUFA diet

led to higher hepatic and plasma concentrations of *n*-3 PUFA-containing bioactive lipids such a phosphatidylcholine, lysophosphatidylcholine and free fatty acids, which could positively influence pathways involved in cardioprotection. The effects of dietary *n*-3 PUFA on obesity at the cellular level was also investigated, using adipocyte hypertrophy as the outcome measure of adipose tissue enlargement. A diet high in *n*-3 PUFA prevented adipocyte hypertrophy in males, with no effect in females. High *n*-3 PUFA diet also led to the downregulation of the mRNA expression of acyl CoA:diacylglycerol acyltransferase 2 (DGAT2), fatty acid binding protein-4 (FABP4), peroxisome proliferator-activated receptor protein γ (PPARγ), and leptin in males, which are key proteins involved in adipocyte hypertrophy; however no effect was observed in females. The last study assessed the effects of dose and duration of exposure to dietary *n*-3 PUFA on DHA accretion in the brain, and the expression of neurotrophins known to have neuroprotective and cardioprotective benefits. A diet high in *n*-3 PUFA led to an accretion of DHA in the brain cortex of the male offspring. Furthermore, dietary *n*-3 PUFA led to an age-dependent increase in the expression of brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase (TrKB), and phosphorylated cAMP response element binding protein (pCREB). Furthermore, there was a positive correlation between the cortical mRNA expression of BDNF and plasma concentrations of triglycerides and non-esterified fatty acids, suggesting a relationship between neurotrophins and regulation of lipid metabolism.

In conclusion, the results from the current thesis demonstrate a sex, dose and age specific effect of *n*-3 PUFA on risk factors of CVD, and on novel regulatory pathways by which *n*-3 PUFA could reduce dyslipidaemia and obesity; the results also suggest that *n*-3 PUFA could be neuroprotective and cardioprotective through a common neurotrophin signalling pathway.

CO-AUTHORSHIP STATEMENT

For the work presented in chapter-2, published in *Prostagladins, Leukotrienes, and Essential Fatty Acids, 2014. (91): p. 39*, I, Kayode Balogun, helped design the study, conducted the postweaning experiments, analyzed all the data and prepared the manuscript.

For the work presented in chapter-3, published in *PLoS One, 2013.* ***8****(11): p. e82399,* I was involved with the design of the study, conduct of the experiments, analysis of the data and preparation of the manuscript.

For the work presented in chapter-4, which is under review for publication in *Lipids*, I helped with the design of the study, conducted the experiments, analyzed the data and prepared the manuscript.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABCA1	ATP binding cassette transporter
ACAT	Acyl-CoA cholesterol acyltransferase
ADA	Adrenic acid
ALA	Alpha linolenic acid
APO	Apolipoprotein
BDNF	Brain-derived neurotrophic factor
c	Cholesterol
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CER	Ceramide
CM	Chylomicrons
CREB	cAMP response element binding protein
COX	Cyclooxygenase
CVD	Cardiovascular disease

DG	Diacylglycerol
DGAT	Acyl CoA:diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FFA	Free fatty acids
FABP	Fatty acid binding proteins
GLC	Gas liquid chromatography
HDL	High-density lipoprotein
H&W	Haematoxylin and eosin
HMG-CoA	3-hydroxy-3-methyl-glutaryl CoA
ICAM	Intercellular adhesion molecule-1
IDL	Intermediate-density lipoprotein
IF	Intrathoracic fat
IL-1	Interleukin-1
IL-6	Interleukin-6
IP	Intraperitoneal

LA	Linoleic acid
LCAT	Lecithin cholesterol-acyltransferase
LDL	Low-density lipoprotein
LDL-r	Low-density lipoprotein receptor
LOX	Lipoxygenase
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
LT	Leukotrienes
LXR	Liver-X receptor
MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NEFA	Non-esterified fatty acids
NF-kB	Nuclear factor kappa-light chain-enhancer of activated B cells
NGF	Nerve growth factor
NT	Neurotrophins
pCREB	Phosphorylated CREB

PPAR	Peroxisome proliferator-activated receptor
PL	Phospholipid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PLA_2	Phospholipase A_2
PS	Phosphatidylserine
PG	Prostaglandins
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RCT	Reverse cholesterol transport
RPLPO	Large ribosomal protein
RXR	Retinoic-X receptor
SFA	Saturated fatty acid
SM	Sphingomyelin
SC	Subcutaneous fat
SMC	Smooth muscle cells
SR-B1	Scavenger-receptor B1

SREBP-1	Sterol-regulatory element binding protein
T2D	Type-2 diabetes
TC	Total cholesterol
TG	Triglyceride
TNF-α	Tumour necrosis factor
TrKB	Tropomyosin receptor kinase
TX	Thromboxanes
VCAM-1	Vascular cell adhesion molecule-1
VLDL	Very-low density lipoprotein
VF	Visceral fat
WHO	World Health Organization

LIST OF APPENDICES

CHAPTER ONE

Introduction and Overview

1.0 Introduction

Cardiovascular disease (CVD) is a disorder of the cardiovascular system; it includes hypertension, coronary heart disease, stroke, cardiac arrhythmia, and heart failure. According to a World Health Organization (WHO) report, 30% of all deaths in 2008 were caused by CVD, and it was projected that more than 23 million people will die yearly from CVD by 2030 (WHO, 2011). Similarly, the Heart and Stroke Foundation of Canada has ascribed 29% of the total death in Canada in 2008 to CVD (Statistics Canada, 2008). It was formerly thought that CVD was a disease of affluence and only rampant in Western society. However, the prevalence of CVD is fast on the rise in developing countries as well (Reddy and Yusuf, 1998). CVD constitutes a huge socio-economic burden with an impact of $403.1 billion in the US, €169 billion in the European Union in 2006 (Leal et al., 2006), and $20.9 billion annually in Canada (Statistics Canada, 2008). The burden of CVD is further complicated by the reduced age of onset, with projected deaths of 6.4 million people between the ages of 30 and 69 as a result of CVD by 2020 (Murray and Lopez, 1996). By the age of 60, 50% of the population will be diagnosed with some type of cardiovascular disorder including atherosclerosis (Holub, 2009). Studies have identified a number of risk factors associated with the morbidities of CVD; these factors are modifiable or non-modifiable.

1.1 Non-modifiable risk factors of CVD

These are factors that cannot be changed or modified by the external environment. Non-modifiable risk factors of CVD include sex, age, and family history.

1.1.1 Sex/gender as a non-modifiable risk factor of CVD

The most characterised risk factor affecting the development of CVD is sex; this has been ascribed to a clear distinction in hormonal regulation between males and females (McNamara et al., 2009). It has been reported that men and women differ significantly in their circulating blood lipids and predisposition to CVD (Johnson et al., 2004). Plasma total cholesterol (TC), a major risk factor of CVD has been shown to increase with age peaking at 50-59 years in men and at 60-69 years in women (Carroll et al., 2005). Sex specific differences in blood lipid parameters have also been reported for plasma high-density lipoprotein cholesterol (HDL-c) and triglycerides (TG) (Carroll et al., 2005). Men are at a higher risk of developing CVD than premenopausal women (Finegold et al., 2013); however, after menopause, the risk is the same in both sexes. The female hormone, oestrogen, is involved in lipid metabolic pathways; it is responsible for the rapid transport and clearance of fat from the blood stream to the liver for excretion in women compared to men and this also partly accounts for the sex specific risk of CVD (Knopp et al., 2005). Oestrogen has also been shown to improve glucose metabolism and endothelial function. After menopause, the concentration of oestrogen decreases, which consequently reduces the concentration of HDL-c and increases low density lipoprotein cholesterol (LDL-c) concentration, which explains the similar risk of CVD between postmenopausal women and men (Jousilahti et al., 1999). The effect of sex in the development of CVD cannot be overemphasized, however, beyond sex differences comes age.

1.1.2 Age as a non-modifiable risk factor of CVD

The risk of CVD increases as an individual ages, and the heart undergoes changes that contribute to this condition. Age remains one of the most prominent risk factors of CVD. The

majority of individuals reported to suffer from heart attack are 65 years or older. The risk of CVD triples with each decade (Finegold et al., 2013). The risk of age has been explained by the reduction of cholesterol catabolism with age (Jousilahti et al., 1999), and the loss of arterial elasticity which consequently leads to CVD (Jani and Rajkumar, 2006). The plasma TC concentration increases with age (Corti et al., 1997) mainly because of the age dependent decrease in the catabolism of cholesterol (Corti et al., 1997). Furthermore, males and females have comparable plasma HDL-c concentration at puberty, after which the differences become apparent as they age (Abbey et al., 1999). There are also documented age effects on the plasma concentration of TG (Castelli, 1984). Although age is a non-modifiable risk factor, studies have shown that modifiable risk factors such as proper diet and physical activity also play important roles in the prevention of CVD in older individuals.

1.1.3 Family history as a non-modifiable risk factor of CVD

There is a genetic component to the development of CVD. Individuals with a family history of cardiovascular complications are at a higher risk of developing CVD. Some genetic aberrations known to promote the risk of CVD are heritable, and these include the mutation in the LDL receptor (LDL-r) gene which is the underlying cause of familial hypercholesterolemia a major risk factor of CVD. Mutation in the leptin or leptin-receptor gene can also be inherited which will cause a defect in leptin signalling and consequent obesity and associated complications.

1.2 Modifiable risk factors of CVD

Modifiable risk factors are risks associated with CVD that could be prevented or increased by altering environmental conditions. Hypertension, defined as the average blood pressure reading

greater than 140 mmHg for systolic pressure and greater than 90 mmHg for diastolic pressure is the principal cause of CVD. There are about 1 billion people worldwide diagnosed with hypertension. Hypertension is principally caused by narrowing of the blood vessels as result of plaque deposition, a process known as atherosclerosis. Another important modifiable risk factor of CVD is diabetes characterised by high circulating concentration of blood glucose as a result of defects in insulin production or regulation, with fasting blood glucose of 126 mg/dl (7 mmol/l) or higher (Abraham et al., 2014). A higher percentage of mortality in people with diabetes is accounted for by CVD. Tobacco smokers are also at a higher risk of developing CVD. Tobacco smoking is estimated to cause approximately 10% of all CVD, and the number of people smoking is fast on the rise with approximately 1.3 billion smokers globally (Thomas et al., 2014). The best characterised modifiable risk factors of CVD are obesity, dyslipidaemia, and nutrition.

1.2.1 Obesity as a risk factor of CVD

Obesity is the accumulation of excess fat in the body. Obesity has been identified as a risk factor of CVD and is one of the top ten global health problems (Madsen et al., 2005). Epidemiological studies have demonstrated a strong association between obesity and cardiovascular events (Wilson et al., 2002). Positive energy balance, which refers to when caloric intake is greater than energy expenditure, and physical inactivity, remain the major causes of obesity. However, some individuals living with obesity have been identified to have a genetic condition that predisposes them to the disease, such as a defect in any of the genes regulating appetite (Farooqi and O'Rahilly, 2004).

In addition to the total body fat, the location and distribution of body fat is also a well-known risk factor of CVD. Body fat is anatomically broadly classified as subcutaneous fat (SC) which are the lower and upper body fat, and the visceral or intra-abdominal fat (VF) and intrathoracic fat (IF) (Sironi et al., 2012). IF are found around the heart, SC fats occur under the skin, and VF occurs around organs in the abdominal region (Sironi et al., 2012). Fat that accumulates inside organs such as liver and heart causing metabolic dysfunction is termed ectopic fat (Gastaldelli and Basta, 2010). Studies have shown that the best predictor of CVD in relation to obesity is not the amount of fat but the location of fat, with VF showing a stronger risk of CVD (Fox et al., 2007). Metabolic dysfunctions seen in obese individuals are linked to an increase in VF as a result of hypertrophy (Despres et al., 2008). Adipose tissue increases in size as a result of adipocyte hypertrophy and/or hyperplasia (Arner and Spalding, 2010). Adipocyte hypertrophy is regulated by the accumulation of TG (Klein et al., 1980). Obese individuals are known to have high TG storage rates (Arner et al., 2011). Adipocyte number is also known to contribute to adipocyte mass (Spalding et al., 2008). An estimated 10% of adipocytes are renewed yearly regardless of body weight in adults (Spalding et al., 2008); nonetheless, the total number of adipocytes is kept constant (Spalding et al., 2008), except in morbidly obese individuals where the number of adipocytes is seen to rise with BMI (Arner et al., 2011). Obese individuals have a larger number of adipocytes than lean individuals, however, these numbers are acquired before adulthood during which the number of adipocytes remain constant even after a weight loss procedure (Spalding et al., 2008).

Adipose tissue inflammation is the major culprit of insulin resistance and type-2 diabetes (T2D) experienced by obese individuals (Lumeng and Saltiel, 2011). Adipose tissue contains a number of cells including adipocytes and macrophages. Obese individuals tend to have the

propensity of accumulating macrophages in the adipose tissue which leads to the secretion of inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α (Weisberg et al., 2003); this is an inflammatory environment that favours the development of pathological conditions associated with obesity. Studies have shown that an increase in adipose mass is accompanied by an increased recruitment of macrophages (Weisberg et al., 2003). Weight loss has been shown to reduce adipose tissue inflammation and improve insulin resistance by reducing macrophage infiltration in the adipose tissue (Cancello et al., 2005). In addition to the lipid storage function of the adipose tissue, the adipose tissue is also functionally an endocrine tissue capable of secreting adipokines that regulate body weight, appetite and insulin resistance. One such adipokine is adiponectin whose expression level decreases with an increase in adipose tissue mass and obesity (Turer et al., 2011). Increased secretion of adiponectin favours insulin sensitivity and reduces the risk of T2D (Li et al., 2009).

The incidence of obesity and related complications are well established at the population level; however, there is still insufficient knowledge on the mechanistic insights at the cellular level. The accumulation of TG is the major cause of adipocyte hypertrophy; therefore, elucidating the molecular processes involved in the formation of TG in the adipose tissue is important in understanding the development of obesity. An excess of calories, especially from fat, are the primary cause of obesity as dietary fat contains more calories per gram compared to carbohydrate and protein. The health effects of fats depend not only on the quantity of fats but also on the quality of fats. Saturated fatty acids (SFA), trans-fats, and cholesterol are the major culprits when it comes to increasing dyslipidaemia, obesity and CVD; however polyunsaturated fatty acids (PUFAs) have been shown to have beneficial effects (Hirafuji et al., 2003). The

mechanism/s by which the quality of fat affects adipose tissue metabolism and whether the effects are similar in both sexes are still not clear.

1.2.2 Dyslipidaemia as a risk factor of CVD

Dyslipidaemia is the abnormal circulating levels of plasma lipids and lipoproteins, which is a major risk factor of CVD. Dobsn *et al*. defined dyslipidaemia as the concentration of TC, LDL-c, apoplipoprotein (APO)-B and lipoprotein (a) (Lp(a)) above the 90^{th} percentile, or circulating concentrations of HDL-c and APO-A1 below the 10^{th} percentile (Dobson et al., 1996). A number of key epidemiological studies have reported the link between dyslipidaemia and CVD. The Framingham Study (Dawber et al., 1951) and the Seven Country Study (Fidanza et al., 1970) reported that dyslipidaemia, especially an increase in LDL-c concentration and a decrease in HDL-c concentration, is a major risk factor of CVD. Dyslipidaemia occurs in different forms ranging from hypercholesterolaemia, which is the elevated circulating concentration of cholesterol; hypertriglyceridaemia, which refers to an increase in circulating concentration of TG; and hyperlipoproteinaemia, an increase in lipoprotein concentration. Dyslipidaemia is caused by a number of factors which represent a blend of genetic and environmental factors.

Dyslipidaemia and the homeostatic regulation of lipid and lipoprotein metabolism are central to the onset of CVD, where LDL-c remains the primary target for lowering blood lipid levels (Choi et al., 2014). However, cardiovascular events still occur despite maintaining optimal levels of LDL-c, indicating the need to consider other factors as well. Therapies have also focussed on increasing the levels of HDL-c, the "good cholesterol", and lowering blood TG levels. Lifestyle modifications, especially dietary modifications, play an important role in dyslipidaemia and CVD. Genetic composition also predisposes an individual to dyslipidaemia, obesity, and

diabetes mellitus; while nutrition, exercise, and smoking are the best studied environmental factors that contribute to the pathogenesis of CVD (Tymchuk et al., 2006). Dyslipidaemia such as hypercholesterolaemia creates an enabling platform for the development of atherosclerosis.

1.2.3 Atherosclerosis and the risk of CVD

Atherosclerosis has been identified as the major cause of CVD. Cholesterol plays an important role in atherosclerotic plaque formation resulting in cardiovascular complications. Cholesterol is important in mammalian cells, where it fulfils important roles in maintaining membrane fluidity, and as a precursor of steroid hormones and bile salts. Despite the functional roles of cholesterol, high circulating cholesterol concentration or a defect in cholesterol metabolism leads to atherosclerosis. Atherosclerosis is an inflammatory disease characterised by the deposition of LDL-c in the arterial wall. Atherosclerotic lesions are predominantly found in muscular and elastic arteries leading to cardiac or brain infarction. Deposition of atherosclerotic plaque in the wall of the arteries is a chronic process; fatty steak lesions have been observed in children (Napoli et al., 1997). Endothelial dysfunction is associated with atherosclerosis and this is caused by high, circulating LDL-c, free radicals from cigarette smoking, diabetes, and hypertension. Endothelial dysfunction leads to a compensatory physiological response aimed at maintaining normal endothelial function. This results in a pro-inflammatory endothelial state, and if persistent and not resolved, it will continue indefinitely with deleterious effects. Prolonged inflammation of the endothelium stimulates the recruitment, proliferation and migration of smooth muscle cells (SMC) leading to the formation of lesions (Glagov et al., 1987). If the inflammation continues, macrophages and lymphocytes are recruited from the blood to populate the lesion and further produce pro-inflammatory cytokines which further damages the artery.

Increased expression of adhesion molecules on the wall of the endothelium marks the onset of atherosclerosis permitting the penetration of monocytes and lymphocytes into the intima of the artery (Frostegard, 2013). Formerly considered a lipid storage disease, evidence of the role of inflammation in atherogenesis has been presented. The dysfunction of the arterial endothelium underlies the inflammation driven atherogenesis. In the intima of the artery, the monocytes differentiate into macrophages which pick up oxidized LDL, become lipid laden and form foam cells (Qiao et al., 1997), which stimulate an overall inflammatory state. Nutrition, especially the quantity and the quality of dietary fats, has been shown to be effective in atheroprotection by reducing obesity and alleviating the markers dyslipidaemia.

1.3 Dietary fats and CVD

The role of dietary fat in the development of CVD is well documented. Dietary fats fall under the main categories of SFA, monounsaturated fatty acids (MUFA) and PUFA. High intake of SFA is generally considered to promote the development of CVD, while both MUFA and PUFA have been shown to be cardioprotective (Hansen and Harris, 2007, Gillingham et al., 2011). Dietary recommendations for fat are: 25-35% of total caloric intake; no more than 7-10% of caloric intake from SFA; less than 1% from trans fat; less than 300 mg/day of cholesterol; and the remainder should be made up of MUFA and PUFA (Perk et al., 2012). While the human body is capable of synthesizing SFA and MUFA, humans lack the enzymes required to synthesize PUFA (Engler and Engler, 2006); thus, PUFAs are essential fatty acids and must be consumed in the diet. Over the past years, there has been a drastic change in the Western diet; this change was promoted by the Industrial Revolution and modern food processing techniques (Grenon et al., 2012), and by the birth of modern day agriculture leading to the production of vegetable oils highly rich in *n*-6 PUFA (Simopoulos, 2002). These changes led to an increased

intake of *n*-6 PUFA in the Western diet and an overall reduction in the dietary consumption of *n*-3 PUFA (Simopoulos, 2002). It has been speculated that these changes in nutritional quality could be responsible for the majority of chronic diseases prevalent in the Western societies, including CVD (Eaton and Konner, 1985).

1.3.1 Metabolism of essential PUFA

The two major essential fatty acids are linoleic acid (LA) of the *n*-6 PUFA class and α-linolenic acid (ALA) of the *n*-3 PUFA class (Seo et al., 2005). LA is abundant in vegetable oils such as safflower and corn oils, while the essential *n*-3 PUFA, ALA, is rich in walnuts and flaxseed oils (Engler and Engler, 2006), all of which are plant-derived sources. The nomenclature of the essential fatty acids is based on the position of the first double bond from the methyl end of the fatty acid chain. *N-3* PUFAs have their first double on carbon number 3 while that of *n*-6 PUFA is on carbon number 6 (Engler and Engler, 2006). The parent 18-carbon LA and ALA can undergo a series of enzyme-catalysed desaturation and elongation steps using desaturase enzymes to produce highly unsaturated longer-chain arachidonic acid (AA) in the *n*-6 PUFA pathway, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the *n*-3 PUFA pathway (de Gomez Dumm and Brenner, 1975, Engler and Engler, 2006). The conversion of ALA to EPA and DHA, the marine derived *n*-3 PUFA, is inefficient with the conversion rate in men estimated to be approximately 0-1% and ~ 9% in women (Brenna, 2002). Studies have shown that the desaturation and elongation processes favour the conversion of ALA to EPA and DHA over the conversion of LA to AA (Hagve and Christophersen, 1984). However, a high consumption of dietary LA could shift the pathway in favour of the production of longer chain *n*-6 PUFA (**Figure. 1.1**). The metabolism of *n*-3 and *n*-6 PUFA also involves the oxygenation of 20 carbon AA and EPA by cyclooxygenases (COX) and lipoxygenases (LOX) to

produce eicosanoids, which are signalling molecules of physiological and therapeutic importance; these include prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). Eicosanoids from AA are pro-inflammatory, pro-thrombotic, and generally promote atherosclerosis (Farooqui et al., 2007). On the other hand, eicosanoids derived from EPA have been shown to be anti-inflammatory, anti-thrombotic, and promote good health (Adkins and Kelley, 2010). It is therefore imperative to maintain an appropriate nutritional balance between *n*-6 and *n*-3 PUFA for optimal body function. Over the years, there has been a decline in the consumption of *n*-3 PUFA in the Western diet forcing the ratio of *n*-6 to *n*-3 PUFA to approximately 20-30:1 which is relatively higher than the 1:1 ratio on which we evolved (Gomez Candela et al., 2011). This decline has been suggested to be responsible for the recent higher prevalence of CVD.

1.3.2 N-3 PUFA and CVD

The scientific inquiry into the health benefits of *n*-3 PUFA originated from the Bang and Dyerberg's observation of the Greenland Inuit (Bang et al., 1971). They observed that the Greenland Inuit had lower concentrations of plasma TG, LDL-c and TC as compared to a Danish cohort; these differences were ascribed to the high fish and marine mammal diet of the Inuit. *N*-3 PUFA such as EPA and DHA occur predominantly in fish and fish oil. An inverse relationship between fish consumption and incidence of CVD has been reported in a meta-analysis of 200,575 subjects (He et al., 2004). Other clinical studies have also corroborated these findings (Burr et al., 1989, Hooper et al., 2006). Dietary supplementation of *n*-3 PUFA (>2g/day) has been shown to alleviate symptoms of dyslipidaemia, improve endothelial function, and resolve inflammation associated with the development of CVD (Balk et al., 2006, Harris, 1997,

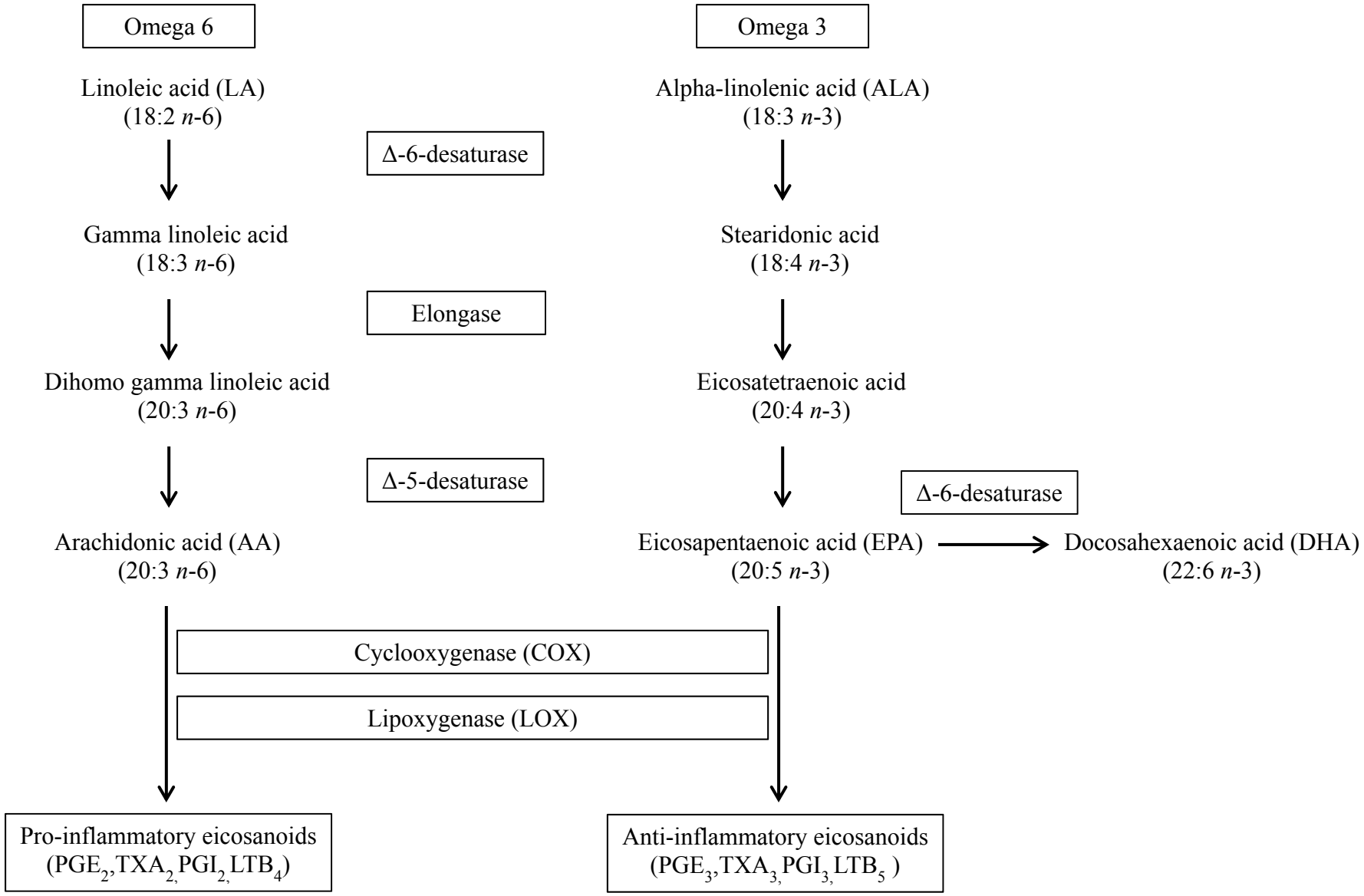

Figure 1.1 Pathways for the synthesis of long chain n-6 and n-3 PUFA . Leukotriene B (LTB); prostaglandin E (PGE); thromboxane A (TXA), prostacyclin (PGI)

Defilippis et al., 2010). There is also evidence supporting the anti-inflammatory, anti-thrombotic, anti-atherogenic and anti-arrhythmic properties of *n*-3 PUFA (Holub, 2002). The North American intake of *n*-3 PUFA is approximately 130-150 mg/day (Holub, 2002, Denomme et al., 2005). With the documented health benefits of *n*-3 PUFA, the consumption of two fatty fish servings per week for the prevention of CVD was recommended by the American Heart Association (Kris-Etherton et al., 2002); this is estimated to provide approximately 450-500 mg/day of EPA and DHA (Kris-Etherton et al., 2002, Kris-Etherton et al., 2007). There is no universally recognized recommendation for the consumption of *n*-3 PUFA due to dietary variations in different countries. However most countries and organisations have made different recommendations based on the recognized health benefits of *n*-3 PUFA and the consensus among the different recommendations is the *n*-6 to *n*-3 PUFA ratio of approximately 5:1 (**Table. 1.1**). The health benefits of *n*-3 PUFA depends on its availability in the body and its accretion in body tissues; hence, the omega-3 index, which is the percentage of highly unsaturated *n*-3 PUFA (EPA+DHA) in the erythrocytes, can be considered as a marker for risk of cardiovascular irregularities (Harris, 2008). An omega-3 index of 4% represents low cardioprotective effects, while an index of 8% signifies relatively high cardioprotection (Jump et al., 2012). The premise for this marker was that the fatty acid composition of the blood can be used as a surrogate for the fatty acid composition of the cardiac muscle (Harris et al., 2004).

1.4 Cardioprotective mechanisms of *n*-3 PUFA

Several mechanisms have been proposed by which *n*-3 PUFA could prevent CVD; the three main mechanisms are: a) regulation of lipid and lipoprotein metabolism; b) alteration of membrane dynamics and production of bioactive lipids; and c) reduction of inflammation. The following sections focus on the involvement of *n*-3 PUFA in the regulation of these pathways.

1.4.1 Cardioprotective effects of n-3 PUFA by regulating lipid and lipoprotein metabolism

Alterations in lipid metabolism underlie the pathology of CVD; thus the regulation of lipid metabolism is crucial for maintaining physiological functions. The following subsections highlight the importance of *n*-3 PUFA in regulating lipid and lipoprotein metabolism and the associated mechanisms.

1.4.1.1 Metabolism of serum lipids and lipoproteins

The liver plays a key role in the trafficking of cholesterol in the body. Three different routes of cholesterol movement have been identified in the body: 1) transport of cholesterol from the intestine to the liver; 2) transport of cholesterol from the liver to extrahepatic or peripheral tissues; and 3) reverse cholesterol transport from peripheral tissues to liver for further metabolism of cholesterol. Approximately half of the ingested cholesterol is absorbed in the small intestine. Subsequent to the uptake of cholesterol by the small intestine, cholesterol is esterified to produce a less polar cholesteryl ester (CE), a reaction catalysed by acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2). The CE formed is packaged into chylomicrons (CM) and travels through the lymph to the liver. CM are rich in TG and also contain free cholesterol, phospholipids and APO A-I, A-IV, and B-48. Upon reaching the circulation, CM acquires APO

Table 1.1: *N*-3 PUFA international intake recommendation

Source	Date	*n*-6:*n*-3 ratio	Other specific recommendations (%en=% of daily energy intake)
National Nutrition Council of Norway	1989	none	0.5% en *n*-3 LCPUFA (1-2 g/day)
NATO Workshop on *n*-3/*n*-6	1989	none	0.8 g/day EPA/DHA (0.27%en)
Scientific Review Committee of Canada	1990	5:1-6:1	*n*-3 PUFA at least 0.5%en
British Nutrition Foundation Task-force	1992	6:1	EPA 0.2-0.5%en:DHA 0.5%en
FAO/WHO Expert Committee on Fats and Oils in Human Nutrition	1994	5:1-10:1	Consider pre-formed DHA in pregnancy
UK Committee on Medical Aspects of Food Policy (COMA)	1994	none	Fish twice/week, one of which should be oily, minimum intake EPA/DHA 200 mg/day
Ad Hoc Expert Workshop	2000	none	EPA+DHA 0.3%en:0.65 g/day minimum
France: AFFSA, CNERNA & CNRS	2001	5:1	500 mg n-3 LCPUFA/day: DHA 120 mg minimum
US National Academy of Science/Institute of Medicine	2002	none	130-260 mg EPA + DHA/day
American Heart Association	2002	none	If no CHD, eat (oily) fish twice/week; if CHD consume 1000mg *n*-3 LCPUFA/day; if high triglycerides, take 2-4g per day, under medical supervision.
UK Scientific Advisory Committee on Nutrition (SACN)	2004	none	Fish twice/week, one should be oily, min intake EPA/DHA 450 mg/day
ISSFAL	2004	none	500 mg *n*-3 lcPUFA/day
Australia and New Zealand Government Recommendations	2005	none	n-3 LCPUFA men 160 mg/day; women 90 mg/day
Superior Health Council of Belgium	2006	none	a minimum of 0.3en% EPA+DHA for adults
Health Council of the Netherlands	2006	none	to achieve the dietary reference intake of 450 mg of *n*-3 PUFA from fish a day, it is necessary to eat two portions of fish a week, at least one of them being oily fish (such as salmon, herring or mackerel).

A summary of the n-3 PUFA intake recommendation worldwide composed by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2010.http://www.issfal.org/statements/pufa-recommendations/recommendations-by-others.

LCPUFA, long chain polyunsaturated fatty acids; CHD, coronary heart disease.

C-1, C-II, C-III, and E, and the fatty acids of the TG are cleaved to produce free fatty acids (FFA). The transport of dietary fats from the intestine to the liver and movement of cholesterol to peripheral tissues in mammals is facilitated by lipoproteins (Genest, 2003). Lipoprotein particles are composed of lipids and APO responsible for their structural integrity (Ridker, 2014). Lipoproteins are categorized according to their particle density and size; the least dense lipoproteins are the CM, followed by very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), LDL and HDL. (Ridker, 2014). The densities of these lipoproteins are affected by the relative concentration of lipids to proteins. The major types of lipoproteins and their properties are given in **Table 1.2**.

During lipid metabolism, ingested dietary fats are transported from the intestine in the form of CM. The TG in the circulating CM is metabolised by lipoprotein lipase (LPL), transforming them into chylomicron remnants, which are cleared by the hepatic LDL-r and the LDL-r-related protein. The released fatty acids are transported to the adipose tissue and muscles where they form TG and are stored (Kwan et al., 2007). There is also an exchange of TG for CE between HDL and CM, and this is facilitated in humans by cholesterol ester transfer protein (CETP) (Barter et al., 2003). The liver synthesizes cholesterol and fatty acids which are packaged and transported in VLDL. Similar to CM metabolism, VLDL also undergoes hydrolysis by LPL to produce FFA, which is once again transported to the adipose tissue for storage as TG. VLDL loses its lipids and transforms to IDL which can be cleared by the liver or further metabolised by LPL resulting in a loss of APO-E to produce LDL. LDL contains APO B-100 and is the main transporter of plasma cholesterol. LDL can be cleared by LDL-r on the membrane of the liver. LDL has been said to carry the "bad cholesterol", an excess of which could be highly atherogenic.

Table 1.2: Properties and functions of lipoprotein classes

Lipoproteins	Density (g/ml)	Apoproteins	Composition (%)				Lipid delivery method	Function
			Protein	TG	c	Phosp		
Chylomicron	<0.95	B-48, C, E	2	90	5	3	Lipoprotein lipase hydrolysis	Transport of dietary TG from intestine to hepatic and extrahepatic tissues
VLDL	0.95-1.006	B-100, C, E	6	60	20	14	Lipoprotein lipase hydrolysis	Transport of TG from liver to extrahepatic tissues and precursor of IDL
IDL	1.006-1.019	B-100, E	18	20	40	22	Receptor mediated hepatic endocytosis and conversion to LDL	Precursor of LDL
LDL	1.019-1.063	B-100	21	7	50	22	Receptor mediated endocytosis by liver and extrahepatic tissues	Primarily transports cholesterol from liver to extrahepatic tissues
HDL	1.063-1.210	A	44	5	25	26	Transfers cholesterol to IDL and LDL	Responsible for reverse cholesterol transport from extrahepatic tissues to the liver

VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL; low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; c, cholesterol; Phosp. Phospholipids. Adopted and modified from (Kwan et al., 2007)

Reverse cholesterol transport (RCT) involves the transport of cholesterol from peripheral tissues to the liver for further metabolism of cholesterol. RCT prevents atherosclerotic plaque formation by reducing the amount of cholesterol available for oxidation in the wall of the artery. Macrophage cholesterol efflux is an integral part of the overall RCT; it involves the regulated expulsion of cholesterol by HDL and APO-A1 from extrahepatic lipid laden macrophages, thereby preventing atherogenesis (Cuchel and Rader, 2006). APO-A1, a major apolipoprotein of HDL, is produced by the intestine and the liver. Lipid-poor APO-A1 acquires lipid by the action of ATP binding cassette transporter (ABCA1) to form nascent HDL particle (Parks et al., 2012). Lecithin:cholesterol acyl transferase (LCAT) esterifies cholesterol in the HDL thereby forming mature HDL (Rader and Hovingh, 2014). In rodents, the free and esterified cholesterol is taken up by the liver through scavenger receptor B1 (SR-B1) without the catabolism of the HDL particles (Acton et al., 1996). The involvement of HDL in RCT makes it functionally atheroprotective. **Figure 1.2** shows a schematic representation of the lipoprotein metabolic pathway.

1.4.1.2 N-3 PUFA and serum lipoprotein metabolism

Plasma TG is associated with atherogenic lipoproteins such as VLDL and LDL. *N-3* PUFA reduces TG concentration chiefly by reducing hepatic VLDL production (Lu et al., 1999). This also leads to an increased conversion of VLDL to LDL (Chan et al., 2003), which explains the increase in large LDL cholesterol concentration upon supplementation with *n*-3 PUFA (Lu et al., 1999). Individuals supplemented with *n*-3 PUFA have an increase in LDL cholesterol concentrations, which is a risk factor of CVD (Harris, 1997, Rivellese et al., 2003). The effects of *n*-3 PUFA on lipoprotein concentrations have been controversial. The current consensus is that *n*-3 PUFA improves the quality of lipoprotein profile to a less atherogenic subclass (large

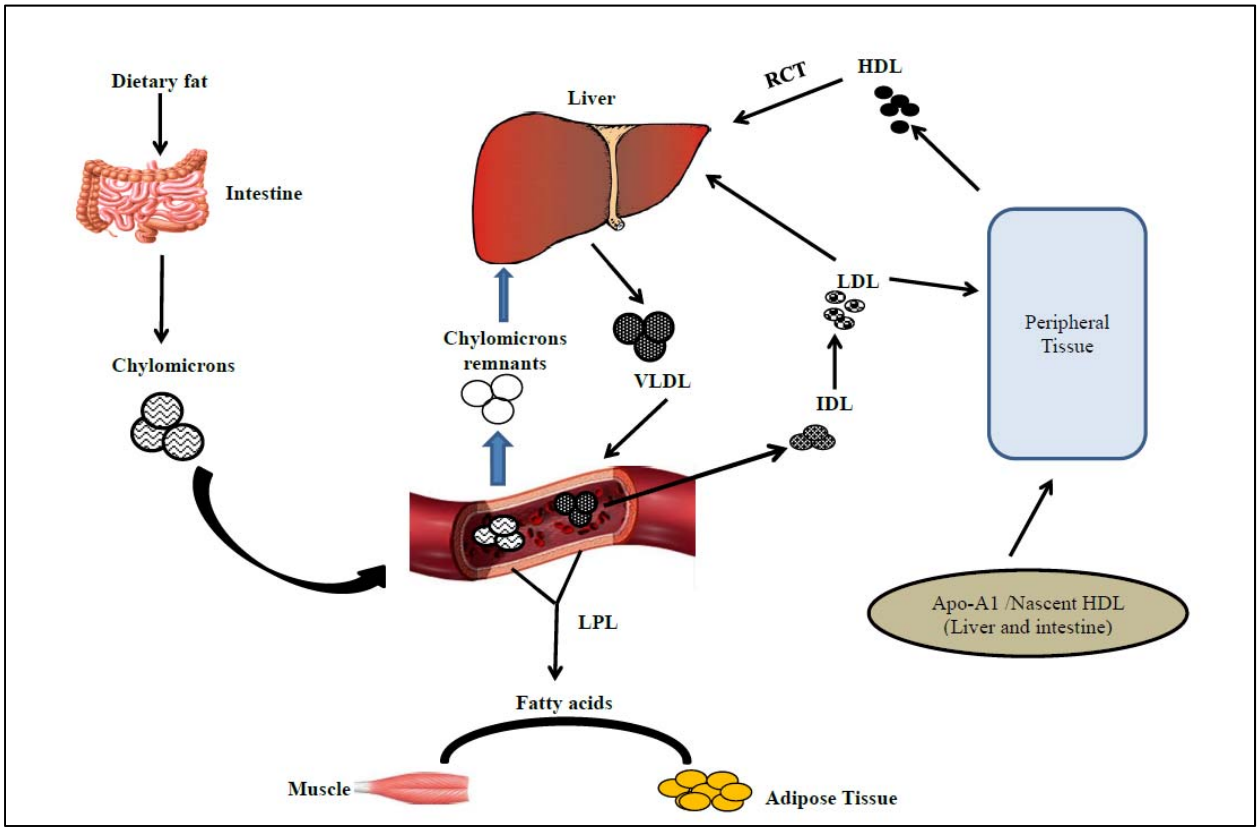

Figure 1.2: Lipoprotein metabolic pathway. VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL; low-density lipoprotein; HDL, high-density lipoprotein; LPL, lipoprotein lipase; Apo-A1, apolipoprotein A1; RCT, reverse cholesterol transport

buoyant LDL particles) without affecting its concentrations (Engler et al., 2005). Studies have shown that small dense LDL is capable of infiltrating the arterial wall and prone to oxidation, making them more atherogenic compared to the large buoyant LDL particles (Tribble et al., 1992). *N*-3 PUFA has been shown to increase the concentration of less atherogenic large LDL particles (Mori et al., 2000); this is facilitated by a decrease in TG concentration which causes an increase in LDL particle size as a result of an increase in hepatic VLDL clearance (Barter and Ginsberg, 2008). With the documented effect of sex and age on LDL-c metabolism, no study has elucidated the effect of *n*-3 PUFA on the age-dependent changes in plasma LDL-c concentration and how this specifically affects the particle sizes of LDL in males and females.

High circulating concentration of TC is also a risk factor for the development of CVD (Glass and Witztum, 2001). *N*-3 PUFA regulates TC concentration by downregulating the expression of sterol regulatory element binding protein (SREBP) thereby supressing the expression of 3-hydroxy-3-methyl-glutaryl CoA (HMG-CoA) reductase, the key enzyme of cholesterol biosynthesis (Le Jossic-Corcos et al., 2005). Liver X receptor (LXR) also prevents cellular cholesterol accumulation by upregulating the expression of 7-α-hydroxylase, cytochrome P450 [CYP7A], an enzyme involved in bile synthesis, thus converting excess cholesterol into bile (Davidson, 2006). There are also reports showing an increase in HDL cholesterol concentration upon supplementation with *n*-3 PUFA (Dunstan et al., 1997). The functionality of HDL depends on its ability to remove cholesterol, known as cholesterol efflux capacity (Khera et al., 2011). HDL is also a heterogeneous lipoprotein with different particle sizes each with different cholesterol efflux capability (von Eckardstein et al., 1994). HDL-2 and HDL-3 are the most studied classes of HDL; they differ by size and functionality with HDL-2 being larger and showing the most cholesterol-efflux potential (Ballantyne et al., 1982). *N*-3 PUFA has been

controversially reported to increase the concentration of larger and buoyant HDL-2 particles without affecting the total HDL cholesterol concentration (Chan et al., 2006), thereby increasing cholesterol efflux which has cardioprotective implication. Though there is plenty of evidence to show the effects of *n*-3 PUFA on lipid and lipoprotein metabolism, no study has accounted for the effects of sex and age on the regulation of lipid and lipoprotein metabolism by *n*-3 PUFA. Furthermore, the functionality of HDL, measured as a function of cholesterol efflux capacity, could be sex dependent, accounting for the reported differences between HDL cholesterol metabolism in females and males.

1.4.1.3 Mechanisms by which N-3 PUFA regulate lipid metabolism

Dyslipidaemia or abnormal concentration of lipids is a major independent risk factor of CVD (Ooi et al., 2013, Liberopoulos et al., 2005). Fish oil has been shown to be potent at treating hypertriglyceridaemia (Harris, 1999). It has been reported that 3-4 g/day EPA and DHA resulted in a 25% reduction of TG levels in normolipidaemic individuals and a 35% reduction in TG in individuals with hyperlipidaemia (Harris, 1997, Kris-Etherton et al., 2002). The mechanisms underlying the TG-lowering effects of *n*-3 PUFA have been explained by the involvement of *n*-3 PUFA in the regulation of genes involved in lipid metabolism; such genes include SREBP, all forms of peroxisome proliferator-activated receptors (PPARs), retinoid X receptor-alpha (RXR-α) and LXR-α. The retinoid X receptor (RXR) heterodimerises with LXR and regulates the gene expression of SREBP1-c by binding to the LXR response element in the SREBP1-c promoter region, thereby suppressing its expression (Yoshikawa et al., 2002). SREBP1-c is the key controller of lipogenesis; its inhibition has been shown to downregulate the expression of fatty acid synthase and acetyl-CoA carboxylase thereby, preventing fatty acid synthesis required for TG production (Strable and Ntambi, 2010). Another classic cardioprotective mechanism by

which *n*-3 PUFA reduce circulating levels of TG is by stimulating fatty acid β-oxidation. *N*-3 PUFA reduces TG concentration by increasing the β-oxidation of non-esterified fatty acids (NEFA) a substrate for TG synthesis (Pegorier et al., 2004). *N*-3 PUFA also regulates the expression of PPAR-α which upregulates acyl coenzyme A oxidase, a rate limiting enzyme in fatty acid catabolism, which further stimulates β-oxidation (Jump and Clarke, 1999). There is an obvious difference in the prevalence of CVD between males and females (Castelli, 1984, Kuhn and Rackley, 1993, Njolstad et al., 1996), and the risk of CVD has also been shown to increase with age in both males and females (Castelli, 1984, Tunstall-Pedoe et al., 1994, Rich-Edwards et al., 1995). To date, the majority of studies have established the cardioprotective effects of *n*-3 PUFA in adult life (Adkins and Kelley, 2010, Massaro et al., 2008), and only a handful of studies have reported the sex specific effects of *n*-3 PUFA (Phang et al., 2009, Phang et al., 2013). However, no study has comprehensively elucidated the possible interactions of sex, age, and *n*-3 PUFA on lipid and lipoprotein metabolism.

1.4.1.3.1 N-3 PUFA and adipocyte metabolism

N-3 PUFA has also been shown to reduce obesity and the majority of the data in this regard have focused on the effect of *n*-3 PUFA on adipocyte differentiation (Okuno et al., 1997, Takahashi and Ide, 2000). Studies have shown that subjecting rodents to a high fat diet increases adipocyte differentiation and adipocyte hypertrophy (Ellis et al., 1990). This has been linked to the upregulation of the expression of CCAAT/enhancer binding protein and PPARγ by high fat diet, the key regulators of differentiation of adipocyte and the storage of lipids (Lopez et al., 2003). The activation of PPARγ is at the heart of adipocyte differentiation and PPARγ deficient mice have been shown to lack adipose tissue (Barak et al., 1999). Mice heterozygous for the PPARγ gene had reduced adipose mass and smaller adipocytes (Yamauchi et al., 2001). Fatty

acids are well known activators of PPARs (Kliewer et al., 1997); however, PPARs respond differently to activation by different fatty acids. It has been reported that PUFAs are better activators of PPARs compared to both MUFA and SFA (Kliewer et al., 1997). A synthetic PPARγ agonist, pioglitazone, has been shown to facilitate an increase in fat mass in rodents by producing new, small fat cells (hyperplasia) (Hallakou et al., 1997). Whereas, activation of PPARγ by high fat diet induces both hypertrophy and hyperplasia; these effects depend on the type, class, and degree of unsaturation of fat used. *N*-3 PUFA has been shown to reduce obesity and body weight (Nakatani et al., 2003).

In-vitro studies using 3T3-L1 cells, a widely used model to study adipocyte differentiation, show that *n*-3 PUFA was less effective at stimulating adipocyte differentiation compared to SFA and MUFA (Madsen et al., 2005). The COX and LOX metabolic products of PUFA called eicosanoids are also known to activate PPARγ (Kliewer et al., 1997). While high *n*-3 PUFA has been shown to reduce obesity, high *n*-6 PUFA diet has the propensity to induce obesity (Massiera et al., 2003). In *in-vitro* studies, *n*-6 PUFA was reported to increase adipocyte differentiation and TG accumulation, whereas *n*-3 PUFA treatment caused less accumulation of TG compared to *n*-6 PUFA (Petersen et al., 2003). Eicosanoids produced by the metabolism of *n*-6 PUFA have been suggested to be responsible for *n*-6 PUFA-induced adipocyte differentiation (Catalioto et al., 1991); inhibiting the expressions of COX-1 and COX-2 has been suggested to rescue adipocyte differentiation induced by *n*-6 PUFA (Petersen et al., 2003). EPA is a poor substrate for COX relative to AA, and *n*-3 PUFAs are known to inhibit the expression of COX, further emphasizing the anti-obesity effects of *n*-3 PUFA (Ringbom et al., 2001).

Adipocyte hypertrophy is the main cause of adipose tissue enlargement and obesity; however, most studies that sought to understand the cellular mechanism of obesity have focused

on adipocyte differentiation. Adipocyte hypertrophy is a consequence of excess accumulation of TG in the adipose tissue; therefore, elucidating the molecular processes involved in the formation of TG in the adipose tissue is important in understanding the development of obesity. Acyl CoA:diacylglycerol acyltransferase (DGAT) is responsible for the synthesis of TG (Bell and Coleman, 1980); it catalyses the covalent binding of acyl group to diacyglycerol (DG), which is the final step of TG synthesis (Chen et al., 2002). Furthermore, fatty acid binding protein-4 (FABP4), also known as adipocyte fatty acid binding protein-2, has been shown to play a vital role in adipocyte hypertrophy (Uysal et al., 2000). Studies have shown that obese individuals have increased expression of FABP4 (Xu et al., 2006, Reinehr et al., 2007), and individuals who lost weight were reported to have a reduced expression of FABP4. However, there is still a paucity of knowledge on the mechanisms involved in adipocyte hypertrophy, and whether the anti-obesity effect of *n*-3 PUFA is elicited by regulating the key genes involved in adipocyte hypertrophy. Furthermore, obesity has been identified as an independent risk factor of CVD in both males and females (Willett et al., 1995, Manson et al., 1990); however, most researchers have excluded the effect of sex and an adequate amount of *n*-3 PUFA in the diet.

1.4.2 Cardioprotective effects of n-3 PUFA by altering membrane dynamics and production of bioactive lipids

N-3 PUFA exists in nature as TG or PL. TG contains three fatty acids bound to a glycerol backbone, and PL contains two fatty acids bound to a glycerol backbone with phosphorus and a head group which could mainly be a choline, ethanolamine, serine, inositol or glycerol. There are three major classes of PLs: the glycerophospholipids, sphingolipids and etherglycerolipids (Burri et al., 2012). Because of their amphipathic nature, PLs are found primarily in plasma membranes where they influence membrane fluidity. The most common membrane PLs are the

glycerophospholipds, mainly phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE).

Membrane fluidity is influenced by the incorporation of long chain *n*-3 PUFA into membrane PL (Hulbert et al., 2005, Leaf et al., 2005), which could alter the functions of transmembrane proteins and their interaction with extracellular ligands (Ma et al., 2004). These alterations indirectly affect signalling pathways and other physiological functions. Furthermore, the fatty acid on the *sn*-2 position of membrane PL could be cleaved by the action of phospholipase A_2 (PLA_2) to produce FFA and lyosphosphatidylcholine (LPC) which are both bioactive molecules (Thies et al., 1992). The released twenty carbon AA and EPA from the membrane phospholipids undergo cyclooxygenases (COX-1 and COX-2) and lipoxygenases-(LOX-5, LOX-12, LOX-15) catalysed oxidation to produce bioactive lipid intermediates and eicosanoids (Jump et al., 2012). AA is a precursor to series-2 prostaglandins and series-4 leukotrienes; these are generally pro-inflammatory, vasoconstrictive and pro-aggregatory, and generally promote CVD (Farooqui et al., 2007). On the other hand, series-3 prostaglandins and series-5 leukotrienes produced from EPA are anti-inflammatory and prevent the development CVD (Adkins and Kelley, 2010). AA-derived eicosanoids include pro-inflammatory PGE_2 (vasodilator), TXA_2 (vasoconstrictor), PGI_2 (platelet aggregator), and LTB_4 (chemotactic factor), while EPA-derived eicosanoids include anti-inflammatory PGE_3 (vasodilator), PGI_3 (inhibit platelet aggregator), and LTB_5 and TXA_3, which are less active than AA-derived eicosanoids (Simopoulos, 1999, Engler and Engler, 2006) **(Figure. 1.1)**.

The second product of hydrolysis of PC is LPC which has been generally considered atherogenic mainly because of its role in promoting arterial inflammation (Kabarowski, 2009). LPC has been shown to increase the expression of adhesion molecules such as vascular cell

adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), promote endothelial dysfunction and increase the expression of inflammatory cytokines and consequently atherogenic plaque formation (Zalewski and Macphee, 2005). It is important to acknowledge that the physiological properties of LPC are heavily dependent on its acyl chain moiety (Ojala et al., 2007). The majority of data that have reported the pro-inflammatory and pro-atherogenic potential of LPC have used SFA or MUFA-containing LPC (Bach et al., 2010). There is, however, new evidence that supports the anti-inflammatory effects of DHA-containing LPC species (Huang et al., 2010). DHA containing LPC has been shown to exhibit its anti-inflammatory property by blocking the activity of 5-LOX responsible for the generation of pro-inflammatory metabolites including LTB_4 (Huang et al., 2008). DHA-LPC is more potent at stimulating the anti-inflammatory process compared to AA-LPC. DHA containing LPC also reduced the concentration of pro-inflammatory cytokines when compared with other LPC-containing fatty acids (Huang et al., 2010). DHA-LPC could be metabolised to release free DHA which could stimulate anti-inflammatory pathways, or the DHA-LPC can be oxygenated by 15-LOX to produce 17S-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17-HPDHA-LPC) which is further metabolised to generate protectin D, a strong anti-inflammatory metabolite (Serhan et al., 2002). In addition to its anti-inflammatory effects, DHA-LPC has been shown to be the preferred carrier of DHA to the brain where it is required for neuronal functions (Bernoud et al., 1999, Thies et al., 1994). Furthermore, LPC has been shown to promote cholesterol efflux from macrophages (Hara et al., 1997). Although the mechanism involved is unclear, it has been suggested that LPC promotes cholesterol efflux through its association with HDL-associated paraoxonase 1(PON1) (Rosenblat et al., 2006). An increase in HDL LPC production increases the binding of HDL to macrophages thereby stimulating increased cholesterol efflux.

The type of fatty acid released from the membrane phospholipids depends on the dietary fatty acid composition. Given the information on the health benefits of bioactive compounds derived from the metabolism of *n*-3 PUFA, including *n*-3 PUFA containing LPC, it will be important to investigate the effect of high *n*-3 PUFA diet on the different classes of bioactive lipids generated from the metabolism of *n*-3 PUFA, and the functional role of these bioactive lipids.

1.4.3. Cardioprotective effects of n-3 PUFA by reducing inflammation

The development of atherosclerosis encompasses a cycle of inflammatory processes, and is now recognised as an inflammatory disease (Hansson, 2005). Studies have reported the ability of *n*-3 PUFA to reduce the expression of adhesion molecules on macrophages (Hughes et al., 1996) and endothelial cells (Weber et al., 1995), thereby preventing the atherosclerotic plaque progression. *N*-3 PUFA also prevents CVD by maintaining vascular endothelial function. The adhesion molecules secreted by endothelial cells are actively involved in platelet adhesion and leukocyte recruitment during inflammation, which are major players in atherogenesis (Adkins and Kelley, 2010). These adhesion molecules are activated by pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8; *n*-3 PUFA has been shown to decrease the expression of these cytokines.

The expression of adhesion molecules on the endothelium is also promoted by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a pro-inflammatory transcription factor (Kumar et al., 2004, Sigal, 2006). NF-kB is involved in a number of cellular responses including inflammation and the immune response to infection. Activation of NF-kB leads to a sequence of reactions that promote inflammation, and consequently atherogenesis. The activated form of NF-kB has been found in atherosclerotic vessel walls and promotes the formation of atherosclerotic

lesions (Brand et al., 1996). *N*-3 PUFA downregulates the expression of NF-kB to suppress inflammation (Chen et al., 2005); this reduces the markers of atherosclerosis, and enhances vascular function (Gupta et al., 2008). *N*-3 PUFA has also been found to impede the translation of genes involved in inflammation by inhibiting NF-kB. Furthermore, EPA was found to inhibit the expression of TNF-α, a classic pro-inflammatory cytokine by preventing the movement of NF-kB into the nucleus (Zhao et al., 2004). NF-kB upregulates the expression of inflammatory cytokines such as IL-6, IL-2, and TNF-α; *n*-3 PUFA has been shown to reduce these cytokines. The expression of PPARs, an important class of nuclear receptor proteins involved in the regulation of inflammation and lipid metabolism has also been shown to be directly regulated by *n*-3 PUFA (Marx et al., 1998). PPARα and γ inhibit NF-kB thereby blocking the production of potent inflammatory cytokines. PPARα has also been shown to exhibit its cardioprotective effect by blocking the production of cellular adhesion molecules, leading to a significant reduction of inflammation (Marx et al., 1998). Moreover, as mentioned previously, *n*-3 PUFA can also alleviate inflammation resulting from atherogenesis by producing anti-inflammatory eicosanoids and docosanoids from EPA and DHA, respectively (Wanten and Calder, 2007).

1.5 *N*-3 PUFA and the brain

Recent evidence suggests a relationship between cardiometabolic irregularities and neurocognitive decline (O'Brien et al., 2003); this proposition is further strengthened by the similarities in the events and timeline leading to the pathological decline of the brain and cardiac functions (Picano et al., 2014). Individuals with neurological abnormalities often have vascular impairment (Snowdon et al., 1997). Emerging evidence reveals that certain trophic factors known as neurotrophins thought to be classically neurotrophic in function also possess metabotropic properties (Chaldakov et al., 2009). The brain's structural component is 50-60%

lipids by dry weight, and 30-35% of brain's total lipids are *n*-3 and *n*-6 PUFA (Youdim et al., 2000). The maintenance of a unique fatty acid profile is very important for brain function and is characterized by a high accretion of SFA, MUFA, DHA, AA, and less of EPA, LA and ALA (Youdim et al., 2000). The brain depends on dietary and hepatic supplies of PUFA to maintain its level (Demar et al., 2005). Although neuronal lipid composition is tightly regulated, dietary supplementation can go a long way in altering neuronal membrane lipid composition. Studies have shown that 6-8 weeks of supplementation with EPA significantly increased the accretion of EPA and its longer chain metabolite, docosapentaenoic acid (DPA), in mouse brain (Luchtman et al., 2012, Meng et al., 2010). Furthermore, a higher intake of DHA increased the accretion of DHA in the brain with a consequent decrease in longer chain *n*-6 PUFA (Bousquet et al., 2008), and a reciprocal effect is observed during DHA deprivation, thereby maintaining the overall level of unsaturation in the brain. However, DHA and AA are functionally different. A high intake of *n*-6 PUFA promotes cognitive impairment (Fedorova and Salem, 2006). The lipid component of the brain is primarily PL, with high enrichment of DHA and AA (Sastry, 1985). DHA is preferentially incorporated into the PE and PS fractions of the brain's membrane PLs (Rapoport, 2001); however, the most abundant PLs in the brain are PE and PC (Rapoport, 2001). PUFAs are released from neuronal membrane PLs by the action of PLA_2. There are three isoforms of PLA_2; 1) the calcium dependent PLA_2 ($cPLA_2$; 2) secretory PLA_2 ($sPLA_2$); 3) calcium independent PLA_2 ($iPLA_2$) (Rapoport, 2013).

DHA is preferentially acylated to the *sn*-2 position of PE and PS (Lee and Hajra, 1991), and is mobilized by the action of iPLA2; while cPLA2 mobilizes AA (Farooqui and Horrocks, 2004). The released DHA is physiologically important in the regulation of signalling cascades (McNamara et al., 2006), production of anti-inflammatory metabolites (Hong et al., 2003), use

for β-oxidation (Yavin et al., 2002) or re-incorporation into membrane PLs. The increased accretion of DHA during foetal development corresponds to the period of neurogenesis, neuronal differentiation and myelination, further emphasizing the importance of DHA during these critical periods (Green et al., 1999). DHA is accumulated in the brain during the third trimester of pregnancy to two years after birth in humans and gestation day 7 to 21 day after birth in rodents (Green et al., 1999, Martinez, 1992). During this period in humans, there is rapid increase in weight of the human brain from approximately 20-1200 g (Carlson et al., 2013). This represents the period of rapid neuronal myelination and maturation of synapses (Dobbing and Sands, 1973). Clandinin *et al* reported that there is a rapid accretion of DHA and AA in the brain during the third trimester of pregnancy and a decrease in the concentration of LA and ALA (Clandinin et al., 1980). Martinez found that the brain accretion of DHA and AA increases 30-fold from the third trimester until about two years after birth (Martinez, 1992).

During pregnancy, the foetus depends on the mother for an adequate supply of DHA, and this is made available through placental transfer (Innis, 2005). DHA is transferred through the placenta in non-esterified form (Dutta-Roy, 2000), which comes from the lipoprotein lipase-catalysed hydrolysis of maternal lipoprotein. Studies have shown that DHA is preferentially transferred across the placental to foetal circulation compared to ALA, LA, AA, oleic and palmitic acid (Larque et al., 2003, Haggarty et al., 1997). The developing foetus has a reduced ability to convert ALA to DHA because of a reduced hepatic expression of the enzymes involved during gestation; therefore the foetus relies on the mother for an optimal supply of the preformed DHA required for brain development (Uauy et al., 2000).

DHA deficiency in the brain during development can be normalized by DHA supplementation; however, relative to other tissues such as the liver and red blood cells, recovery

from DHA deficiency is slow (Moriguchi et al., 2001, Xiao et al., 2005). DHA composition in adult brain is resistant to dietary *n*-3 PUFA deficiency (Bourre et al., 1992), as shown by an increase in the half-life of DHA as a result of a deficiency in *n*-3 PUFA (DeMar et al., 2004). Ageing is a natural process accompanied by a number of physiological changes. During ageing, there is a decrease in DHA concentration in the brain which coincides with the alteration in neurotransmission and other vital functions of the brain (Favrelere et al., 2000), including the development of neurological disorders such as Alzheimer's disease (Soderberg et al., 1991).

The protective role of *n*-3 PUFA in mental health is well documented. *N*-3 PUFA supplementation has been shown to reduce the symptoms of attention deficit hyperactivity disorder (ADHD) by 25% in patients supplemented with 174 mg DHA and 558 mg EPA for 3 months (Johnson et al., 2009). The efficacy of *n*-3 PUFA in treating autism has also been reported. Autistic children supplemented with 700 mg DHA and 840 mg EPA for four weeks saw significant improvement of their symptoms compared to the control (Amminger et al., 2007). The protective effects of *n*-3 PUFA in some neurological diseases such as Alzheimer's disease (Morris et al., 2003) and multiple sclerosis (Mehta et al., 2009) have also been reported The beneficial effect of *n*-3 PUFA is essential in developing brain as well as in mature brain (Kitajka et al., 2004). Studies have also documented that mothers that consumed high amount of *n*-3 PUFA during pregnancy had children with better cognitive abilities than non-*n*-3 PUFA consuming mothers (Daniels et al., 2004), suggesting *n*-3 PUFA is vital for brain function. However, the exact mechanisms by which *n*-3 PUFA regulate the function of the developing brain as well as the mature brain are still not clear. The function of the brain is regulated by specific neurotrophins, and it has been suggested that *n*-3 PUFA likely alter the expression of neurotrophins.

1.5.1 Neurotrophins and the brain

The term neurotrophins refers to a group of trophic factors required for neuronal differentiation and survival (Huang and Reichardt, 2001). The major classes of neurotrophins are the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT 4/5). The activities of neurotrophins are turned on during the embryonic stage, and may be restricted to a particular developmental period (Birling and Price, 1995). There is high heterogeneity in the expression of neurotrophins in the nervous system, and they generally decrease with age (Zermeno et al., 2009). Neurotrophins are vital to the proper functioning of the central nervous system where they play an important role in neuronal survival, cell differentiation, synaptogenesis, and synaptic plasticity (Lu et al., 2008, Reichardt, 2006). Among the neurotrophins, the activity of BDNF is the best characterised, and an alteration in the expression of BDNF has been shown to have a significant impact on neuronal activities and predisposition to cognitive decline, and neuropsychiatric disorders in both humans and rodents (Castren and Rantamaki, 2008, Chen et al., 2006). The expression of BDNF is regulated both at the mRNA and protein levels (Greenberg et al., 2009). The endoplasmic reticulum synthesizes BDNF as a precursor protein preproBDNF which is then converted to proBDNF and transported to the Golgi, where it is converted to mature BDNF (mBDNF) by the action of furin and endoprotease, or in the secretory granules by convertases (Mowla et al., 1999).

Neurotrophins are released as their precursor proneurotrophins approximately 30kDa in size, and are cleaved to form the mature neurotrophins of approximately 13kDa in size. All neurotrophins bind with low affinity to P75 ^{NTR} receptor (Rodriguez-Tebar et al., 1990), while neurotrophins binds specifically with high affinity to TrK receptors. NGF binds to TrKA (Klein et al., 1991a); the ligands for TrKB are BDNF (Klein et al., 1991b) and NT-4/5 (Klein et al.,

1992) while TrKC is the receptor for NT-3 (Lamballe et al., 1991). Binding of neurotrophins to TrK receptors initiates a series of downstream phosphorylation of proteins including PI3K/AKT pathway, Ras kinase pathway (Kaplan and Miller, 2000), and the phosphorylation of cAMP response element binding protein (CREB), which consequently leads to the activation of genes involved in neuronal survival. Signalling activities of neurotrophins elicited through TrK receptors promote neuronal survival; on the contrary, signalling through the $p75^{NTR}$ promotes neuronal apoptosis (Bibel and Barde, 2000).

1.5.1.1. Neurotrophins and n-3 PUFA: brain and heart connection

The secretion of neurotrophins is not limited to the central nervous system as they are released in the peripheral system as well, where they have been shown to play a role in atherosclerosis and lipoprotein metabolism among others (Tasci et al., 2012). Interestingly, neurotrophins and *n*-3 PUFA share some functional similarities; in addition to their effects on neural and cardiovascular functions, with age, there is a significant decrease in tissue accretion of *n*-3 PUFA (Lommatzsch et al., 2005), as well as neurotrophins (Golden et al., 2010), and a consequent increase in the risk of cardiovascular and neuropsychiatric disorders. Approximately 80% of the secreted BDNF comes from the brain; however, there is evidence of secretion in peripheral tissues (Suliman et al., 2013). There are reports of the effects of BDNF in both neuropsychiatric and metabolic disorders suggesting a brain-body regulatory role of BDNF (Yoshida et al., 2012). There is also evidence of the involvement of BDNF in inflammation which is a major player in CVD (Cai et al., 2006); low circulating BDNF concentration has been reported in patients with CVD and metabolic syndrome (Golden et al., 2010). Mice expressing aberrant BDNF receptor TrKB have been shown to develop cardiovascular abnormalities (Palko et al., 1999). Heterozygous knockout mice for BDNF are hyperphagic, with the propensity to

become obese and insulin resistant (Kernie et al., 2000). Laboratory findings have shown that the administration of BDNF improves glucose metabolism in diabetic mice models (Tonra et al., 1999), and the circulating BDNF concentration is age and sex-specific (Golden et al., 2010). Studies have also shown inverse correlation between plasma BDNF and circulating blood lipid concentrations including TG, cholesterol and LDL-c (Golden et al., 2010, Jung et al., 2011, Jiang et al., 2011). BDNF has been shown to lower plasma NEFA, TC and blood glucose in mice (Tsuchida et al., 2002). Similar to neurotrophins, *n*-3 PUFA is well established to be both neuroprotective (Georgieff, 2007) and cardioprotective (Saravanan et al., 2010); however, the effects of *n*-3 PUFA on the causal relationship between these two conditions have not been elucidated. In addition to the neuroprotective effects of *n*-3 PUFA, neurotrophins have also been shown to be important in neuronal development. With the reported cardioprotective and neuroprotective properties of BDNF and *n*-3 PUFA, it will be important to investigate whether they both share a regulatory relationship. No study has investigated the age-dependent role of *n*-3 PUFA in regulating neurotrophin signalling in the brain as all evidence points towards the speculation that the cardioprotective and neuroprotective effects of *n*-3 PUFA are centrally mediated by neurotrophin signalling.

1.6 Controversies in the cardioprotective effects of *n*-3 PUFA

There are numerous reports on the beneficial health effects of *n*-3 PUFA, however there are also controversial reports on the cardioprotective efficacy of *n*-3 PUFA. A recent systematic review and meta-analysis on the association between *n*-3 PUFA supplementation and risk of CVD revealed that *n*-3 PUFA was not associated with lower mortality from cardiovascular events (Rizos et al., 2012). Furthermore, a recent study reported no significant effect on markers of CVD after a 6 year supplementation with *n*-3 PUFA (Investigators et al., 2012). These

controversies could be due to differential effects of *n*-3 PUFA based on sex, dose, duration of exposure and the type of *n*-3 PUFA used.

1.6.1 Sex, age and the cardioprotective effects of n-3 PUFA

There are sex-specific differences in the regulation of *n*-3 PUFA metabolism. Studies have reported a higher tissue accretion of DHA in females compared to males (Decsi and Kennedy, 2011, Extier et al., 2010), which is due to a higher conversion rate of ALA to longer chain EPA and DHA in women (Decsi and Kennedy, 2011). This is explained mechanistically by the sex-specific effect of the liver desaturase enzyme responsible for the conversion of ALA to EPA and DHA, with female rats showing a higher hepatic expression of δ-5 and δ-6 desaturases compared to males (Extier et al., 2010). Interestingly, oestrogen has been shown to affect tissue distribution of *n*-3 PUFA with females showing a higher concentration in plasma and tissues, further explaining sex specific differences (Childs et al., 2008). Although controversial, the role of oestrogen in LDL metabolism has been reported; oestrogen decreased the circulating concentration of atherogenic LDL (Campos et al., 1997). There are also sex differences in the concentration of HDL particles between men and women (Knopp et al., 2005). The mechanism underlying the sex-specific difference in lipid metabolism has been explained by the interaction between PPAR-α and the oestrogen receptor. Studies have shown that a signal cross talk exists between PPAR-α and oestrogen receptor (Souidi et al., 1999, Wang and Kilgore, 2002). There is also evidence of the sex specific effect of PPAR-α on the regulation of cholesterol metabolism (Costet et al., 1998).

The effect of sex in the development of CVD cannot be overemphasized; however, age may be equally important. The prevalence of CVD is different between male and female, and this has

also been shown to be affected by age (Castelli, 1984); these differences have been linked to the age-dependent variation in cholesterol concentration between males and females (Rossouw, 2002). The plasma TC concentration increases with age (Corti et al., 1997) mainly because of the age-dependent decrease in the catabolism of cholesterol (Corti et al., 1997). Furthermore, males and females have similar plasma HDL cholesterol concentration at puberty after which differences develop with age (Abbey et al., 1999). There are also documented age effects on plasma concentration of TG concentration (Castelli, 1984). Given the sex-specific effects on the distribution and accretion of *n*-3 PUFA, and the clear distinction in lipid and lipoprotein metabolism between males and females, it is important to elucidate the sex specific effects on *n*-3 PUFA on markers of CVD before a solid recommendation of *n*-3 PUFA can be made. Furthermore, it will also be important to consider the effect of age on the sex specific effect of *n*-3 PUFA as this clearly will provide information on how these factors will affect the cardioprotective effects of *n*-3 PUFA.

1.6.2 Dose and the cardioprotective effects of n-3 PUFA

It is imperative to administer an appropriate dose of *n*-3 PUFA to get the desired cardioprotective benefits. The recent systematic review and meta-analyses that discredited the cardioprotective effects of *n*-3 PUFA (Rizos et al., 2012) considered studies that used a lower than the recommended dosage of *n*-3 PUFA; the mean intake of EPA and DHA in their study was approximately half the recommended dosage of 3 g/day to treat cardiovascular complications. The beneficial effects of *n*-3 PUFA on cardiovascular events have been shown to be dose dependent. Moertl *et al* found a 2.5% improvement in left ventricular ejection fraction in patients with coronary heart failure after administering 1g/day of *n*-3 PUFA for 3 months; however, a 5.5% improvement was recorded with a higher dose of 4g/day of *n*-3 PUFA (Moertl

et al., 2011). In the same cohort, only higher doses were effective at resolving inflammation and alleviating endothelial dysfunction, further emphasizing the importance of dose in the health benefits of *n*-3 PUFA (Moertl et al., 2011). Furthermore, studies have also reported the beneficial effect of *n*-3 PUFA dosage greater than 1g/day at treating endothelial dysfunction (Wright et al., 2008). To obtain a full cardioprotective effect of *n*-3 PUFA, a dosage in excess of the currently recommended 1g/day will be required (Calder, 2009). The immunomodulatory effects of *n*-3 PUFA has also been shown to be dose dependent, with observable benefits within the range of 1.65 and 3.3 g/day (Calder, 2009). The importance of dosage in the health benefits of *n*-3 PUFA has also been corroborated by animal studies with significant improvement from cardiovascular events observed at a higher dosage of about 7% of energy intake from EPA + DHA (Duda et al., 2009). Several clinical trials have reported the beneficial effects of consuming fish and *n*-3 PUFA supplements at different dosages. Subjects who were asked to consume 200-400 g oily fish per week or the equivalent of fish oil capsules (EPA; 180 mg and DHA; 120 mg) saw significant cardioprotective effects (Burr et al., 1989). In another study, individuals who received approximately 882 mg of EPA and DHA (1:2) had up to 20% reduction in the risk of CVD (Marchioli et al., 2002) compared to those without *n*-3 PUFA supplements (1999). Analyses of prospective cohort studies have shown that the consumption of 250-500 mg/day EPA and DHA is sufficient to prevent the risk of CVD (Harris et al., 2009). Another study reported that the upper limit of 500 mg/day provided the most beneficial effect (Harris et al., 2008), and that even greater intake will confer additional protection (Makhoul et al., 2010). It was also reported that 4 g/day of *n*-3 PUFA reduced TG concentration by 25-30 %, and a dose response relationship was also observed (Harris, 1997). Another study of 42 participants revealed a 45% decrease in TG after supplementing patients with 4 g/day *n*-3 PUFA (Harris et al., 1997). The cardioprotective

benefits of *n*-3 PUFA have been well documented; however, the optimal dose remains to be established. There is no firm recommended dietary intake for *n*-3 PUFA; however, different international organisations have made dietary recommendations with a common theme of *n*-6 to *n*-3 PUFA ratio of approximately 5:1 as shown in **Table 1.1.** The *n*-6 to *n*-3 PUFA ratio of 5:1 has been suggested as an optimal ratio for whole body homeostasis, which is different from the 30:1 ratio which approximately represents the current *n*-6: *n*-3 PUFA ratio in a typical Western diet. No study has comprehensively assessed the effect of *n*-3 PUFA dosage with reference to the *n*-6: *n*-3 PUFA ratio on the markers of CVD.

1.7 Mouse as an animal model

Mouse genetics are the most characterised among mammals (Paigen et al., 1990). The mouse has also been favoured as a model of animal experimentation because of its small size and short reproduction time (Fazio and Linton, 2001), which would allow for studies involving the monitoring of transfer of genetic information through generations. We chose the C57BL/6 mouse for our study because it has been established as a model for the study of diet-induced atherosclerosis (Paigen et al., 1990, Paigen et al., 1987), diabetes and obesity (Surwit et al., 1988, Surwit et al., 1991). This model is susceptible to high-fat diet-induced hyperlipidaemia, atherosclerosis, and obesity, making it suitable for the study of lipid and lipoprotein metabolism. C57BL/6 is also a strain of choice used to generate numerous transgenic models to study pathological conditions related to lipid and lipoprotein metabolism (Getz and Reardon, 2006), thus leaving the option of extending our study with different transgenic models in the future. We acknowledge the difference in the regulation of lipid and lipoprotein metabolism between mice and humans. In contrast to humans, mice do not have CETP; therefore, 70% of TC occurs in HDL in mice, while the major carrier of cholesterol in human is LDL (Kako et al., 2002).

However, easily obtainable information from mice would provide a greater understanding of the regulation of the lipid and lipoprotein metabolism in humans.

1.8 Rationale and hypotheses

There are numerous reports supporting the cardioprotective effects of *n*-3 PUFA; however, there are also inconsistent reports. Given the complexity and physiological variation of the human population, it is pertinent to consider the various factors that could potentially affect the metabolism and hence the health benefits of *n*-3 PUFA before a strong recommendation can be made. The studies outlined in this thesis were carried out to better understand the effects of *n*-3 PUFA on the risk factors of CVD such as dyslipidaemia and obesity, with particular emphasis on the regulation of lipid and lipoprotein metabolism, and factors such as age, sex, and dose that could potentially affect the health benefits of *n*-3 PUFA. These studies also sought to identify novel mechanisms by which *n*-3 PUFA could reduce the risk of CVD using C57BL/6 mouse model. The specific aims and the underlying hypotheses of the study were:

Aim 1: To investigate dose-, age- and sex-specific effects of dietary *n*-3 PUFA on the regulation of plasma lipids and lipoprotein concentrations (Chapter 2)

***Hypothesis*:** There is an obvious difference in the prevalence of CVD between males and females, and the risk of CVD has also been shown to increase with age in both male and female. However, no study has comprehensively elucidated the possible interactions of sex, age, and *n*-3 PUFA on lipid and lipoprotein metabolism. It was hypothesized that the effects of *n*-3 PUFA on lipid and lipoprotein concentrations will be dose-dependent, and longer exposure to *n*-3 PUFA diet will significantly enhance the effects of *n*-3 PUFA. It was further hypothesized that the effects of *n*-3 PUFA on lipids and lipoproteins will be higher in females compared to males.

Aim 2: To investigate the effects of dietary *n*-3 PUFA on the lipidomic profile of plasma and liver (Chapter 3)

Hypothesis: Cells, tissues and biological fluids consist of numerous bioactive lipid mediators involved in cellular processes, which are likely altered by dietary *n*-3 PUFA. It was hypothesized that a diet high in *n*-3 PUFA will increase the proportion of longer chain *n*-3 PUFA in bioactive lipids.

Aim 3: To investigate the sex-specific effects of dietary *n*-3 PUFA on adipocyte metabolism (Chapter 4)

Hypothesis: Adipocyte hypertrophy is a consequence of excess accumulation of TG in the adipose tissue, therefore, elucidating the molecular process involved in the formation of TG in the adipose tissue is important in understanding the development of obesity. It was hypothesized that high dietary *n*-3 PUFA will prevent adipocyte hypertrophy by downregulating the mRNA expression of key proteins involved in adipocyte hypertrophy in a sex-dependent fashion.

Aim 4: To investigate whether dose and duration of exposure to dietary *n*-3 PUFA will cause alterations in DHA accretion in the brain and the expression of neurotrophins (Chapter 5)

Hypothesis: *N*-3 PUFA and neurotrophins are important to the proper functioning of the central nervous system. However the mechanism/s by which *n*-3 PUFA regulate neurotrophin signalling are still not clear. It was hypothesized that perinatal and sustained post-weaning diet high in *n*-3 PUFA will cause an accretion of DHA in the brain of the offspring, and consequently increase the mRNA expressions of BDNF, NGF, TrKB, and CREB in an age dependent fashion.

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CHAPTER TWO

Dose, age, and sex-specific effects of dietary n-3 PUFA on the regulation of plasma lipids and lipoproteins metabolism

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2.1 Abstract

There is clear evidence of the effects of sex and age on the prevalence of cardiovascular disease. We investigated the interactions of dietary *n*-3 PUFA, sex, and age on plasma lipids and lipoproteins in the offspring of C57BL/6 mice exposed to varying amounts of *n*-3 PUFA at weaning and 16 weeks postweaning. There was an increase in plasma TG from weaning to 16 weeks in both male and female offspring; however, the high *n*-3 PUFA group showed a reduction in TG in both sexes at 16 weeks. High *n*-3 PUFA caused an increase in plasma LDL-c from weaning to 16 weeks in male offspring; however, the LDL particle size was significantly larger in the high *n*-3 PUFA group. Plasma from male mice showed higher cholesterol efflux compared to females; high *n*-3 PUFA increased cholesterol efflux. Thus the effects of *n*-3 PUFA are age and sex dependent.

2.2 Introduction

CVD is a complicated and multifarious disease, and is the number one cause of mortality worldwide. A report from WHO has shown that 30% of the global deaths in 2008 were caused by CVD, and by 2030, more than 23 million people will die annually from CVD (WHO, 2011). Even though CVD is the leading cause of death between men and women, there is a clear evidence of the influence of sex in the prevalence of CVD (Mosca et al., 2011). Several independent factors are associated with the morbidities of CVD, such as high circulating TG and LDL-c (Carmena et al., 2004, St-Pierre et al., 2005), hyperglycaemia (Eguchi et al., 2007, Wahab et al., 2002), hyperinsulinaemia (Ingelsson et al., 2005), and elevated makers of inflammation (Engler et al., 2003). In addition to the aforementioned risk factors, age and sex also play an important role in the development of CVD (Castelli, 1984).

Growing evidence suggests that the predisposition to CVD begins *in utero* and progresses through adulthood (Barker, 1995, Barker, 1997, Barker, 2004a, Barker, 2004b). Research findings have also shown that a mother's nutritional status during pregnancy may affect the future predisposition of the offspring to the onset of diseases in later years (de Boo and Harding, 2006). Moreover, the hallmarks of metabolic and cardiovascular diseases such as dyslipidaemia, obesity, and diabetes have been linked to poor maternal diet and placental dysfunction (Hales and Barker, 2001). Previous studies from our laboratory demonstrated that a maternal diet high in SFA caused an increase in offspring plasma lipid and lipoprotein levels, and induced endothelial dysfunction (Chechi and Cheema, 2006, Chechi et al., 2009). SFA are known to increase the risk of CVD (Keys, 1997), while PUFAs are considered beneficial (Kushi et al., 1995). There is an increased intake of PUFA, mainly *n*-6 PUFA, in the typical North American

diet (Simopoulos, 2002). On the other hand, the intake of highly unsaturated long chain *n*-3 PUFA has declined in the recent years (Simopoulos, 2002).

N-3 PUFA such as EPA, and *n*-6 PUFA such as AA, are primary substrates for eicosanoid production. Eicosanoids derived from AA are considered pro-inflammatory, pro-thrombotic, and generally promote atherosclerosis (Simopoulos, 2002). On the contrary, diets rich in *n*-3 PUFA have been shown to be anti-inflammatory, anti-thrombotic, and prevent CVD (Simopoulos, 2002). Furthermore, *n*-3 PUFA has been reported to alleviate markers of CVD such as high TG, endothelial dysfunction, cardiac arrhythmia, and inflammation (Calder, 2006, Harris, 1997, Leaf et al., 2003, Goodfellow et al., 2000, Hirafuji et al., 2003). The cardiovascular benefits of *n*-3 PUFA are mediated through modifications of lipoprotein profile, and by increasing LDL particle size (Sanders et al., 1997, Kelley et al., 2007). The metabolism of *n*-3 PUFA has been shown to be sex-specific, with sex hormones influencing the desaturation and elongation enzymes involved in the synthesis of long chain *n*-3 PUFA (Burdge and Wootton, 2002, Pawlosky et al., 2001). However, it is not known whether the effect of *n*-3 PUFA on the regulation of lipid and lipoprotein profiles is sex and age specific.

There is an obvious difference in the prevalence of CVD between males and females (Castelli, 1984, Kuhn and Rackley, 1993, Njolstad et al., 1996), and the risk of CVD has also been shown to increase with age in both males and females (Castelli, 1984, Tunstall-Pedoe et al., 1994, Rich-Edwards et al., 1995). To date, the majority of studies have established the cardioprotective effects of *n*-3 PUFA in adult life (Adkins and Kelley, 2010, Massaro et al., 2008), and a handful of studies have also reported the sex-specific effects of *n*-3 PUFA (Phang et al., 2009, Phang et al., 2013). However, no study has comprehensively elucidated the possible interactions of sex, age, and *n*-3 PUFA on lipid and lipoprotein metabolism. In the present study,

we hypothesized that the effects of *n*-3 PUFA on lipid and lipoprotein concentrations will be dose-dependent, and longer exposure to *n*-3 PUFA diet will significantly enhance the effects of *n*-3 PUFA. We further hypothesized that the effects of *n*-3 PUFA on lipids and lipoproteins will differ between males and females. We therefore investigated the effect of varying the quantity of dietary exposure to *n*-3 PUFA during gestation and lactation on lipid and lipoprotein concentrations of the male and female offspring of C57BL/6 mice at weaning. We further evaluated whether longer exposure to postweaning diet high in *n*-3 PUFA will cause further alterations in the concentrations of plasma lipids and lipoproteins, and whether the effect is sex-specific. Our findings revealed that longer exposure to high dietary *n*-3 PUFA during perinatal and postweaning periods reduced plasma circulating concentrations of lipids, and atherogenic very small low density lipoprotein particles in the offspring. We further report for the first time that a diet high in *n*-3 PUFA increased cholesterol efflux *ex-vivo*. Our results also suggest sex- and age-specific effects of *n*-3 PUFA on the regulation of lipid and lipoprotein concentrations.

2.3 Materials and methods

2.3.1 Diets

The experimental diets were prepared from a base semi-synthetic diet with the fat sources omitted and designed to allow the control of fat at 20% w/w (MP Biomedicals, USA). Information on the macronutrient composition of the semi-synthetic diet and oil mixtures are presented in **Table 2.1**. Fish oil (Menhaden), safflower oil, extra-virgin olive oil, and lard were used as sources of *n*-3 PUFA, *n*-6 PUFA, MUFA and SFA respectively. The oils were used to make three different mixtures containing approximately 10% (High *n*-3), 4% (Medium *n*-3), and 2% (Low *n*-3) *n*-3 PUFA of the total dietary fat. Total SFA, MUFA, and PUFA were kept constant in the experimental diet. The medium and low *n*-3 PUFA diets were designed to

contain an *n*-6:*n*-3 PUFA ratio of 15:1 and 30:1 respectively which approximately represent the current *n*-6:*n*-3 PUFA ratios in a typical Western diet; while the high *n*-3 PUFA diet contains an *n*-6:*n*-3 PUFA ratio of 5:1, which is suggested to be an optimal ratio for whole body homeostasis (Gomez Candela et al., 2011). The diets were isocaloric and contained the same amount of protein, carbohydrate and fat; the only difference was the ratio of *n*-6 to *n*-3 PUFA. The diets were designed to test the quality of dietary fat consumed in regards to the ratio of *n*-6:*n*-3 PUFA composition. Gas-liquid chromatography (GLC) was used to determine the fatty acid composition of the experimental diets (**Table 2.2**). Fresh diets were prepared by mixing the semi-synthetic powder with the oil mixture at 20% w/w as the only source of fat and treated with nitrogen gas to prevent oxidation, then stored frozen at -20°C.

2.3.2 Animals and experimental design

All experimental procedures involving animals were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee. Male and female C57BL/6 mice (seven weeks old) were purchased from Charles Rivers Laboratories (MA, USA), and were housed in separate cages (2 mice/cage) under controlled temperature (21± 1°C) and humidity (35 ± 5%) conditions with a 12-hour light/12-hour dark period cycle. Mice were kept on standard rodent chow pellets (Prolab RMH 3000) (PMI nutrition, MO, USA) for a one week acclimatization period. After this period, female mice were randomly divided into three groups. Each group was fed one of the three experimental diets that differed only in their *n*-3 PUFA composition, and designated as "high *n*-3", "medium *n*-3", or "low *n*-3" diets for two weeks before mating, after which one male mouse was introduced into a cage with two female mice for a one week period. Vaginal plug formation was used to confirm pregnancy, and pregnant mice were moved to a clean cage

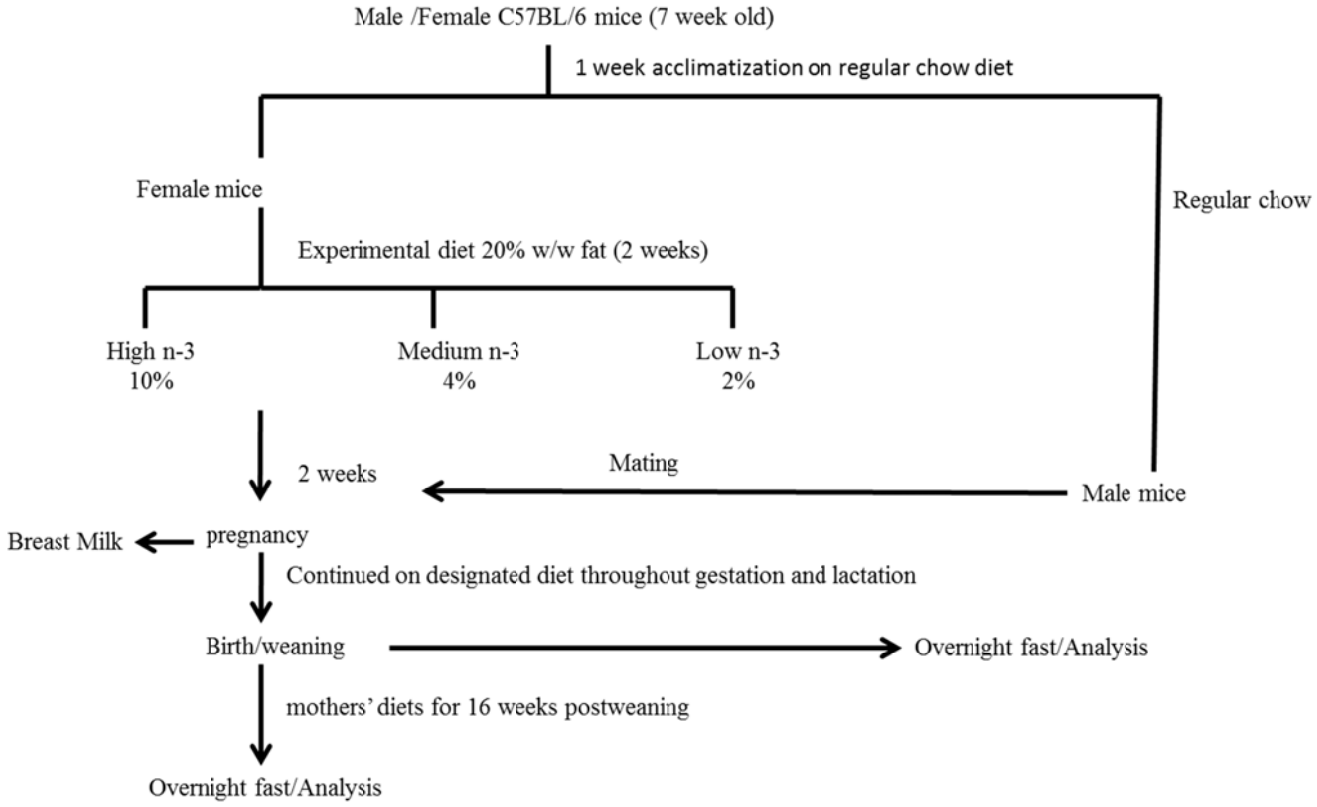
throughout gestation and until weaning. Mothers were fed experimental diets throughout gestation, lactation, and until weaning. Pups were counted on the first day of delivery as postnatal day one, after which pups and mothers were not disturbed to prevent cannibalism. Fresh food and water were provided *ad-libitum* every day, and body weight was recorded every week. At weaning, pups were sexed, culled, and housed separately according to sex (2 mice/cage). Half of the offspring (male, n=10 and female, n=10) were studied at weaning, while therest of the offspring (male, n=8 and female, n=8) were continued on their mothers' designated diet for 16 weeks (healthy young adult mice). Mice were sacrificed after an overnight fast either at weaning or after 16 weeks on specified diets using isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4), and plasma was separated immediately. Tissues were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80ºC until further analyses.

At weaning, dams were milked after anesthetizing with pentobarbital (35mg/kg/ intraperitoneal (IP) / (0.1ml/100g) and milk secretion was stimulated using oxytocin (4 IU/kg IP). Throughout the experimental period, animals were provided with water and fresh food *ad-libitum*, every other day. Body weights were recorded once a week and food intake was recorded every other day. No significant differences were observed in both body weight and food intake amongst the groups (*Appendix II*).

2.3.3 Analyses of biochemical parameters

Plasma lipid and lipoprotein concentrations were quantified using commercially available kits according to the manufacturers' instructions: plasma TG kit #236-17 (Genzyme Diagnostics, PEI, Canada); TC kit #234-60 (Genzyme Diagnostics, PEI, Canada); NEFA kit # 999-34691

Schematic representation of the experimental design



(Wako Chemicals, VA, USA). HDL-c was precipitated from plasma using kit #200-26A (Diagnostic Chemicals Ltd, Canada) and the HDL-c concentration was determined using TC assay kit #234-60 (Genzyme Diagnostics, PEI, Canada). Plasma LDL-c concentration was calculated according to the method of Friedewald *et al.* (Friedewald et al., 1972, Fraulob et al., 2010). Plasma LDL particle size was analyzed by LipoScience Inc. (NC, USA) using NMR techniques. LDL particle size was reported in particle concentration unit (nanomoles of particles per liter) (Jeyarajah et al., 2006).

2.3.4 Fatty acid analyses of breast milk by gas liquid chromatography

Lipids were extracted from breast milk using the method of Folch *et al* (1957). Fatty acid methyl esters were prepared by heating lipid extracts with 2 ml of trans-methylation reagent (6% concentrated sulfuric acid and 94% methanol + few crystals of hydroquinone added as an anti-oxidant) for 2 h at 65°C. Extraction of methyl esters was performed using hexane and water at -20°C overnight. The hexane layer of the sample was dried the following morning under gentle stream of nitrogen gas and the residue was dissolved in carbon disulfide prior to GLC analysis. Samples were run on an Omegawax X 320 (30 m x 0.32 mm) column from Supelco (Sigma-Aldrich, Canada) using a flame ionization detector for 60 minutes. The GLC parameters were set as: oven, 200°C; injector, 240°C; detector 260°C; helium was used as the carrier gas. PUFA standards -2 and -3 (Sigma-Aldrich, Canada) were used as standards for identification of fatty acids by retention time.

2.3.5 Macrophage cholesterol efflux assay

Macrophage cholesterol efflux assay was performed according to previously published methods with minor modifications (Adorni et al., 2007, Boone et al., 2011). Briefly, J774 cells

were seeded in 12 well plates at a density of 2 x 10^5 cells/well in RPMI medium supplemented with 10% foetal bovine serum (FBS) and 1 X antibiotic/antimycotic. The following day, labelling was performed in RPMI supplemented with 1% FBS, 1 μCi/ml ^{3}(H)-cholesterol (Perkin Elmer, MA, USA), 2 μg/ml ACAT (acyl-CoA: cholesterol acyl transferase) inhibitor 58-035 (Sandoz, QC, CA), and 1X antibiotic/antimycotic for 24 hours. Cells were equilibrated for 18 hours in RPMI medium in the presence of LXR agonist and ABCA1 agonist, T0901317 (1μM) (Sigma, MO, USA), and Retinoic X receptor (RXR) agonist, retinoic acid (1μM) (Sigma, MO, USA). Cholesterol efflux was initiated by treating cells with RPMI containing 2% plasma samples from either the high (n=6) or low (n=6) *n*-3 PUFA group as the efflux acceptor; or 0.2% bovine serum albumin (BSA) as the control, for 5 hours. At the end of the 5 hour efflux interval, the medium was collected and centrifuged at 2000 rpm for 5 minutes. Supernatants were removed for liquid scintillation counting. Wells were washed twice with 1X PBS, and residual radioactivity in the cells was determined after scraping the cells in 1X PBS. Cholesterol efflux was calculated as [^{3}H]-cholesterol in medium/([^{3}H]-cholesterol in medium + [^{3}H]-cholesterol in cells) x 100. All efflux values were corrected by subtracting the % efflux at time zero (before active/passive efflux).

2.3.6 Statistical analysis

Data were analyzed using IBM SPSS Statistics (Version 20.0). Differences between means were compared using three way analysis of variance (ANOVA) to determine the main effects and interactions of diets, age, and sex. Two way ANOVA was used to analyze the very small LDL particle size and cholesterol efflux data with diet and sex as factors. Pairwise comparison using Bonferroni correction was used to determine differences when an interaction was observed. Breast milk fatty acid composition was expressed as weight percentage of the total

Table 2.1. Composition of the semi-purified diet with 20% (w/w) fat level

Ingredients	High *n*-3 diet	Medium *n*-3 diet	Low *n*-3 diet
		g/kg	
Casein	200	200	200
DL-methionine	3	3	3
Sucrose	305.8	305.8	305.8
Corn starch	200	200	200
Alphacel non-nutritive bulk	50	50	50
DLA tocopherol powder	1.2	1.2	1.2
Mineral mix*	40	40	40
Fat	200	200	200
Menhaden oil	42.3	10.7	2.1
Safflower oil	131.9	135.55	136.55
Olive oil	11.75	8.2	7.2
Lard	14.05	45.55	54.15

Supplied in quantities adequate to meet NRC requirements (National Research Council, 1995).

Ψ Vitamin Mix *(1 kg): Thiamine hydrochloride, 0.6 g; riboflavin, 0.6 g; pyridoxine hydrochloride, 0.7 g; nicotinic acid, 3.0 g; d-calcium pantothenate, 1.6 g; folic acid, 0.2 g; d-biotin, 0.02 g; cyanocobalamin (vitamin B_{12}), 0.001 g; retinyl palmitate (vitamin A) pre-mix (250,000 IU/g), 1.6 g; DL-α-tocopherol acetate (250 IU/g), 20 g; cholecalciferol (vitamin D_3, 400,000 IU/g), 0.25 g; menaquinone (vitamin K_2), 0.005 g; sucrose, finely powdered, 972.9 g*

****Mineral Mix:*** *Calcium phosphate dibasic, 500.0 g/kg; sodium chloride, 74.0 g/kg; potassium citrate monohydrate, 220.0 g/kg; potassium sulfate, 52.0 g/kg; magnesium oxide, 24.0 g/kg; manganese carbonate (43-48% Mn), 3.50 g/kg; ferric citrate (16-17% Fe), 6.0 g/kg; zinc carbonate (70% ZnO), 1.6 g/kg; cupric carbonate (53-55% Cu), 0.30 g/kg; potassium iodate, 0.01 g/kg; sodium selenite, 0.01 g/kg; chromium potassium sulfate, 0.55 g/kg; sucrose, finely powdered, 118.0 g/kg*

Table 2.2 Fatty acid composition of the experimental diets*

Fatty Acids	Low *n*-3	Medium *n*-3	High *n*-3
14:0	0.11	0.39	1.26
16:0	6.32	7.43	8.71
18:0	5.35	4.53	2.67
∑ **SFA**	11.77	12.35	12.64
16:1*n*-7	0.36	0.09	2.41
18:1*n*-9 + C18:1*n*-7	27.82	25.81	25.14
20:1*n*-9	ND	0.54	0.61
∑ **MUFA**	28.18	26.43	28.16
18:2*n*-6	57.73	57.03	47.86
20:4*n*-6	0.11	0.14	0.23
18:3*n*-6	0.04	0.04	0.10
22:4*n*-6	0.09	ND	0.54
∑ **Omega-6**	57.92	57.18	48.90
18:3*n*-3	0.55	0.64	0.78
20:5*n*-3	0.31	1.37	3.64
22:6*n*-3	0.39	1.16	3.19
18:4*n*-3	0.15	0.20	0.87
22:5*n*-3	0.46	0.32	0.63
20:4*n*-3	0.08	0.12	0.66
∑**Omega-3**	1.93	3.81	9.76

**Data are expressed as weight percentage of the total extracted fatty acids. Σ SFA= sum of saturated fatty acids, Σ MUFA= sum of monounsaturated fatty acids, Σ PUFA= sum of polyunsaturated fatty acids, Σ Omega-6= sum of omega-6 fatty acids, Σ Omega-3= sum of omega-3 fatty acids, ND= Not detected.*

extracted fatty acids; fatty acid composition data were then arcsine transformed before subjecting to one way ANOVA and Newman-Keuls post hoc statistical tests. Values were expressed as mean ± SD. Differences were considered to be statistically significant if the associated *P* value was < 0.05.

2.4 Results

2.4.1 Effect of perinatal exposure to diets varying in n-3 PUFA on breast milk fatty acid composition

Breast milk fatty acid composition of female mice fed high, medium, or low *n*-3 PUFA diet during gestation and lactation is given in **Table 2.3**. There was no significant difference in total and individual SFA and MUFA among the three dietary groups. Furthermore, there was no significant difference in the total composition of *n*-6 PUFA between the three dietary groups. However, the high *n*-3 PUFA group showed a significantly lower concentration of AA compared to both medium and low *n*-3 PUFA groups ($P<0.01$). There was a significant difference in total *n*-3 PUFA and DHA among the three dietary groups ($P<0.01$), with the high *n*-3 PUFA group showing the highest concentration, followed by the medium and low *n*-3 PUFA groups. There was a significant increase in breast milk concentrations of EPA and docosapentaenoic acid (22:5n3; DPA) in the high *n*-3 PUFA group compared to the medium *n*-3 PUFA group ($P<0.01$). Red blood cells phospholipids fatty acid composition was measured at weaning and 16 weeks, which also confirmed dietary incorporation of essential fatty acids.

Table 2.3 Total breast milk fatty acid composition of mothers fed experimental diets during pregnancy and lactation*

Fatty Acids	Low *n*-3	Medium *n*-3	High *n*-3
C14:0	2.52 ± 1.28	3.41 ± 2.06	2.85 ± 1.64
C16:0	15.53 ± 1.99	14.95 ± 2.75	14.13 ± 2.50
C18:0	5.28 ± 0.48	5.84 ± 0.89	6.92 ± 4.53
∑ **SFA**	23.32 ± 2.83	24.19 ± 5.05	23.91 ± 3.84
C16:1*n*7	1.05 ± 0.71	1.57 ± 0.83	1.90 ± 1.23
C18:1*n*9	26.47 ± 2.05	29.51 ± 1.57	21.84 ± 6.65
C20:1*n*9	0.87 ± 0.24	0.82 ± 0.09	0.59 ± 0.05
∑ **MUFA**	34.13 ± 3.02	32.78 ± 4.96	27.34 ± 9.14
C18:2*n*6	39.63 ± 4.37	36.92 ± 1.51	39.60 ± 5.67
C18:3*n*6	0.45 ± 0.19	0.40 ± 0.11	0.50 ± 0.18
C20:4*n*6	2.02 ± 0.05^{a}	1.89 ± 0.12^{a}	1.44 ± 0.13^{b}
∑ **Omega-6**	42.18 ± 4.46	39.97 ± 1.90	41.76 ± 5.79
C18:3*n*3	0.23 ± 0.20	0.32 ± 0.01	0.36 ± 0.16
C20:5*n*3	ND	0.42 ± 0.10^{b}	1.68 ± 0.56^{a}
C22:5*n*3	ND	0.66 ± 0.11^{b}	1.30 ± 0.26^{a}
C22:6*n*3	0.12 ± 0.21^{c}	1.22 ± 0.37^{b}	2.69 ± 0.61^{a}
∑ **Omega-3**	0.36 ± 0.37^{c}	2.80 ± 0.56^{b}	6.46 ± 1.73^{a}

**Data are expressed as weight percentage of the total extracted fatty acids. Values are expressed as mean ± SD, n = 3. Data were assessed using one-way ANOVA after arcsine transformation. Significant effects were further analysed using Newman-Keuls post hoc test. Mean values within a row with unlike superscript letters were significantly different (P < 0.05). Σ SFA= sum of saturated fatty acids, Σ MUFA= sum of monounsaturated fatty acids, Σ PUFA= sum of polyunsaturated fatty acids, Σ Omega-6= sum of omega-6 fatty acids, Σ Omega-3= sum of omega-3 fatty acids, ND= Not detected.*

2.4.2 Effect of diets varying in n-3 PUFA on the concentrations of plasma lipids of male and female offspring at weaning and 16 weeks postweaning

There was an independent effect of age on plasma concentration of TG ($P < 0.0001$; Figure 2.1A), with plasma TG increasing significantly from weaning to 16 weeks in both male and female offspring. There was also a significant independent effect of diet on plasma TG concentration ($P < 0.0001$; Figure 2.1A). The male offspring showed no significant difference in plasma TG among the three experimental groups at weaning (Figure 2.1A); however, at 16 weeks, there was a significant decrease in plasma TG in the high *n*-3 PUFA group compared to medium and low *n*-3 PUFA groups ($P<0.001$: Figure 2.1A). The female offspring on the other hand showed significantly lower plasma TG in the high *n*-3 PUFA groups at both weaning and 16 weeks compared to the low *n*-3 PUFA group ($P<0.01$; Figure 2.1). No significant interaction of age and diet, age and sex, and diet and sex were observed in the plasma TG concentration (Figure 2.1A). Furthermore, it was interesting to note that there was no significant difference in plasma TG concentration between low and medium *n*-3 PUFA diet groups in male and female offspring at 16 weeks (Figure 2.1A).

A significant interaction of diet and sex was observed in plasma TC cholesterol concentration ($P = 0.015$; Figure 2.1B). There was a significant diet dependent increase in plasma TC in the low *n*-3 PUFA group compared to the medium and high *n*-3 PUFA group in the male offspring at weaning and 16 weeks ($P < 0.05$; Figure 2.1B). At weaning, the female offspring in the low *n*-3 PUFA group showed a significantly higher plasma TC concentration ($P < 0.05$; Figure 2.1B) compared to the medium and high *n*-3 PUFA groups, while no significant effect of diet was observed at 16 weeks. Furthermore, a significant interaction of age and sex was observed in plasma TC concentration ($P < 0.0001$; Figure 2.1B). There was an age-dependent increase in the

concentration of plasma TC concentration in male offspring; interestingly, this effect was not observed in female offspring (Figure, 2.1B).

There was a significant interaction of age and diet in plasma concentration of NEFA ($P < 0.0001$; Figure 2.1C). A significant effect of age and sex was also observed in plasma concentration of NEFA ($P < 0.003$; Figure 2.1C). Perinatal exposure to diet high in *n*-3 PUFA had no effect on plasma circulating NEFA at weaning both in male and female offspring (Figure 2.1C). However, there was a significant decrease in plasma NEFA concentration after sustained exposure to high *n*-3 PUFA diet for 16 weeks in both male ($P<0.0001$; Figure 2.1C) and female ($P<0.0001$; Figure 2.1C) mice compared to both medium and low *n*-3 PUFA diets. Interestingly, there was no difference in NEFA concentrations in medium and low *n*-3 PUFA groups at 16 weeks.

2.4.3 Effect of diets varying in n-3 PUFA on the concentrations of plasma lipoproteins of male and female offspring at weaning and 16 weeks post-weaning

Plasma HDL-c revealed a significant interaction between age and sex ($P < 0.01$; Figure 2.2A). Perinatal and postweaning diets high in *n*-3 PUFA had no effect on HDL-c concentrations among the three dietary groups both at weaning and 16 weeks in male and female offspring (Figure 2.2A). Cholesterol efflux showed significant interaction of diet and sex ($P = 0.006$; Figure 2.2B). The female offspring had lower cholesterol efflux with respect to diet as compared to the male offspring ($P = 0.006$; Figure 2.2B). The high *n*-3 PUFA group had higher cholesterol efflux compared to the low *n*-3 PUFA group in male offspring at 16 weeks ($P<0.05$; Figure 2.2B). However, there was no effect of diet on cholesterol efflux in female offspring at 16 weeks (Figure 2.2B).

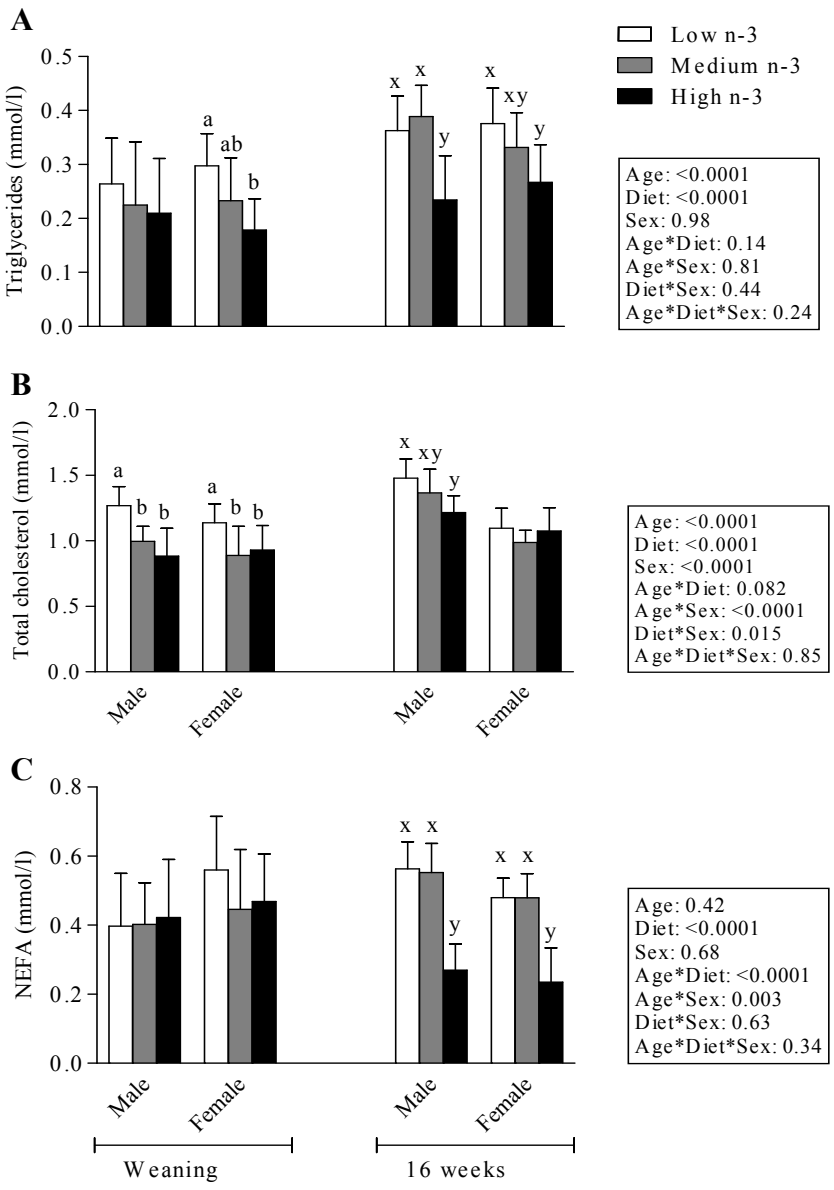


Figure 2.1. Effects of diets varying in the quantity of n-3 PUFA on plasma lipids of offspring at weaning and 16 weeks: *Plasma triglycerides (A), total cholesterol (B), and non-esterified fatty acids, NEFA (C) concentrations of male and female offspring were measured at weaning and 16 weeks as explained under methods section. Values are expressed as means ± SD, n =10 (weaning) and n=8 (16 weeks). Data were assessed using three way ANOVA to determine the main effects and interactions of diets, age and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a,b) were used to denote significant differences between various dietary groups at weaning, and letters (x,y) represent significant differences between various dietary groups at 16 weeks. $P < 0.05$ was considered significant.*

Plasma LDL-c concentration showed significant interactions of age and diet (P = 0.002; Figure 2.3A); age and sex (P = 0.012; Figure 2.3A); and diet and sex (P = 0.004; Figure 2.3A). There was a marked reduction of LDL-c concentration in the high and medium *n*-3 PUFA groups compared to the low *n*-3 PUFA group at weaning in male offspring (P < 0.001; Figure 2.3A). These differences in LDL-c concentration however, disappeared at 16 weeks, with the three dietary groups showing no statistically significant differences (Figure 2.3A). There was however an increase in LDL-c concentration at 16 weeks compared to weaning in male offspring. Interestingly, the female mice showed a somewhat distinct LDL-c profile. At weaning the female offspring showed no difference in LDL-c concentration between the high and low *n*-3 PUFA groups (Figure 2.3A); however, the medium *n*-3 PUFA group was significantly lower than the low *n*-3 PUFA group (P<0.01; Figure 2.3A). At 16 weeks, the female offspring in the high *n*-3 group showed an increase in LDL-c compared to both the medium and low *n*-3 PUFA groups (P<0.01; Figure 2.3A), the medium *n*-3 PUFA group had the lowest LDL-c concentration groups (P<0.01; Figure 2.3A).

Since the high *n*-3 PUFA group showed higher LDL-c concentrations at 16 weeks, the LDL particle size of the high and low *n*-3 PUFA groups was measured. Although there was no difference in plasma LDL-c concentration in the male offspring at 16 weeks between the high and low *n*-3 PUFA groups, the high *n*-3 PUFA group showed a significantly lower concentration of very small LDL particles compared to the low *n*-3 PUFA group (P<0.05; Figure 2.3B). The female offspring in the high *n*-3 PUFA group also showed a significantly lower concentration of very small LDL particles compared to the high *n*-3 PUFA group at 16 weeks (P<0.05; Figure 2.3B). There was also an effect of sex on the plasma concentration of very small LDL particles,

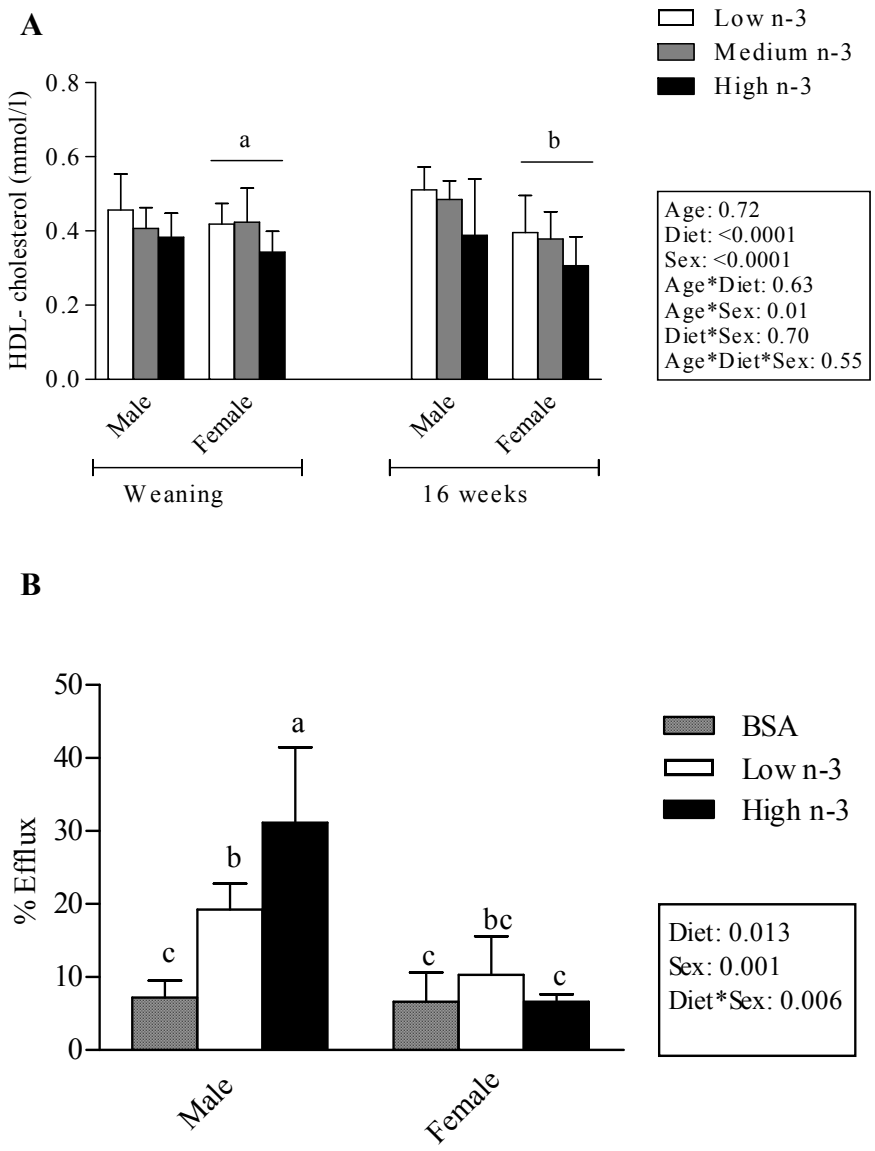


Figure 2.2. Effects of diets varying in the quantity of n-3 PUFA on plasma HDL-cholesterol (HDL-c) concentration and cholesterol efflux.

Plasma HDL-c concentration (A) of male and female offspring were measured at weaning and 16 weeks as explained in the methods section. Values are expressed as means ± SD, n =10 (weaning) and n = 8 (16 weeks). Data were assessed using three way ANOVA to determine the main effects and interactions of diets, age and sex for plasma HDL-cholesterol; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Cholesterol efflux (B) was measured from plasma from male and female

mice as explained in the methods section. Cholesterol efflux was calculated as ([^{3}H]-cholesterol in medium/[^{3}H]-cholesterol in medium + [^{3}H]-cholesterol in cells) x 100. All efflux values were corrected by subtracting the % efflux at time zero (before active/passive efflux). Values are expressed as % cholesterol efflux mean ± SD, n = 5. Data were assessed using two way ANOVA to determine the main effects and interactions of diets and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a,b,c) represent significant differences where P < 0.05 was considered significant.

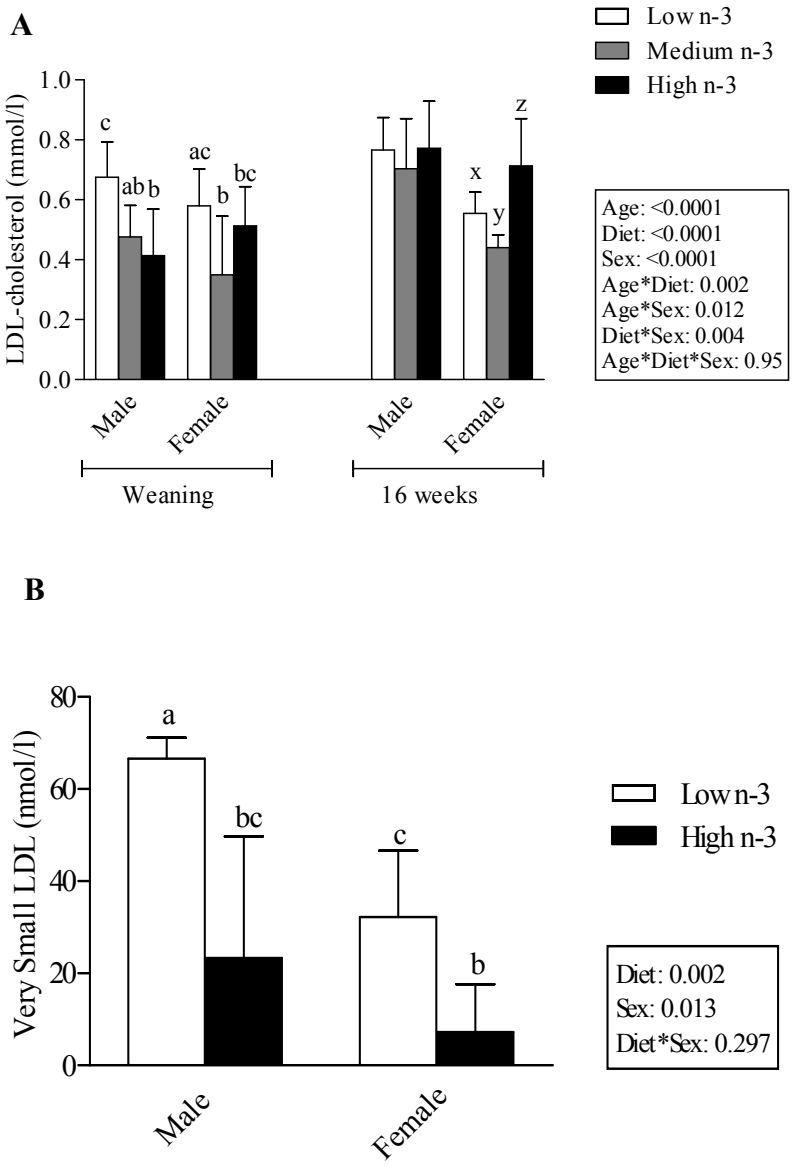


Figure 2.3 Effects of diets varying in the quantity of n-3 PUFA on plasma LDL-cholesterol (LDL-c) and very small LDL particle size concentrations

Plasma LDL-c concentration (A) of male and female offspring was measured at weaning and 16 weeks as explained in the methods section. Values are expressed as means ± SD, n =10 (weaning) and n = 8 (16 weeks). Data were assessed using three way ANOVA to determine the main effects and interactions of diets, age and sex; pairwise comparison using Bonferroni

correction was used to determine differences when there was an observed interaction. Letters (a,b,c) were used to denote significant differences between various dietary groups at weaning, and letters (x,y,z) represent significant differences between various dietary groups at 16 weeks. $P < 0.05$ was considered significant. Plasma concentration of very small LDL particle size (B) of male and female offspring was measured at 16 weeks as explained in the methods section. LDL particle size was reported in particle concentration units (nanomoles of particles per litre) and values are expressed as means ± SD. Data were assessed using two way ANOVA to determine the main effects and interactions of diets and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed interaction. Letters (a,b,c) represent significant differences between various dietary groups where $P<0.05$ was considered significant.

with the female offspring showing a lower concentration of very small LDL particles compared to male offspring ($P = 0.013$; Figure 2.3B).

2.5 Discussion

The predisposition to CVD has been shown to be affected by age and sex, and the difference in sex predisposition to CVD has been linked to disparities in age-dependent fluctuation of cholesterol concentration in males and females (Rossouw, 2002). The cardiovascular benefits of *n*-3 PUFA are mediated by the modification of lipid and lipoprotein profiles; however, there is a paucity of information on sex- and age-specific effects of *n*-3 PUFA (Kelley et al., 2007, Sanders et al., 1997). In the current study, the effect of sustained exposure to perinatal and postweaning diets high in *n*-3 PUFA on plasma lipid and lipoprotein concentrations was investigated in the male and female offspring of C57BL/6 mice at weaning and 16 weeks postweaning. Our findings reveal for the first time, that a perinatal diet high in *n*-3 PUFA reduced the markers of dyslipidaemia in the offspring, and that the effects of *n*-3 PUFA on lipids and lipoproteins are augmented by sustained exposure to a postweaning diet rich in *n*-3 PUFA. Our findings also established that the effect of *n*-3 PUFA on the concentration of plasma lipids and lipoproteins is age- and sex-dependent.

Breast milk fatty acids, especially long chain PUFA are required for optimal development of the offspring (Novak and Innis, 2011). The breast milk fatty acid composition was therefore measured to confirm that the essential fatty acids in the perinatal diet fed to the mothers are incorporated in their breast milk. There was a positive correlation between diet and breast milk fatty acid composition *(Appendix III),* confirming that maternal dietary *n*-3 PUFA supplemented during pregnancy and lactation is reflected in the breast milk. The breast milk samples from the

high *n*-3 PUFA group were highly enriched with DHA, followed by the medium *n*-3 PUFA group, and the low *n*-3 PUFA group showed the least breast milk concentrations of DHA. Others have also shown that supplementing maternal diet with *n*-3 PUFA during lactation led to an increase in breast milk EPA and DHA composition (Novak and Innis, 2011, Arterburn et al., 2006, Brenna et al., 2007, Jen et al., 2009). EPA and DPA were not detected in the low *n*-3 PUFA which could be as a result of the low concentration of fish oil in the low *n*-3 PUFA diet. Others have reported a low concentration of EPA and no detection of DPA in breast milk of rats fed low *n*-3 PUFA diet (Novak and Innis, 2011).

Elevated plasma TG is an independent risk factor of CVD, and a long standing association exists between TG and coronary heart diseases (Austin et al., 1998, Sarwar et al., 2007). Both epidemiological and animal studies support the TG-reducing effect of *n*-3 PUFA (Imaichi et al., 1963, von Lossonczy et al., 1978, Mori and Woodman, 2006, Kelley et al., 2007, Zampolli et al., 2006, Niot et al., 1994). However, the majority of studies that assessed the effects of *n*-3 PUFA on plasma TG used adults. We report an independent effect of age and diet on the plasma concentration of TG. There was an age-dependent increase in the concentration of TG in both male and female offspring. This is not surprising as TG concentration increases with age as a result of increase in body weight and accumulation of fat from weaning to adulthood (Castelli, 1984). No significant difference in plasma TG concentration was found in the male offspring at weaning. This is consistent with the findings of Korotkova *et al* (Korotkova et al., 2005, Korotkova et al., 2004), who reported that the offspring of mothers fed a high *n*-3 PUFA diet showed no change in plasma TG at three weeks. However, our findings showed that after 16 weeks of sustained exposure to the experimental diets, the male offspring on high *n*-3 PUFA diet showed a significant reduction in plasma TG concentrations compared to both medium and low

n-3 PUFA groups. On the other hand, female offspring in the high *n*-3 PUFA group showed a significant reduction in plasma TG concentration both at weaning and 16 weeks compared to the low *n*-3 PUFA group. Joshi *et al* reported a lower TG level at 6 months in male offspring of dams fed an *n*-3 PUFA rich diet (Joshi et al., 2003). Other studies also support our findings that longer exposure to *n*-3 PUFA has TG lowering effects in adult rats (Niot et al., 1994, Froyland et al., 1997). It was interesting to note that plasma TG concentration was not different between low and medium *n*-3 PUFA groups in both male and female offspring at 16 weeks. Thus, our data strongly suggests that a longer exposure to *n*-3 PUFA and a higher dose of *n*-3 PUFA is necessary to elicit its TG reducing effects.

Several mechanisms have been proposed by which TG levels are reduced by *n*-3 PUFA (Kawakami et al., 2006). TG is synthesized in the liver in response to the circulating concentrations of NEFA (Kersten et al., 1999, Pegorier et al., 2004), and it has been suggested that the TG lowering effect of *n*-3 PUFA could be due to an increased stimulation of β-oxidation, which leads to a decrease in available NEFA for TG formation (Mizushima et al., 1997). We found a significantly lower plasma NEFA concentration in both male and female offspring fed a high *n*-3 PUFA diet at 16 weeks, which could be responsible for the lower TG concentration in this group. *N*-3 PUFA is well known to increase the gene expression of PPARα (Wahli et al., 1995), which upregulates acyl coenzyme A oxidase to increase β-oxidation (Jump and Clarke, 1999). Furthermore, TG synthesis is controlled by the transcription factor sterol regulatory element binding protein-1c (SREBP-1c), the key gene in lipogenesis. PPARα has been shown to regulate the expression of SREBP-1c (Fernandez-Alvarez et al., 2011, Knight et al., 2005), thus, *n*-3 PUFA controls TG concentration by downregulating the gene expression of SREBP-1c (Pegorier et al., 2004), which in turn inhibits lipogenesis, and by stimulating β-oxidation.

There was an age dependent increase in plasma concentration of TC in male offspring from weaning to 16 weeks, however, this was not observed in female offspring. An increase in plasma TC with age is well documented (Richter et al., 2004, Corti et al., 1997, Shepherd, 2001), which results from an age-dependent decrease in catabolism of cholesterol (Corti et al., 1997). Furthermore, there was a significant interaction of diet and sex on the concentration of plasma TC; the high and medium *n*-3 PUFA groups showed lower plasma TC concentration compared to the low *n*-3 PUFA group in both male and female offspring at weaning. The novel interactive effect of *n*-3 PUFA and sex on the concentration of TC could be attributed to hormonal influence. It has been suggested that the regulation of lipid metabolism by *n*-3 PUFA is affected by oestrogen. Clinical and laboratory findings have shown that there is a regulatory relationship between PPARα and oestrogen receptor, and this relationship is influenced by *n*-3 PUFA and oestrogen (Souidi et al., 1999, Wang and Kilgore, 2002). The sex specific effects of PPARα have been demonstrated in mice where cholesterol concentration was shown to be greater in male mice compared to female mice (Costet et al., 1998, Linden et al., 2001); interestingly, this was also observed in our results. Reports from studies that measured plasma TC in offspring of mothers fed *n*-3 PUFA diet are controversial. Yessoufou *et al* observed a low TC concentration in male rats offspring fed high *n*-3 PUFA diet for 90 days postweaning (Yessoufou et al., 2006); however, other studies reported no change in total TC in offspring after feeding *n*-3 PUFA rich diet for 3 weeks (Korotkova et al., 2002, Korotkova et al., 2005). As stated before, *n*-3 PUFA is well known to downregulate the gene expression of SREBP (Fernandez-Alvarez et al., 2011, Knight et al., 2005), thereby inhibiting the expression of hydroxylmethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol synthesis (Le Jossic-Corcos et al., 2005, Li et al., 2006). Our findings suggest that the time of exposure is critical in determining an

effect of dietary *n*-3 PUFA on plasma TC levels, and that the effect is sex specific, where only male offspring on high *n*-3 PUFA showed reduction in TC. Thus, future studies will focus on the sex and age dependent effects of *n*-3 PUFA on the regulation of SREBP and HMG-CoA reductase. Furthermore, we suspect a cross-talk between *n*-3 PUFA and estrogen signalling that needs to be investigated.

There was no effect of diet on plasma HDL-c in male and female offspring both at weaning and 16 weeks. Studies in adult mice have also reported no change in plasma HDL-c after supplementing with *n*-3 PUFA for 4 weeks (Zampolli et al., 2006), 20 weeks (Magdeldin et al., 2009), and for 32 weeks (Wang et al., 2009). Interestingly, there was an age dependent difference in the concentration of HDL-c between the male and female offspring, where females showed lower levels. Studies have shown that men and women have a similar concentration of HDL-c concentration at puberty, after which hormonal influences result in a marked difference of HDL-c concentration between males and females (Abbey et al., 1999, Kirkland et al., 1987, Matthews et al., 1989). Furthermore, the effects of *n*-3 PUFA on HDL-c has been shown to be more pronounced in men compared to women (Okuda et al., 2005). Thus, it will be important to investigate the effect of *n*-3 PUFA and sex hormones on HDL-c concentration.

HDL elicits its atheroprotective effect mainly through reverse cholesterol transport (RCT), a process involving the transportation of excess cholesterol from peripheral tissue to the liver for further metabolism of cholesterol. Studies have reported that the cardioprotective effect of HDL-c depends on its efflux capacity (Morgan et al., 2004, Khera et al., 2011). Although we did not observe a significant effect of diet on HDL-c, we investigated the cholesterol efflux capacity of plasma obtained from mice fed high and low *n*-3 PUFA diets and report for the first time an interaction of *n*-3 PUFA and sex with cholesterol efflux. Plasma from the male offspring showed

a significantly higher cholesterol efflux capacity compared to the female offspring. Furthermore, plasma samples from the high *n*-3 PUFA male offspring caused an increase in cholesterol efflux compared to the low *n*-3 PUFA group. However, there was no difference in cholesterol efflux in the female offspring between the two dietary groups. To our knowledge, there are only two other studies that evaluated the effect of *n*-3 PUFA on cholesterol efflux in adult hamster (Kasbi Chadli et al., 2013), and rat (Marmillot et al., 2000). However, these studies did not elucidate the sex specificity of *n*-3 PUFA on cholesterol efflux. It is well established that there are different subclasses of HDL with different capabilities to mediate cholesterol efflux (von Eckardstein et al., 1994). HDL-2 and HDL-3 are the most characterised HDL subclasses, and HDL-2 has been shown to be involved in scavenging more cholesterol from the peripheral tissues compared to HDL-3 (Ballantyne et al., 1982, Asayama et al., 1990). *N*-3 PUFA has been shown to increase the concentration of HDL-2 particles (Chan et al., 2006, Wilkinson et al., 2005), even without obvious change in total HDL concentration (Agren et al., 1996). It is likely that *n*-3 PUFA caused an increase in cholesterol efflux by increasing the concentration of HDL-2 subclass, which needs to be further investigated.

We observed a significant interaction between: age and diet; age and sex; and diet and sex on plasma concentration of LDL-c. The plasma concentration of LDL-c increased with age in the male offspring. Furthermore, there was a significant increase in plasma LDL-c concentration in the high *n*-3 PUFA group from weaning to 16 weeks in both male and female offspring. The increase in LDL-c with age has been well documented (Heiss et al., 1980, Moulopoulos et al., 1987, Schaefer et al., 1994), which is due to reduced catabolism of LDL with ageing (Grundy et al., 1985). *N*-3 PUFA may potentially increase LDL-c concentration by promoting the conversion of VLDL to LDL through rapid TG clearance by lipoprotein lipase (Park and Harris,

2003). The female offspring, showed a very interesting LDL-c profile. Diet high in *n*-3 PUFA increased the concentration of LDL-c in female offspring compared to the other groups at 16 weeks. This is an exciting observation, opening a new frontier of research on the sex specificity of *n*-3 PUFA in regulating lipid metabolism. (Rossouw et al., 2012, Kavanagh et al., 2009). Reports on the effect of *n*-3 PUFA on LDL-c have been contradictory; some have proposed a reducing effect (Yamashita et al., 2005, Vasandani et al., 2002, Magdeldin et al., 2009), some assert there is no effect (Mori et al., 2000, Conquer and Holub, 1996), while others believe *n*-3 PUFA increase LDL-c concentration (Theobald et al., 2004, Sanders et al., 2006). There are also noteworthy studies that linked oestrogen receptor to LDL-c and the risk of CVD, thus, sex specific regulation of LDL-c needs to be investigated in future studies.

Since we observed an increase in plasma LDL-c concentration, we measured LDL particle size which is used as a predictor of LDL related risk of CVD (Sanders et al., 2006, Griffin, 1999). Individuals with a high concentration of small LDL particles are more at risk of developing CVD compared to those with equal concentration of large LDL particle size (Rosenson et al., 2002, Berneis and Krauss, 2002, Sacks and Campos, 2003). Small LDL particle penetrate the arterial wall easily, and are more prone to oxidation (Rajman et al., 1999, Hurt-Camejo et al., 2000). Oxidative modification of LDL could eventually lead to the formation of foam cells, recruitment of adhesion molecules, stimulation of inflammation, and a consequent atherosclerotic plaque formation (Yui et al., 1993, Toshima et al., 2000, Lusis, 2000). Our NMR data show that *n*-3 PUFA supplementation led to a reduction in the concentration of the more atherogenic small LDL particle. Others have also indicated that *n*-3 PUFA increase LDL particle size (Suzukawa et al., 1995, Lee et al., 2013).

In conclusion, our findings demonstrate that sustained exposure of the offspring to perinatal diet high in *n*-3 PUFA reduces the concentration of atherogenic lipoproteins, and increases cholesterol efflux. In addition, our findings indicate that the effect of perinatal exposure to diet high in *n*-3 PUFA could be augmented if the offspring is exposed for a longer time period to a postweaning diet high in *n*-3 PUFA. This would consequently lead to a less atherogenic lipid profile to reduce the risk of CVD. We also report novel sex specific effects of *n*-3 PUFA on the concentration of plasma lipids and lipoproteins. As summarized in figure 2.4, there are different effects of *n*-3 PUFA on plasma lipids and lipoproteins of males and females; these sex specific effects are very important in the recommendation of *n*-3 PUFA for treating dyslipidaemia.

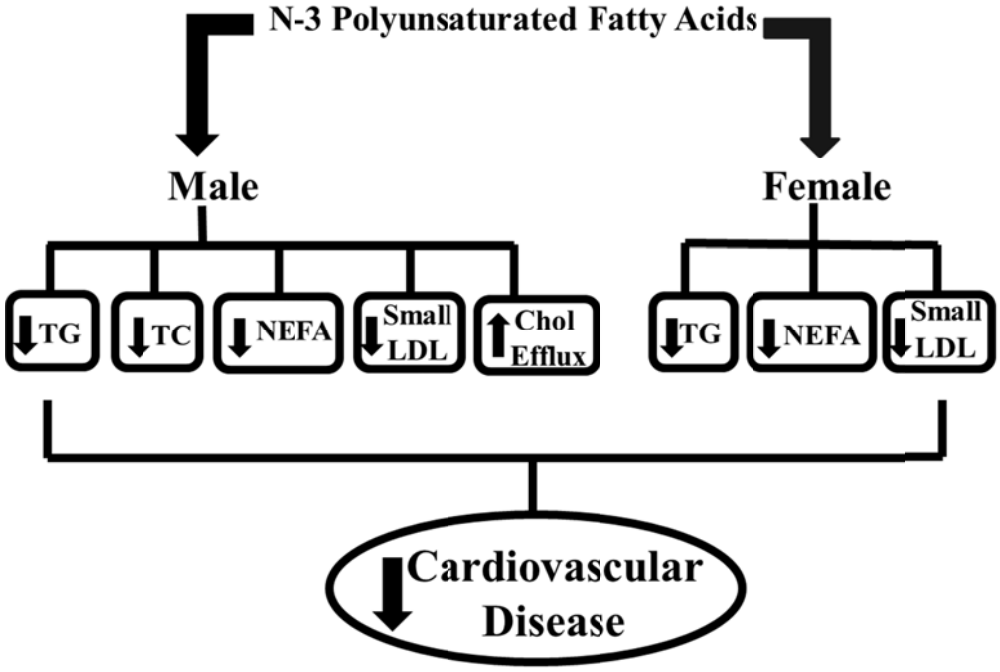


Figure 2.4 Schematic representation of sex-specific effects of n-3 PUFA on plasma lipids and lipoproteins

N-3 PUFA regulates plasma lipids and lipoproteins differently in male and female C57BL/6 mice. Chol efflux = cholesterol efflux; NEFA = Non-esterified fatty acids; Small LDL = small low density lipoprotein particles; TC = Total cholesterol; TG = Triglycerides.

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CHAPTER THREE

The effects of dietary n-3 PUFA on the fatty acid composition of hepatic and plasma bioactive lipids

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3.1 Abstract

N-3 PUFAs are converted to bioactive lipid components that are important mediators in metabolic and physiological pathways; however, which bioactive compounds are metabolically active, and their mechanisms of action are still not clear. We investigated using lipidomic techniques, the effects of diets high in *n*-3 PUFA on the fatty acid composition of various bioactive lipids in plasma and liver. Female C57BL/6 mice were fed semi-purified diets (20% w/w fat) containing varying amounts of *n*-3 PUFA before mating, during gestation and lactation, and until weaning. Male offspring were continued on their mothers' diets for 16 weeks postweaning. Hepatic and plasma lipids were extracted in the presence of non-naturally occurring internal standards, and tandem electrospray ionization mass spectrometry methods were used to measure the fatty acyl compositions. There was no significant difference in total concentrations of phospholipids in both groups. However, there was a significantly higher concentration of EPA-containing PC, LPC, and CE ($p < 0.01$) in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group in both liver and plasma. Plasma and liver from the high *n*-3 PUFA group also had a higher concentration of free *n*-3 PUFA ($p < 0.05$). There were no significant differences in plasma concentrations of different fatty acyl species of PE, TG, sphingomyelin and ceramide. Our findings reveal for the first time that a diet high in *n*-3 PUFA caused enrichment of *n*-3 PUFA in PC, LPC, CE and free fatty acids in the plasma and liver of C57BL/6 mice. PC, LPC, and nonesterified free *n*-3 PUFA are important bioactive lipids, thus altering their fatty acyl composition may have important metabolic and physiological roles.

3.2 Introduction

Essential PUFAs of the *n*-3 and *n*-6 classes are important in the regulation of metabolic processes. *N*-3 PUFAs such as DHA and EPA have attracted a lot of attention in the past years as a result of their potential health benefits (Calder, 2004, Schmidt et al., 2005, Belluzzi et al., 2000). *N*-3 PUFA has been shown to prevent atherosclerosis (Wassall and Stillwell, 2009, Riediger et al., 2009), regulate nuclear transcription factors involved in gene expression of inflammatory markers, and stimulate cognitive development (Cottin et al., 2011, Banni and Di Marzo, 2010). Markers of cardiovascular disease such as high TG, endothelial dysfunction, cardiac arrhythmia, and inflammation are also reduced by the administration of *n*-3 PUFA (Calder, 2004, Harris, 1997, Leaf et al., 2003, Goodfellow et al., 2000, Hirafuji et al., 2003).

In vivo, *n*-3 PUFA can either exist as free products of enzyme hydrolysis or bound to PL or TG. Reports have shown that dietary *n*-3 PUFA are preferentially incorporated into PL compared to TG (Holub, 1978). PLs are important constituents of the cellular membrane bilayers, with PC and PE being the most abundant (Gundermann et al., 2011). Membrane fluidity can be influenced by the incorporation of dietary long chain *n*-3 PUFA into membrane PL, which enhances the functions of transmembrane proteins and their interactions with extracellular ligands (Hulbert et al., 2005, Leaf et al., 2005, Stillwell and Wassall, 2003). This indirectly affects signalling pathways and other physiological functions of the membrane. PC has been implicated in an array of physiological functions, and their functional properties depend on their fatty acyl chains.

The liver is principally involved in the metabolism and release of PC into circulation. PC is metabolised by the enzyme PLA_2, releasing the fatty acid at the *sn*-2 position, and LPC into the

plasma pool for distribution to extrahepatic tissues (Thies et al., 1992, Huwiler and Pfeilschifter, 2009). Upon hydrolysis of the *sn*-2 fatty acid of PC, the released FFA is further metabolised to form bioactive compounds with either pro-inflammatory or anti-inflammatory properties (Huwiler and Pfeilschifter, 2009). AA, a long chain *n*-6 PUFA released from the metabolism of PC, is further metabolised by the COX or LOX enzyme pathways to produce the inflammatory series-2 prostaglandins or series-4 leukotrienes, respectively (Farooqui et al., 2007). On the contrary, the COX and LOX metabolic products of EPA, an *n*-3 PUFA, are generally anti-inflammatory (Adkins and Kelley, 2010). Furthermore, protectins and resolvins, the products of oxygenation of DHA and EPA, have been reported to exhibit anti-inflammatory properties (Ariel and Serhan, 2007, Bannenberg and Serhan, 2010, Kohli and Levy, 2009).

LPC, the other product of enzyme hydrolysis of PC, is an important lipid mediator involved in cellular metabolism. Direct hepatic secretion is an important source of the abundant unsaturated LPC found in the plasma (Croset et al., 2000). LPC has been controversially linked with the development of atherosclerosis (Wu et al., 2005, Lucas et al., 2008, Schmitz and Ruebsaamen, 2010). However, there is evidence suggesting that the biological properties of LPC depend on the acyl chain of the molecule (Hayashi et al., 2001, Yoshida et al., 2003). Studies have reported an increase in plasma concentration of saturated LPC in diseases conditions such as obesity, diabetes and rheumatoid arthritis (Fuchs et al., 2005). In another line of evidence, polyunsaturated LPC significantly reversed saturated LPC-induced inflammation (Hung et al., 2012). However, the importance of specific LPC fatty acyl chains in metabolic regulation is still not clear.

Most studies to date have explored the health benefits of *n*-3 PUFA esterified to ethyl esters or TG (Schuchardt et al., 2011, Galli et al., 2012, Tang et al., 2012). Moreover, the majority of

the reported findings that support the pro-inflammatory and the atherogenic actions of LPC were established using saturated or monounsaturated LPC (Wu et al., 2005, Lucas et al., 2008, Portman and Alexander, 1969, Thukkani et al., 2003, Aiyar et al., 2007). Cells, tissues, and biological fluids contain numerous bioactive lipid mediators involved in cellular processes, which are likely altered by dietary *n*-3 PUFA. The aim of this study was to employ high throughput lipidomic techniques to evaluate the effect of diets high or low in *n*-3 PUFA content on the fatty acid composition of various lipids in the plasma and liver of C57BL/6 mice. Our findings reveal for the first time that diets high in *n*-3 PUFA caused enrichment of *n*-3 PUFA in PC, LPC, CE and FFA in the plasma and liver of C57BL/6 mice, which will likely have important physiological roles.

3.3 Materials and Methods

3.3.1 Ethics Statement

All experimental procedures were approved by Memorial University Animal Care Committee in accordance with the principles and guidelines of Canadian Council on Animal Care (approval no: 10-09-SK).

3.3.2 Animals and diets

Animals were treated, housed and acclimated as described in section 2.3.2. After the period of acclimatization, female mice were randomly divided into two groups. Each group was fed either of the two experimental diets that differed only in their *n*-3 PUFA composition, and designated as "high *n*-3" and "low *n*-3" diets for two weeks before mating. The experimental diets were made as described in section 2.3.1 and the fatty acid composition of the experimental diet is given in **Table 2.2.** Females were continued on the experimental diets throughout

gestation, lactation, and until weaning. Just before weaning, breast milk samples were collected from the mothers, to ascertain that the dietary essential fatty acid is reflected in the breast milk. There was a positive correlation between dietary and breast milk fatty acids composition (*Appendix III*). At weaning, male offspring (*n*=6) were continued on their mothers' designated diet for 16 weeks (i.e. offspring obtained from mother's fed a high *n*-3 PUFA diet continued on high *n*-3 PUFA diet). Animals were provided with water and fresh food *ad libitum*, every other day. Body weights were recorded once a week, and food intake was recorded every other day. No significant differences were observed in both body weight and food intake (*Appendix II*). At 16 weeks, male offspring were fasted overnight and sacrificed using isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4), and plasma was separated immediately. Tissues were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80ºC until further analyses.

3.3.3 Lipidomic analysis

3.3.3.1 Lipid extraction, standards, and solvents

Plasma or liver samples were flash frozen at the temperature of liquid nitrogen at collection. 10µl plasma was directly extracted into organic solvent, while 100 mg liver tissue was pulverised and homogenised and then immediately lipids were extracted into organic solvent. Lipid extraction into organic solvent was performed using the Bligh-Dyer method (Bligh and Dyer, 1959) with high performance liquid chromatography-mass spectrometry grade solvents in the presence of non-naturally occurring internal lipid standards. The standards used were Δ9-trans-triheptadecenoin (tri-17:1 TG), 1, 2-diarachidoyl-*sn*-glycero-3-phosphocholine (di-20:0 PC), *N*-heptadecanoyl-D-*erythro*-sphingosylphosphorylcholine (17:0 sphingomyelin), 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (17:0 LPC), 1, 2-dimyristoyl-*sn*-glycero-3-

phosphoethanolamine (di-14:0 PE), cholesteryl heptadecanoate (17:0 CE), and *N*-heptadecanoyl-D-*erythro*-sphingosine (17:0 ceramide). The extracted lipids in the chloroform phase were dried down under a gentle stream of N_2 gas, and were re-suspended in 500 µl of chloroform. 50 µl aliquot of the suspension was then mixed with 200µl methanol and 2µl of 10 mM methanolic NaOH and injected into a Thermo Fisher TSQ Quantum Ultra tandem electrospray ionization mass spectrometry (ESI-MS) system for lipid analyses. High pressure liquid chromatography-mass spectrometry grade methanol and chloroform were used for all extractions; these solvents were purchased from Burdick and Jackson (NJ, USA).

3.3.3.2 Electrospray ionization-mass spectrometry (ESI-MS)

Samples were analysed using direct-infusion ESI-MS in positive or negative ion mode using a Thermo Fisher TSQ Quantum Ultra system with XCalibur data acquisition software (Han and Gross, 2005). The tune parameters for sample analyses were optimized and set as follows: spray voltage = 3500 V, flow rate = 3 µl/min, ion sweep gas pressure = 0.2 (arbitrary units), sheath gas = 12 (arbitrary units), auxiliary gas pressure = 6 (arbitrary units), and capillary temperature = 270^{o}C. The collision energies for the analyses of PC, LPC, and sphingomyelins (SM) were set at 28 eV. The collision energies for analyses of CE was set at 25 eV, and for ceramide (CER) was set at 32 eV. CEs were detected in positive ion mode by scanning for neutral loss (NL) of cholestadiene (*m/z* 368.5). Sodiated species of SM, PC, and LPC were detected in positive ion mode by scanning for the NL of choline (*m/z* 59.1). FFA and PE were identified in negative ion mode by survey scan for $[M-H]^-$ between *m/z* 200 and 900. Sodiated species of TG were identified in positive ion mode by survey scan for $[M+Na]^+$ between *m/z* 800 and 1000. All data analyzed were corrected for ^{13}C isotope effects as described by Han *et al.* (Han and Gross, 2005).

3.3.4 Statistical Analysis

Data were analysed using GraphPad Prism software (version 5.0). Statistical significance for differences between groups was determined by unpaired t-test. Results are expressed as mean ± standard deviation (SD). Differences were considered to be statistically significant if the associated *P* value was < 0.05.

3.4 Results

3.4.1 PC fatty acyl composition of mice fed high and low n-3 PUFA

There were no significant differences in the total concentrations of PL in both plasma and liver between high and low *n*-3 PUFA fed mice (*Appendix IV*). However, there was a significantly higher concentration of 16:0-20:5 PC in the plasma ($P < 0.01$; Figure 3.1A) and liver ($P < 0.05$; Figure 3.1B) of the high *n*-3 PUFA group compared to the low *n*-3 PUFA group. Interestingly, 20:4 containing PC (16:0-20:4 and 18:0-20:4) increased significantly in the plasma of the low *n*-3 PUFA group compared to the high *n*-3 PUFA group ($P < 0.05$; Figure 3.1A). A similar effect was observed in the liver, with 16:0-20:4 and 18:0-20:4 showing a significant increase in the low *n*-3 PUFA group ($P < 0.001$; Figure 3.1B). This signifies a higher incorporation of EPA and a lower incorporation of AA into plasma PC in the high *n*-3 PUFA group. The high *n*-3 PUFA group also showed higher incorporation of DHA into hepatic PC compared to the low *n*-3 PUFA group ($P < 0.05$; Figure 3.1B); however, there were no differences in plasma DHA-containing PC in both groups (Figure 3.1A).

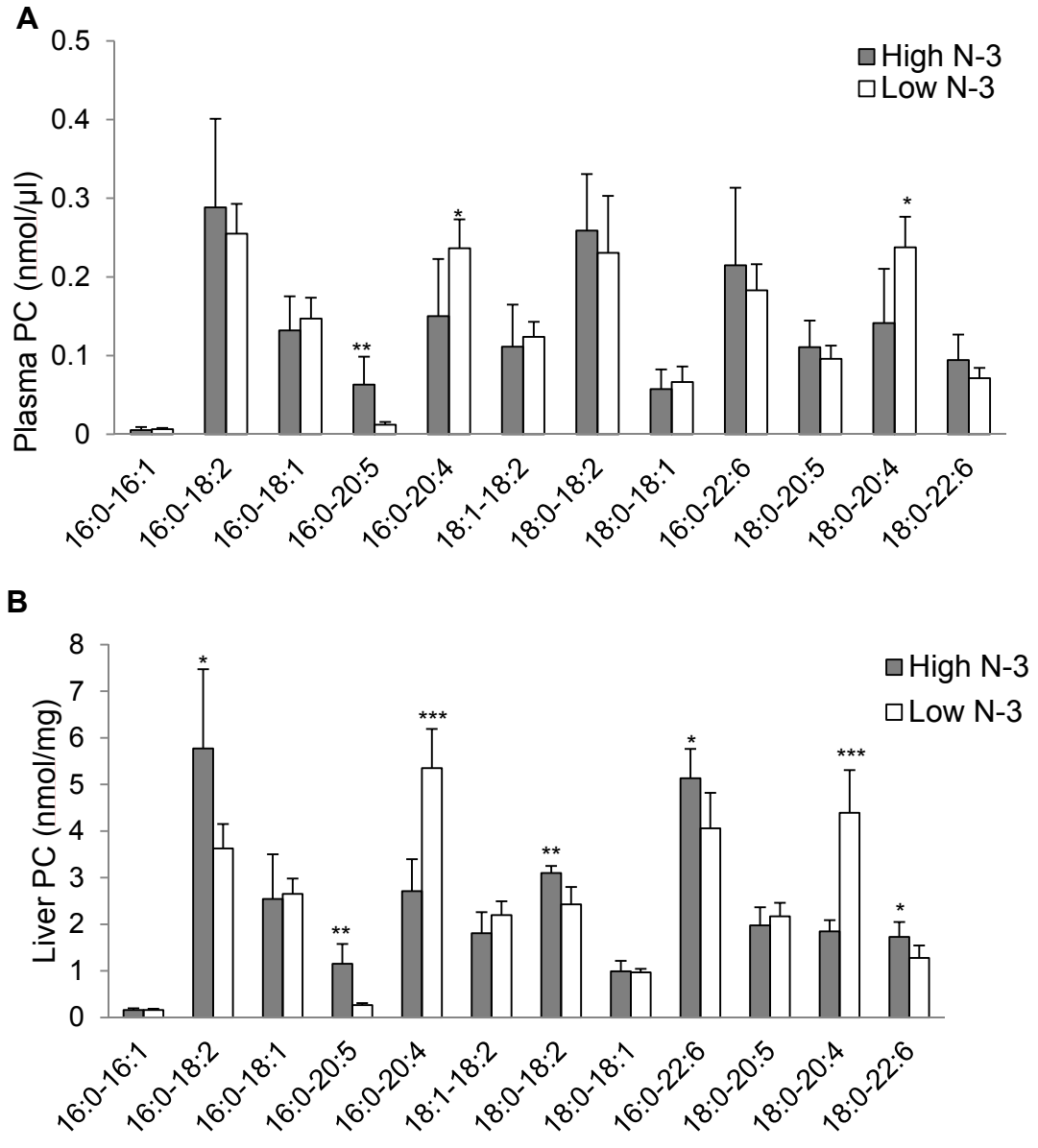

Figure 3.1. Phosphatidylcholine (PC) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) PC species were quantified by measuring the sodiated adducts of PC using ESI-MS in positive ion mode by NL scanning for m/z 59.1, as described in the "Methods". Data were corrected for ^{13}C isotopic effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

3.4.2 FFA of mice fed high and low n-3 PUFA

There was a significant accretion of AA in the plasma of the low *n*-3 PUFA group compared to the high *n*-3 PUFA group ($P < 0.05$; Figure 3.2A). The plasma concentration of EPA was considerably higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group ($P < 0.05$; Figure 3.2A). However, no significant difference was observed in the plasma concentration of DHA between groups. Livers of the mice fed a low *n*-3 PUFA diet were significantly enriched with 18:1 and 20:4 FFA ($P < 0.01$; Figure 3.2B) compared to the high *n*-3 PUFA group. Similarly, the liver concentrations of EPA ($P < 0.01$; Figure 3.2B) and DHA ($P < 0.05$; Figure 3.2B) were significantly higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group.

3.4.3 LPC fatty acyl composition of mice fed high and low n-3 PUFA

Similar to the PC data, the concentration of 20:5 LPC was significantly higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group, in both plasma ($P < 0.01$; Figure 3.3A) and liver ($P < 0.01$; Figure 3.3B). However, there was a significant accretion of 20:4 LPC in the plasma ($P < 0.01$; Figure 3.3A) and liver ($P < 0.05$; Figure 3.3B) of the low *n*-3 PUFA group compared to the high *n*-3 PUFA group. Irrespective of the differences observed in LPC, there was no significant difference in the total concentration of LPC in both groups (*Appendix III*).

3.4.4 PE fatty acyl composition of mice fed high and low n-3 PUFA

No significant differences were observed in the fatty acyl species of PE in the plasma (Figure 3.4A) of the high *n*-3 PUFA and low *n*-3 PUFA groups. The liver showed no differences amongst PE species containing EPA; however, there was a significant increase of hepatic 18:0-

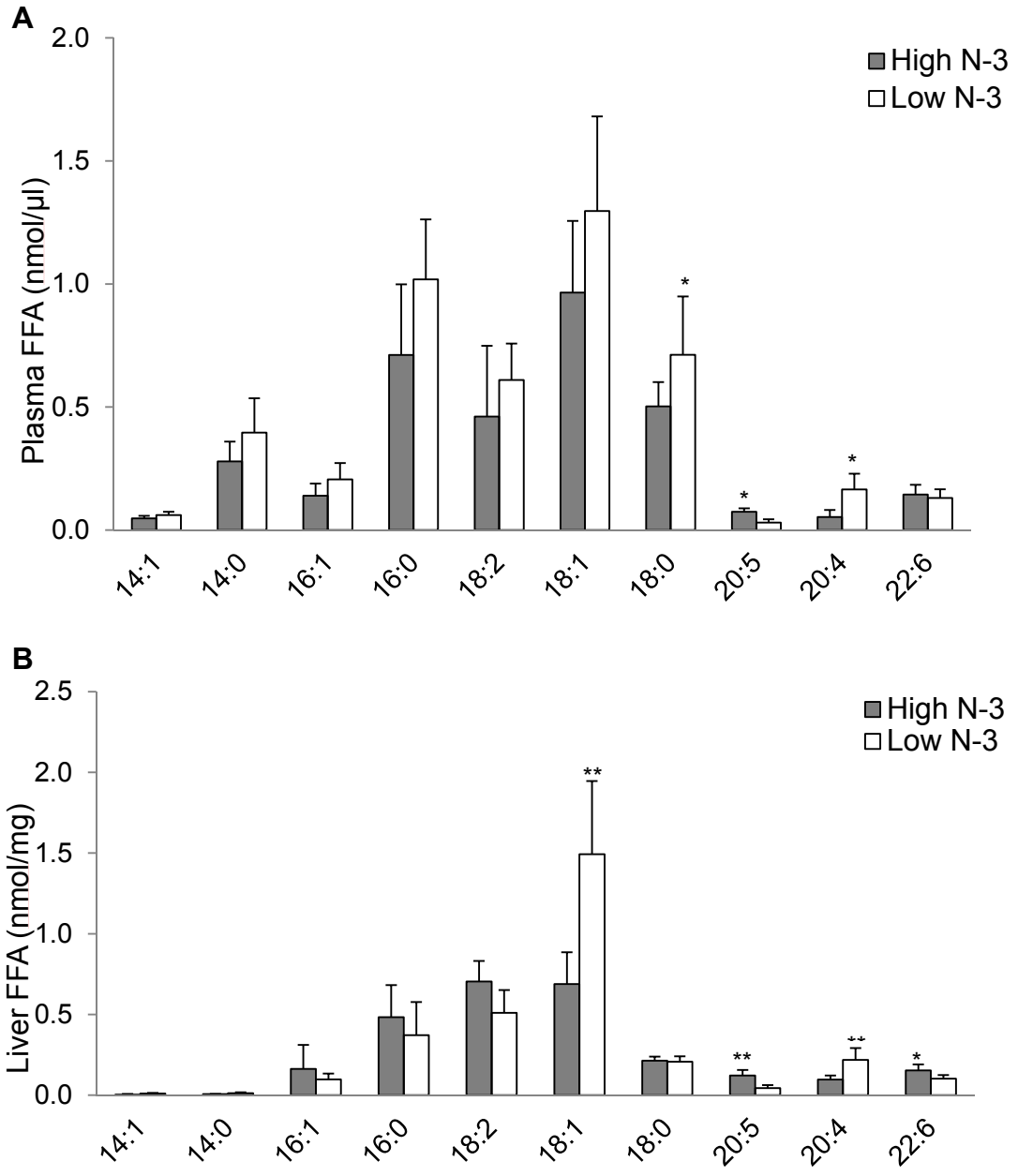

Figure 3.2. Free fatty acid (FFA) composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) FFA composition was quantified by measuring $[M-H]^-$ using ESI-MS in negative ion mode between m/z 200 and 900. Data were corrected for ^{13}C isotopic effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

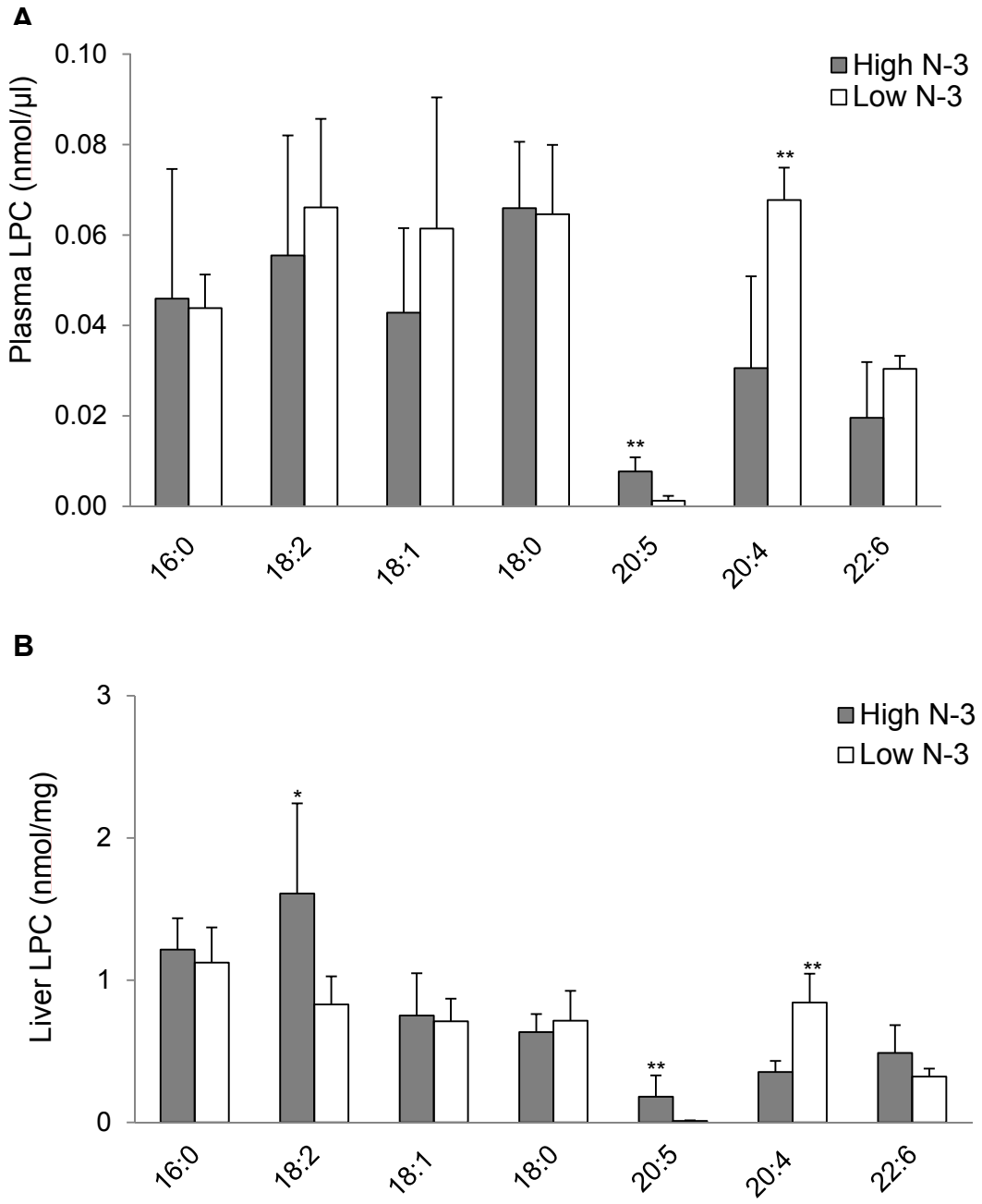

Figure 3.3. Lysophosphatidylcholine (LPC) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) LPC species were quantified by measuring the sodiated adducts of PC using ESI-MS in positive ion mode by NL scanning for m/z 59.1, as described in the "Methods". Data were corrected for ^{13}C isotopic effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

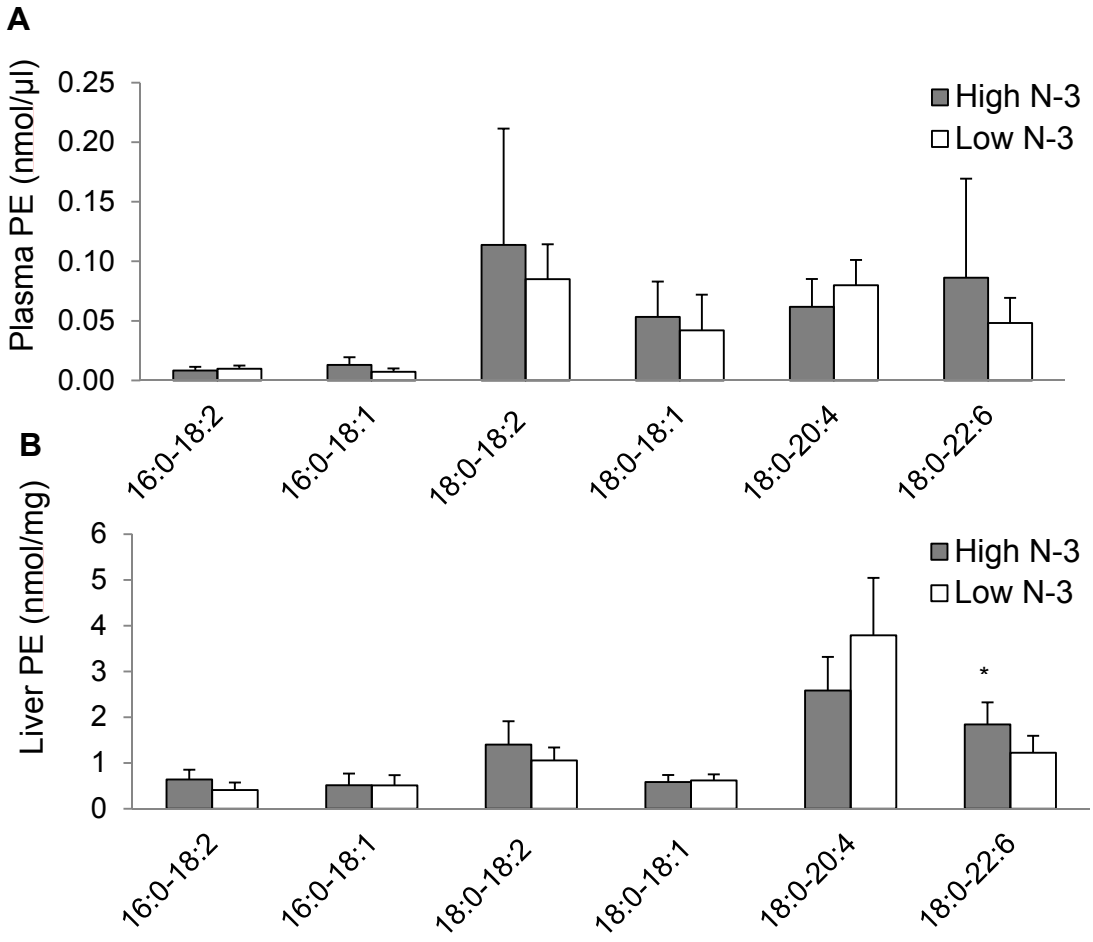

Figure 3.4. Phosphatidylethanolamine (PE) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) levels of PE species were quantified by measuring $[M-H]^-$ using ESI-MS in negative ion mode between m/z 200 and 900. Data were corrected for ^{13}C isotopic effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

22:6 PE in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group ($P < 0.05$; Figure 3.4B).

3.4.5 Sphingolipid and ceramide fatty acyl composition of mice fed high and low n-3 PUFA

Treatment with high *n*-3 PUFA did not significantly modify the fatty acyl species of SM in the plasma (Figure 3.5A) and liver (Figure 3.5B) of mice fed diet high in *n*-3 PUFA. Plasma of mice from high *n*-3 PUFA group showed an increase in 16:0 CER compared to the low *n*-3 PUFA group ($P < 0.05$ Figure 3.6A); however, there was no effect of diet on different fatty acyl species of CER in the liver (Figure 3.6B).

3.4.6 CE and TG fatty acyl composition of mice fed high and low n-3 PUFA

The high *n*-3 PUFA group showed a significant increase in plasma 20:5 CE compared to the low *n*-3 PUFA group ($P < 0.05$; Figure 3.7A). Conversely, there was a high accumulation of plasma 20:4 CE in the low *n*-3 PUFA group compared to the high *n*-3 PUFA group ($P < 0.05$; Figure 3.7A). The liver of the high *n*-3 PUFA group was enriched in 20:5 CE ($P < 0.001$; Figure 3.7B) and 22:6 CE ($P < 0.01$; Figure 3.7B) compared to the low *n*-3 PUFA group. The liver concentration of 20:4 CE was significantly higher in the low *n*-3 PUFA group ($P < 0.01$; Figure 3.7B) compared to the high *n*-3 PUFA group.

No differences were observed in the concentrations of the different fatty acyl species of TG found in the plasma between experimental groups (Figure 3.8A). Nevertheless, the liver showed a distinctive difference in the concentration of fatty acyl species profile for TG. The concentrations of 54:6, 56:7, and 58:8 TGs were significantly greater in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group ($P < 0.01$; Figure 3.8B). The low *n*-3 PUFA group

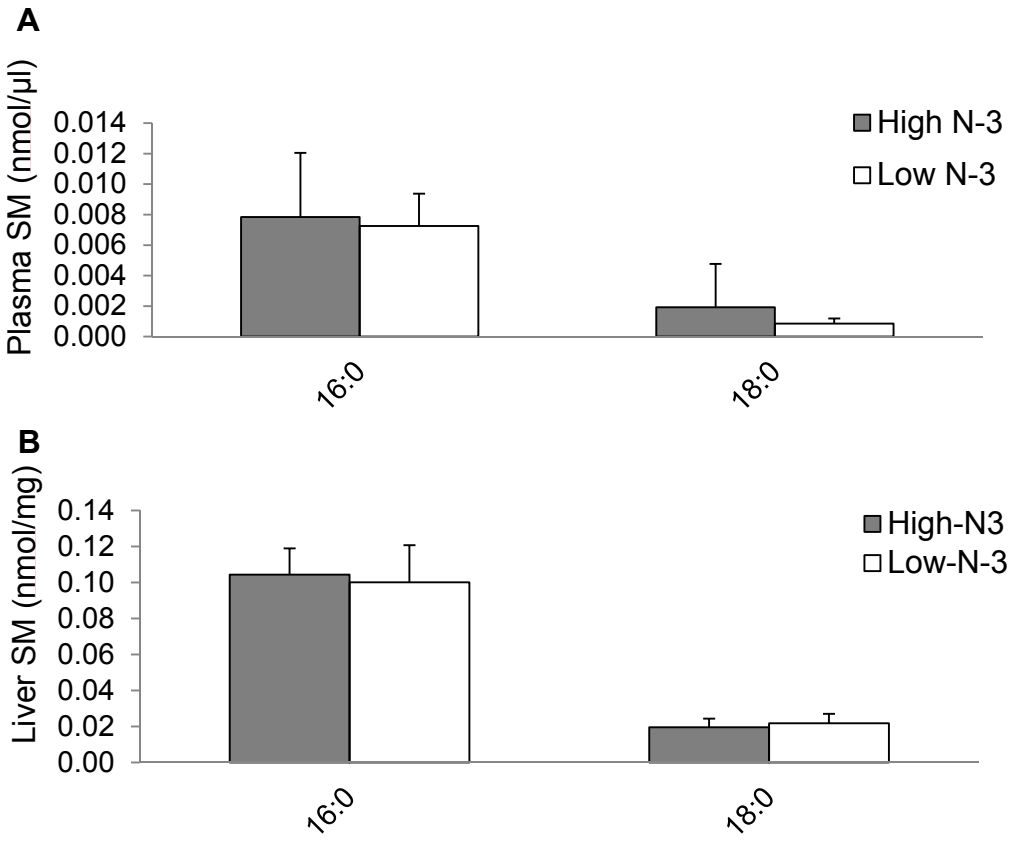

Figure 3.5. Sphingomyelin (SM) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) levels of SM species were quantified by measuring the sodiated adducts of PC using ESI-MS in positive ion mode by NL scanning for m/z 59.1, as described in the "Methods". Data were corrected for* ^{13}C *isotopic effects. Data* are presented as *mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

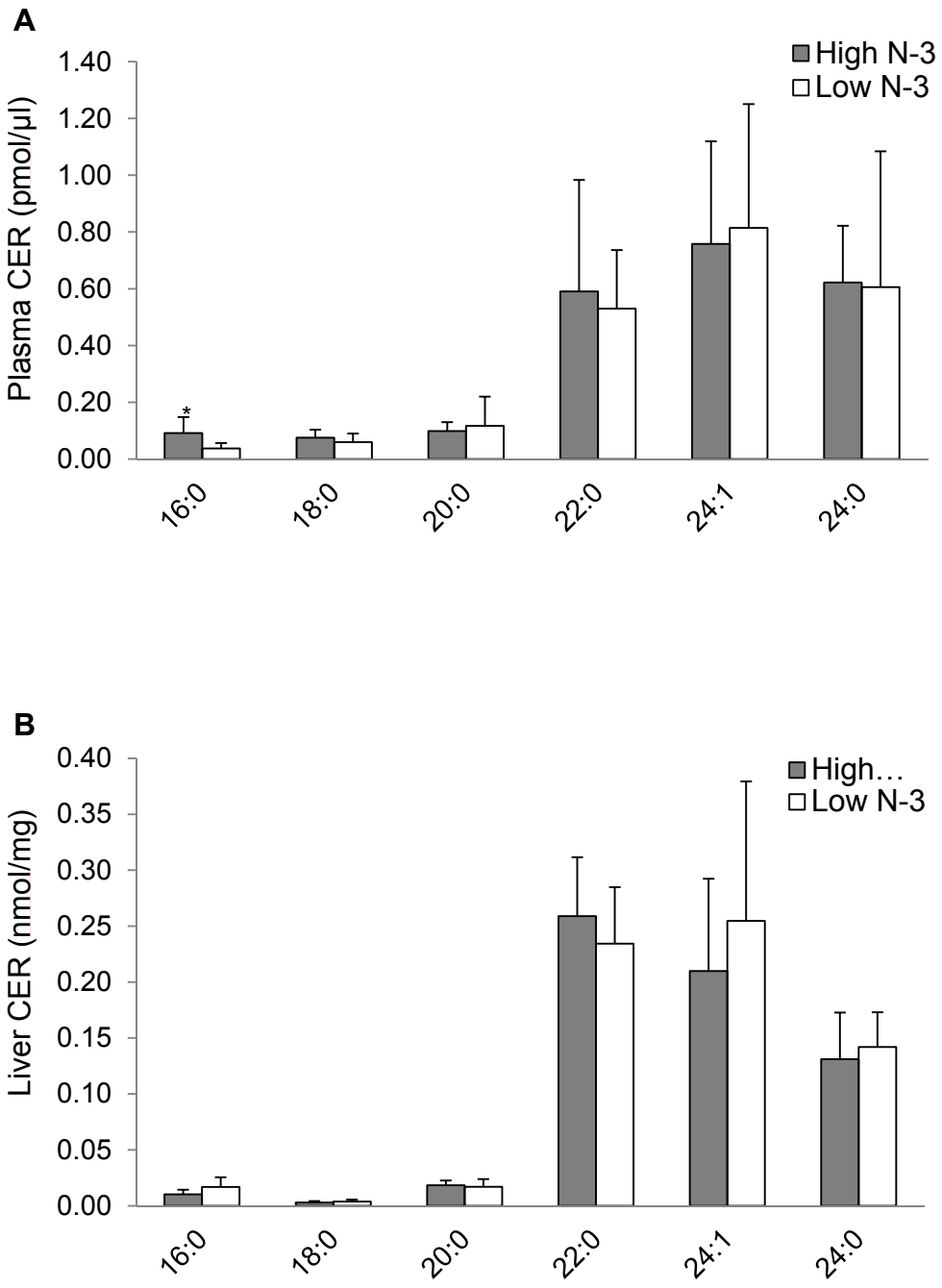

Figure 3.6. Ceramide (CER) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) CER species were quantified using ESI-MS in negative ion mode by NL scanning for m/z 256.2. Data were corrected for ^{13}C isotope effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

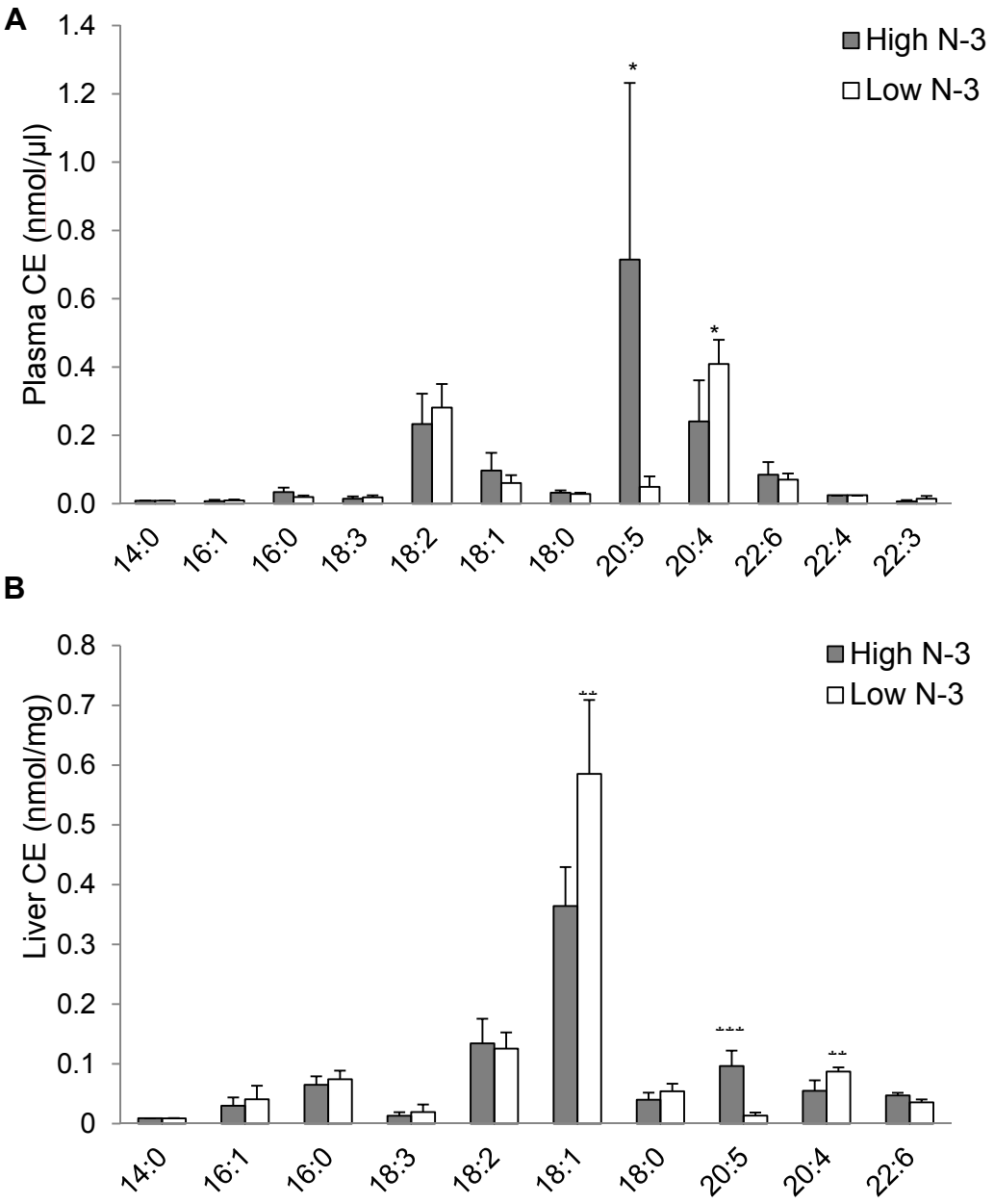

Figure 3.7. Cholesteryl ester (CE) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) CE species were quantified using ESI-MS in positive ion mode by NL scanning of m/z 368.5 as described in the "Methods". Data were corrected for ^{13}C isotope effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

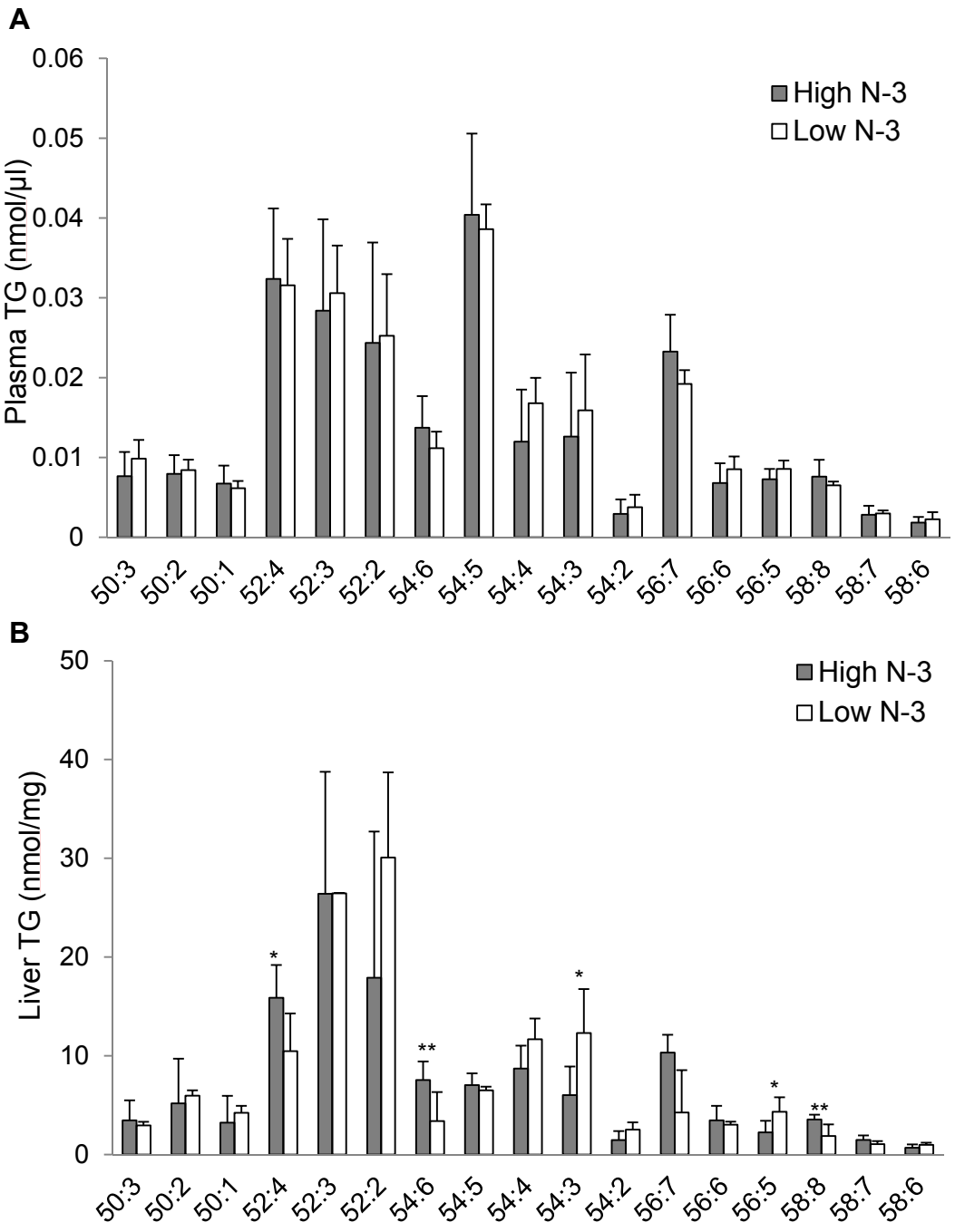

Figure 3.8. Triglyceride (TG) fatty acyl composition of mice fed high and low n-3 PUFA enriched diets. *Plasma (A) and liver (B) TG species were quantified by measuring $[M+Na]^+$ using ESI-MS in positive ion mode between m/z 800 and 1000 described in the "Methods". Data were corrected for ^{13}C isotope effects. Data are presented as mean (n=6) ± SD and P-values calculated using unpaired t-test. *P < 0.05; **P < 0.01; ***P < 0.001.*

showed a significantly higher 54:3 and 56:5 TG species compared to the high *n*-3 PUFA group ($P < 0.05$; Figure 3.8B).

3.5 Discussion

N-3 PUFA has been shown to reduce plasma TG (Hartweg et al., 2008, Harris, 1997), prevent atherosclerosis (von Schacky, 2003), and alleviate inflammation (Calder, 2006). It has been proposed that the beneficial effects of *n*-3 PUFA are mediated through the actions of bioactive lipid components; however, which bioactive lipids are metabolically active, and their mechanisms of action are still not clear. We performed lipidomic analyses on plasma and liver samples of high and low *n*-3 PUFA fed male C57BL/6 mice. There was an evident distinction in the fatty acid profiles of different lipids present in the plasma and liver of the two dietary groups. Feeding a high *n*-3 PUFA diet led to a marked reduction of AA-containing PC, with a concomitant increase of EPA-containing PC. Although plasma samples from mice fed a high or low *n*-3 PUFA diet showed no difference in DHA-containing PC, there was a trend towards an increase in the high *n*-3 PUFA group. Browning *et al.* reported that habitual supplementation of fish oil led to an increased incorporation of EPA in plasma PC (Browning et al., 2012); however, they did observe a significant incorporation of DHA into PC after dietary supplements of DHA (Browning et al., 2012). A plausible explanation for the disparity in our findings is that DHA-containing PC is rapidly converted to LPC for delivery to extrahepatic tissues. The liver PC fatty acyl composition is consistent with changes in plasma PC, where high dietary *n*-3 PUFA increased the concentrations of hepatic EPA and DHA species of PC. On the contrary, there was a significant reduction of hepatic AA-containing PC in mice fed high *n*-3 PUFA diet. PC is an important phospholipid involved in lipid metabolism. PC has been shown to play a major role in cellular proliferation, degeneration, and membrane fluidity and functions (Gundermann et al.,

2011). The physiological properties of PC are heavily dependent on their fatty acid composition and *n*-3 PUFA has been shown to be preferentially incorporated into PC (Browning et al., 2012). PC from marine sources are rich in *n*-3 PUFA and have been shown to significantly reduce markers of inflammation (Deutsch, 2007), by the inhibition of TNF-α induced activity of NF-κB (Treede et al., 2007). In addition to their anti-inflammatory effects, *n*-3 PUFA rich PC are also known to possess lipid lowering effects (Klimov et al., 1995, Bunea et al., 2004, Taylor et al., 2010).

Plasma PC is rapidly metabolised by PLA_2 to produce FFA and LPC. These bioactive metabolites of PC can also elicit beneficial effects depending on the type, chain length, and degree of unsaturation of their fatty acids. Feeding diets enriched in *n*-3 PUFA caused an increase in the plasma concentration of EPA and a decrease in plasma concentration of AA. PC is an important source of FFA in plasma (Browning et al., 2012). We found a high concentration of EPA-containing PC in the high *n*-3 PUFA group; it is thus reasonable to assume that the high plasma concentration of EPA found in the high *n*-3 PUFA fed mice is partly a product of enzyme hydrolysis of EPA rich PC. This is in line with previous findings that support the incorporation of *n*-3 PUFA into the plasma pool when given as dietary supplements (Browning et al., 2012, Hodson et al., 2008). We observed that the dietary supplementation with high *n*-3 PUFA caused an increase in the concentrations of hepatic EPA and DHA, and a decline in the hepatic concentration of AA. Our observation is in line with the study of Lamaziere *et al.*, who reported an increase in hepatic EPA and DHA after rats were administered fish oil for 30 days (Lamaziere et al., 2013). In addition to the aforementioned health benefits of *n*-3 PUFA, high levels of hepatic *n*-3 PUFA are also involved in inhibiting lipogenesis, thereby preventing the development of non-alcoholic fatty liver disease (NAFLD) (Lamaziere et al., 2013).

Mechanistically, *n*-3 PUFA has been shown to regulate lipogenesis by inhibiting LXR and SREPB-1c (Xu et al., 1999, Yoshikawa et al., 2002). Chronic low-grade inflammation underlies the pathology of most metabolic disorders, and free *n*-3 PUFA has been shown to possess potent anti-inflammatory properties (Massaro et al., 2008). *N*-3 PUFA alleviates inflammation by directly regulating transcription factors involved in inflammation (Jump et al., 1994, Pawar and Jump, 2003, Pawar et al., 2003) and indirectly by producing series-3 and series-5 eicosanoids (von Schacky et al., 1985, Wanten and Calder, 2007). In addition to their inflammation-resolving properties, free unesterified *n*-3 PUFA have also been shown to improve symptoms of dyslipidaemia (Hirafuji et al., 2003, Goodfellow et al., 2000, Leaf et al., 2003).

Another important bioactive product of enzyme hydrolysis of PC is LPC. We found that feeding a diet high in *n*-3 PUFA considerably increased plasma circulating levels of 20:5 LPC, and drastically reduced plasma concentration of 20:4 LPC. Similar to our plasma data, we found a higher concentration of hepatic 20:5 LPC and a low concentration of hepatic 20:4 LPC in the high *n*-3 group. Interestingly, there was no significant difference in hepatic 22:6 LPC, although there was a trend towards an increase in the high *n*-3 PUFA group. Ottestad *et al.* recently reported a similar finding where fish oil supplementation to healthy humans significantly increased plasma 20:5 LPC and 22:6 LPC (Ottestad et al., 2012). Another study by Block *et al.* also found that fish oil supplementation increased EPA and DHA-containing LPC (Block et al., 2010). However, we only found a change in plasma EPA containing LPC, while there was no difference in DHA containing LPC between the two groups. LPC has been suggested as the major carrier of DHA to the brain tissues (Lagarde et al., 2001). Tracer studies revealed that labelled LPC-DHA and unesterified DHA injected into the blood of rat disappeared within 20 s and labelled LPC-DHA was recovered in different organs including the brain (Thies et al., 1992).

It would thus be logical to believe that the DHA-containing LPC was rapidly cleared from circulation.

Studies have linked LPC with the development of atherosclerosis (Wu et al., 2005, Lucas et al., 2008, Schmitz and Ruebsaamen, 2010). This is possibly due to their association with oxidized LDL, and promotion of inflammation (Fuentes et al., 2002, Eguchi et al., 2007) by generating reactive oxygen species and nitric oxides in different types of cells (Park et al., 2009, Colles and Chisolm, 2000). However, the studies that linked LPC with the pathogenesis of obesity, diabetes, and rheumatoid arthritis have reported an increase in saturated LPC (Fuchs et al., 2005). Of paramount importance to the biological functions of LPC are acyl length and degree of saturation of their fatty acids (Block et al., 2010). LPC rich in *n*-3 PUFA have been shown to possess beneficial properties. *N*-3 PUFA species of LPC were found to reduce inflammation (Chen et al., 2005, Yan et al., 2004), and LPC containing DHA at *sn*-1 position exhibits significantly higher anti-inflammatory properties compared to LPC with either LA or AA (Hung et al., 2011b, Hung et al., 2011a, Hung et al., 2012). It has been proposed that LPC containing *n*-3 PUFA exhibit their anti-inflammatory properties through hydrolytic cleavage of the *n*-3 PUFA moiety, or oxygenation by 15-lipooxygenase (15-LOX) to produce inflammation resolving lipids such as 1-(15-hydroperoxyeicosatetraenoyl)-LPC and 1-(17-hydroperoxydocosahexaenoyl)-LPC (Hung et al., 2011a). Most of the available data on the health benefits of *n*-3 PUFA rich LPC were shown using LPC containing *n*-3 PUFA at *sn*-1 position. We have not investigated the position of the acyl group of our LPC; however, it is known that there is rapid isomerization of acyl group from *sn*-2 to a more stable *sn*-1 position in LPC (Pluckthun and Dennis, 1982). It has been reported that after separation of blood from plasma, 90% of the unsaturated fatty acids were found at *sn*-1 position in LPC (Croset et al.,

2000). We can therefore speculate that a significant percentage of the plasma 20:5 LPC contain EPA at *sn*-1 position.

Also noteworthy is the fact that, despite the changes in fatty acyl species of PC and LPC in response to diet, there was no difference in the total concentrations of PC and LPC between the two experimental groups. A similar observation was reported by Ottestad *et al.*, who found no difference in total concentrations of PC and LPC despite the apparent changes in individual fatty acyl species in response to fish oil supplementation (Ottestad et al., 2012). This interesting observation suggests that the functional properties of *n*-3 PUFA involve remodelling and improving the quality of bioactive lipid mediators without affecting their concentrations.

A diet rich in *n*-3 PUFA had no effect on different fatty acyl species of plasma TG, confirming preferential incorporation of *n*-3 PUFA into PC. However, the high *n*-3 PUFA diet led to an increase in plasma and liver concentrations of *n*-3 PUFA CE, and a decrease in 20:4 CE. CE is less polar than free cholesterol and it functions as an inert storage molecule. The high incorporation of *n*-3 PUFA in CE could be explained as a response to the high *n*-3 PUFA in the diet. This would simply indicate that *n*-3 PUFA are stored and will be later released for other physiological functions. A cross sectional study has reported a positive correlation between dietary PUFA and CE (Ma et al., 1997)

Although we have shown that there is high incorporation of *n*-3 PUFA in the liver and plasma PC and LPC of mice fed high *n*-3 diet, it was also imperative to ascertain the effect of high *n*-3 PUFA diet on PE, which is also abundant in the membranes. There was no difference in plasma PE between the two dietary groups; the only difference detected in the liver was an increase in 18:0-22:6 PE in the high *n*-3 group. Our findings confirm that fish oil

supplementation leads to a preferential incorporation of *n*-3 PUFA into PC and LPC compared to other phospholipids (Ottestad et al., 2012).

Sphingolipids, such as CER and SM, are important signalling molecules. CER is a sphingolipid linked with inflammation and the pathogenesis of CVD (Pfeiffer et al., 2001, de Mello et al., 2009). There are also speculations on the involvement of CER in insulin signalling, although available information is scanty (Wymann and Schneiter, 2008, Turpin et al., 2006). We found no difference in total concentration of CER in plasma and liver. Our findings are similar to Ottestad *et al.*, who found that fish oil supplement had no effect on plasma concentration of CER. Interestingly, we found an increase in 16:0 CER in the high *n*-3 PUFA group. The functional roles of this CER species are currently unknown and need to be explored. There was no difference in plasma and liver SM concentrations between the two groups. The physiological functions of SM have not been extensively studied. However, it is known that SM is involved in the formation of specialized membrane microdomains known as lipid rafts involved in signalling.

In conclusion, our findings have shown that diets high in *n*-3 PUFA alter plasma and liver lipidomic profile of the offspring. We found that *n*-3 PUFA is preferentially incorporated into PC and LPC, and despite the changes in lipidomic profile, the total concentrations of these lipids were not altered. Additionally, we found that dietary *n*-3 PUFA is capable of remodelling the fatty acyl moieties of PC, LPC, and CE, which may have important physiological implications, and needs to be further investigated. Future studies will be undertaken to investigate the mechanism(s) by which *n*-3 PUFA remodelled bioactive lipids regulate metabolic pathways.

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CHAPTER FOUR

The sex-specific effects of dietary n-3PUFA and adipocyte hypertrophy

A version of this chapter is under revision for publication in Lipids

4.1 Abstract

Obesity is characterised by an increase in fat mass primarily as a result of adipocyte hypertrophy. Diets enriched in *n*-3 PUFA are suggested to reduce obesity; however, the mechanisms are not well understood. We investigated the effect of *n*-3 PUFA on adipocyte hypertrophy and the key genes involved in adipocyte hypertrophy. Female C57BL/6 mice were fed semi-purified diets (20% w/w fat) containing high *n*-3 PUFA before mating, during pregnancy, and until weaning. Male and female offspring were continued on high *n*-3 PUFA (10% w/w), medium *n*-3 PUFA (4% w/w), or low *n*-3 PUFA (2% w/w) diet for 16 weeks postweaning. Adipocyte area was quantified using microscopy, and gonadal mRNA expression of DGAT2, FABP4 and leptin were measured. The high *n*-3 PUFA group showed higher levels of total *n*-3 PUFA in gonadal TG compared to the medium and low *n*-3 PUFA groups ($P<0.001$). The high *n*-3 PUFA male group had a lower adipocyte size compared to the medium and low *n*-3 PUFA group ($P<0.001$); however, no difference was observed in females. The high *n*-3 PUFA male group showed lower mRNA expression of FABP4, DGAT2 and leptin compared to the low *n*-3 PUFA group, with no difference in females. Plasma lipid levels were lower in the high *n*-3 PUFA group compared to the other groups. Our findings show for the first time that *n*-3 PUFA prevents adipocyte hypertrophy by downregulating FABP4, DGAT2 and leptin; the effects are however sex specific.

4.2 Introduction

The incidence of obesity is on the rise in both developed and developing countries (Dunstan et al., 2002, Flegal et al., 1998). Obesity is defined as the accumulation of excess fat, and is associated with pathophysiological conditions such as T2D, CVD, and dyslipidaemia (Grundy, 2004, Zou and Shao, 2008). The phenotype of obesity is characterised at the cellular level by an increase in adipose tissue mass, mainly as a result of the enlargement of adipocytes (hypertrophy) (van Harmelen et al., 2003). Adipocyte hypertrophy is accompanied by changes in metabolic activities such as lipid metabolism, which could exacerbate the development of obesity (Engfeldt and Arner, 1988). Larger adipocytes are known to both release more fatty acids and produce more TG relative to smaller adipocytes (Jamdar, 1978). The size of an adipocyte is a great predictor of a healthy metabolic profile (Grundy, 2004, Zou and Shao, 2008). Studies have linked adipocyte hypertrophy to dyslipidaemia and T2D (Bernstein et al., 1975, Haller et al., 1979).

Adipocyte hypertrophy is a consequence of excess accumulation of TG in the adipose tissue, and it is highly dependent on the nuclear receptor protein, PPARγ (Lopez et al., 2003). Therefore, elucidating the molecular process involved in the formation of TG in the adipose tissue is important in understanding the development of obesity. DGAT is responsible for the synthesis of TG (Bell and Coleman, 1980); it catalyses the covalent binding of acyl CoA to diacyglycerol (DG), which is the final step of TG synthesis (Chen et al., 2002). DGAT2, one of the two isoforms of the enzyme has been shown to be the main DGAT isoform involved in TG synthesis (Stone et al., 2004). Furthermore, the size of an adipocyte affects its endocrine functions (Kubota et al., 1999). Larger adipocytes secrete more inflammatory cytokines and NEFA, and increase lipolysis, which could lead to the development of metabolic syndrome

(Kubota et al., 1999). FABP4, also known as adipocyte fatty acid binding protein 2 (aP2), and accounts for around 6% of cellular protein (Xu et al., 2006) and has also been implicated in the regulation of glucose and lipid metabolism (Xu et al., 2006). Mice lacking the FABP4 gene are genetically protected from developing obesity and its complications (Uysal et al., 2000). Adipose tissue also secretes adipokines such as leptin. Leptin is a hormone produced primarily by adipocytes and it plays a vital role in food intake and metabolism (Ahima et al., 1996). Both human and rodent studies have reported a positive correlation between plasma leptin concentration and body fat (Frederich et al., 1995). The secretion of leptin has been positively correlated with the size of adipocytes (Guo et al., 2004), and clinical studies have also shown a positive correlation between the size of adipocyte and circulating leptin concentration (Couillard et al., 2000).

The aetiology of obesity remains elusive; however obesity has been suggested to arise as a result of the interplay between genetic and environmental factors (Orio et al., 2007). The development of obesity is closely linked with nutrition; hence, nutritional intervention is a viable option of tackling the obesity epidemic. High dietary fat consumption has been suggested to be a major risk factor of obesity (Astrup et al., 2008). Due to the high caloric content of fat, it has been proposed that a reduction in the consumption of dietary fat could be a viable option of alleviating the obesity epidemic; however, this has not been successful, and obesity continues to be on the rise (Connor and Connor, 1997, Weinberg, 2004). Besides the quantity of dietary fat, the quality of fat has also been suggested to play an important role in the development of obesity. Studies using rodents have shown that a high fat diet promotes adipose tissue differentiation and hypertrophy (Ellis et al., 1990, Shillabeer and Lau, 1994). *N*-3 PUFA has been shown to elicit several health benefits, including but not limited to the prevention of T2D, CVD, and alleviation

of inflammation (Connor, 2000). The Western diet has witnessed a transition to lower consumption of *n*-3 PUFA (Gomez Candela et al., 2011), which may be part of the reason for the rise in the prevalence of obesity. There is burgeoning evidence of the anti-obesity effects of *n*-3 PUFA. Rodent studies have shown that *n*-3 PUFA can prevent the development of insulin resistance and obesity (Ruzickova et al., 2004, Kuda et al., 2009). Studies in rats have also shown that *n*-3 PUFA could reduce obesity by reducing the size of an adipocyte without affecting the adipocyte number and the body weight (Parrish et al., 1990).

The incidence of obesity and related complications are well established at the population level, however, there is still a lack of information on the mechanism at the cellular level. Furthermore, obesity has been identified as an independent risk factor of CVD in both males and females (Willett et al., 1995, Manson et al., 1990); however, most researchers have excluded the effect of sex and an adequate amount of *n*-3 PUFA in the diet. In the present study, we hypothesized that high dietary *n*-3 PUFA will prevent adipocyte hypertrophy by downregulating the mRNA expression of DGAT2 and FABP4 in a sex dependent fashion in C57BL/6 mice. The specific objectives were to investigate the sex specific effects of diets varying in the quantity of *n*-3 PUFA on: adipocyte TG fatty acid composition; on adipocyte size; 3) on the mRNA expression of adipocyte DGAT2, FABP4, leptin, PPARγ; and on plasma lipids and lipoproteins of C57BL/6 mice. Our findings show for the first time that *n*-3 PUFA prevents adipocyte hypertrophy by downregulating the mRNA expression of DGAT2, FABP4, and leptin; however the effects were sex dependent.

4.3 Materials and Methods

4.3.1 Experimental Diets

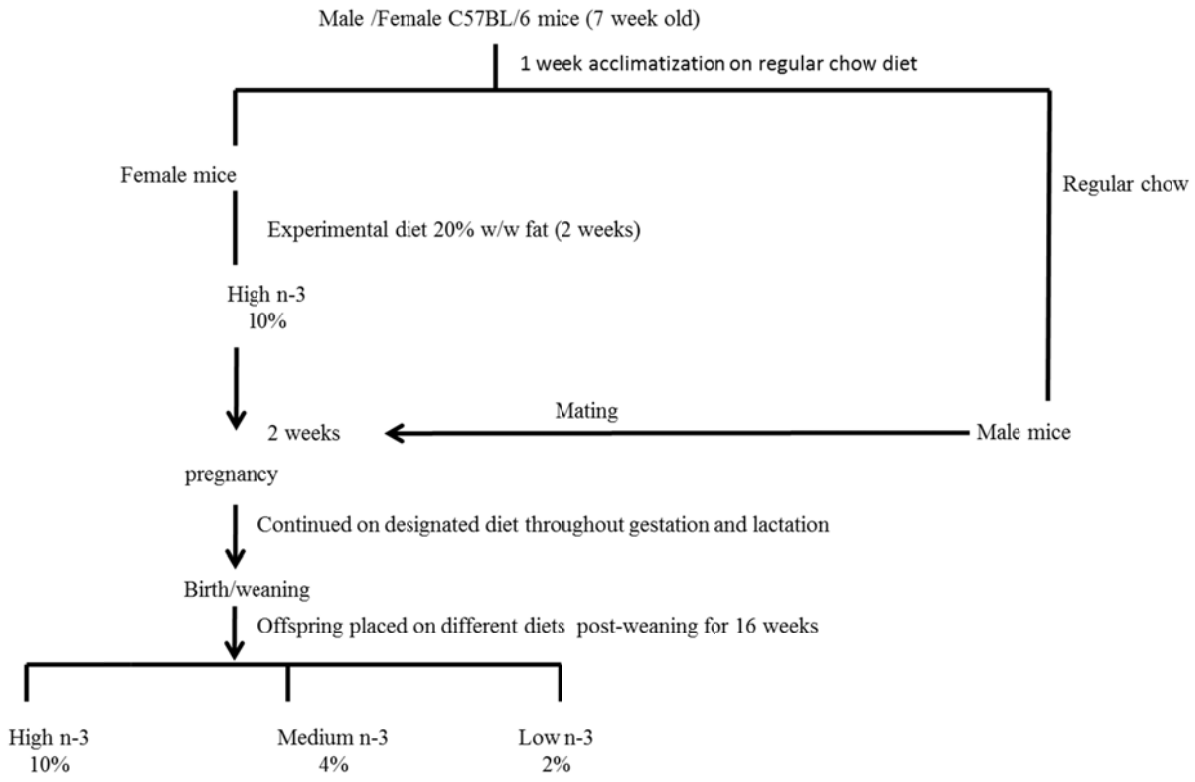
The experimental diets were prepared as previously described in section 2.3.1. The composition of the semi-synthetic diet is provided in **Table 2.1**. The fatty acid composition of the experimental diet was analysed by GLC and is given in **Table 2.2.**

4.3.2 Animals and experimental design

All the experimental procedures were done in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee. Seven week old male and female C57BL/6 mice were obtained from Charles Rivers Laboratories (MA, USA) and were housed in separate cages under regulated temperature (21± 1°C) and humidity (35 ± 5%) conditions with a 12-hour light/12-hour dark period cycle. Mice were acclimatized on standard rodent chow pellets (Prolab RMH 3000) (PMI nutrition, MO, USA) for one week. After this period, female mice were fed the high *n*-3 PUFA diet for two weeks before mating. Female mice were continued on this diet throughout gestation, lactation, and until weaning to ensure an optimum level of *n*-3 PUFA for maintaining whole body homeostasis (Gomez Candela et al., 2011). At weaning, the offspring (male and female) were divided into three separate groups. Each group was fed one of the three experimental diets that differed in *n*-3 PUFA levels, and designated as "High *n*-3", "Medium *n*-3", and "Low *n*-3" diets, for 16 weeks postweaning. At the end of the experimental period, the animals were sacrificed after an overnight fast using isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4), and plasma was separated immediately. Tissues were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80ºC

until further analyses. Animals were provided with fresh food *ad-libitum* every other day during the period of the experiment, and water. Body weights of the animals were documented weekly, and food consumption was recorded every two days.

Schematic representation of the experimental design



4.3.3 Fatty acid analyses of adipocyte TG

Total lipids were extracted from gonadal fat pads using the method of Folch *et al.* (Folch et al., 1957) as per our previous publication (Chechi et al., 2010). Adipocyte TG were separated on thin layer chromatography (TLC) plates using hexane: ethyl ether: acetic acid (70:30:2 v/v) (Keenan MHJ, 1982). The TG spots were identified in comparison with known standard. The TG

spots were scraped and the fatty acid composition was determined using GLC according to our previously published method (Chechi et al., 2010).

4.3.4 Adipocyte histology and imaging

Adipocyte area was measured using computer image analysis according to the method of Chen and Farese (Chen and Farese, 2002) with minor modifications. Briefly, gonadal fat was harvested from mice and fixed in 4% paraformaldehyde for 24 hours. Fixed tissues were transferred to phosphate-buffered saline (PBS) and stained using standard haematoxylin and eosin (H&E) staining procedure. The stained sections were visualized using the Epiflouresence microscope (E600) at 10X magnification. All the pictures were taken at the same time, using the same settings and magnification. The analyses of cell area were performed using the same scale bar (50μm) to ensure uniformity. Adipocytes areas were determined using Image J software. A total of 4 images per animals was obtained, and the sizes of 40 cells in two different microscopic fields were obtained per image giving a total of 800 cells per group.

4.3.5 RNA extraction and real-time qPCR

Total RNA was extracted from gonadal fat pads using Trizol method (Chomczynski and Sacchi, 1987) and contaminating genomic DNA was removed by treating with DNAse enzyme (Promega, USA). RNA concentration and purity were measured using NanoDrop 2000 (Thermo Scientific, USA). Primers for DGAT2, FABP4, leptin, and PPARγ used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/), and obtained from IDT technologies (IA, USA); primer sequences are given in **Table 4.1**. Amplification was performed using iQ SYBER Green Supermix (Biorad, USA). The reactions were run in a reaction volume of 20 μl and 100 ng cDNA per reaction. The Δ Ct was recorded for each gene of interest, and

normalized with RPLPO (large ribosomal protein) as the housekeeping gene. The expression levels between the two groups were compared using the Pfaffl's method (Pfaffl, 2001). The Pfaffl's method is based on the relative quantification of mRNA expression level of the target gene relative to a reference gene. This method takes into account the difference in the efficiency between the target and reference genes. The Ct value of the target gene is normalized to that of the reference gene, and the change in Ct is normalized to a change in Ct of chosen calibrator sample; the expression ratio is calculated by the published Pfaffl's equation (Pfaffl, 2001)

4.3.6 Plasma biochemical parameters

Plasma biochemical parameters were measured using commercially available kits according to the manufacturer's instruction (kit #236-17 and 234-60 for plasma TG and TC respectively, Genzyme Diagnostics, PEI, Canada; and kit # 999-34691 for non-esterified fatty acids (NEFA), Wako Chemicals, VA, USA). HDL-c was precipitated from plasma using kit #200-26A (Diagnostic Chemicals Ltd, Canada) and the HDL-c concentration was determined using total cholesterol assay kit #234-60 (Genzyme Diagnostics, PEI, Canada). Plasma LDL-c concentration was calculated according to the method of Friedewald *et al.* (Friedewald et al., 1972, Fraulob et al., 2010). The current study on the effects of high *n*-3 PUFA on adipocytes was conducted at the same time as our previous study describing the sex-specific effects of perinatal and postnatal dietary *n*-3 PUFA on lipids and lipoproteins (Chapter 2), and therefore shares the same high *n*-3 PUFA group for plasma biochemical parameters.

Table 4.1. Sequences of primers used for qPCR*

Gene	Primers
DGAT2 (S)	ctgctgttggctggtttcac
DGAT2 (AS)	caggaggatatgcgccagag
FABP4 (S)	cataaccctagatggcgggg
FABP4 (AS)	ccagcttgtcaccatctcgt
Leptin (S)	accaggcacccttggagggg
Leptin (AS)	tgtggggccctcactccctg
PPARγ (S)	tgttatgggtgaaactctggg
PPARγ (AS)	agagctgattccgaagttgg
RPLPO (S)	tcactgtgccagctcagaac
RPLPO (AS)	aatttcaatggtgcctctgg

**All primers were designed using NCBI primer blast, and obtained from IDT technologies. Abbreviation: S, sense primer; AS, anti-sense primer; DGAT2, acyl CoA:diacylglycerol acyltransferase 2; FABP4, fatty acid binding protein 4; PPARγ, peroxisome proliferator activator receptor protein gamma; RPLPO, large ribosomal protein (P0)*

4.3.7 Statistical Analysis

Data were analysed using IBM SPSS Statistics (version 20.0). Sample means were compared using two way analysis of variance (ANOVA) to determine main effects of sex and diet, and the interactions between them. Pairwise comparison using Bonferrroni correction was used to determine differences among the groups when there was an observed statistical significant difference. Results are expressed as mean ± standard deviation (SD). Gonadal fat TG fatty acid compositions were expressed as weight percentage of the total extracted fatty acids. Fatty acid composition data were then arcsine transformed and real-time qPCR data were $\log_{10}$ transformed prior to statistical analyses. Differences were considered to be statistically significant if the associated *P* value was < 0.05.

4.4 Results

No significant differences were observed in body weight, fat pad weight, and food intake amongst various dietary groups, and between males and females (*Appendix V*).

4.4.1 Fatty acid composition of gonadal TG of mice fed varying quantity of dietary n-3 PUFA

The gonadal TG fatty acid composition of both male and female mice fed varying amounts of dietary *n*-3 PUFA is presented in **Table 4.2.** There was a concentration dependent significant increase in the gonadal levels of myristic acid (C14:0), with the high *n*-3 PUFA diet showing the highest amount ($P<0.0001$) in both male and female mice. There was however, no effect of sex on C14:0 or an interaction between sex and diet. The male mice showed a significant independent increase in the levels of palmitic acid (C16:0), stearic acid (C18:0), and total saturated fatty acids (SFA) compared to the female mice ($P<0.001$). The levels of C16:0 and total SFA were significantly higher in the high *n*-3 PUFA group compared to the low and

medium *n*-3 PUFA groups in male offspring ($P<0.001$). The level of C18:0 was higher in the high *n*-3 PUFA group compared to the medium *n*-3 PUFA group, but was not different from the low *n*-3 PUFA group. On the other hand, females showed higher level of C18:0 on the high *n*-3 PUFA diet compared to both the low and medium *n*-3 PUFA groups ($P<0.001$). Similar to the males, females also showed the highest total SFA level followed by the medium and the low *n*-3 PUFA groups ($P<0.001$).

Female mice showed a significantly higher level of palmitoleic acid (C16:1n7) compared to male mice ($P<0.001$); however, no effect of sex was observed in the TG fatty acid levels of C18:1n9/C18:1n7, C20:1n9, and total MUFA. High *n*-3 PUFA group showed the lowest level of C18:1n9/C18:1n7 compared to the medium and low *n*-3 PUFA groups in both male and female mice. The medium *n*-3 PUFA group showed significantly lower level of C18:1n9/C18:1n7 than the low *n*-3 PUFA group ($P<0.001$). No difference was observed in the level of C20:1n9 among the three dietary groups in male mice. The female mice fed the high *n*-3 PUFA diet however showed significantly lower levels of C20:1n9 compared to the medium and low *n*-3 PUFA groups ($P<0.01$). A lower concentration of total MUFA was observed in the high *n*-3 PUFA mice compared to the low *n*-3 PUFA mice for both male and female mice ($P<0.001$).

There was no effect of sex on individual and total gonadal *n*-6 PUFA levels, and no interaction between diet and sex was observed; however, there was a significant independent effect of diet in both male and female mice ($P<0.01$). Interestingly, there was a significantly higher amount of linoleic acid (LA; C18:2n6) and total *n*-6 PUFA in the high *n*-3 PUFA group compared to both medium and low *n*-3 PUFA groups in male mice ($P<0.05$). A similar trend was also observed in female mice; however, there was no statistically significant difference in LA between high and medium *n*-3 PUFA groups. AA was significantly lower in the high and

medium *n*-3 PUFA groups compared to the low *n*-3 PUFA group in the male mice; however, no difference was observed in the female mice among the three dietary groups.

There was a significant interaction of sex and diet in the levels of EPA ($P<0001$); however, no interaction was observed in DHA (C22:6n3) and total *n*-3 PUFA. As expected, the high *n*-3 PUFA fed male and female mice had a significantly higher accretion of EPA, DPA, DHA and total *n*-3 PUFA compared to the low *n*-3 PUFA group ($P<0.001$). There was however no difference in DHA and total *n*-3 PUFA between the medium and low *n*-3 PUFA group in the female mice. Interestingly, female mice had a significantly higher level of DHA and total *n*-3 PUFA compared to male mice ($P<0.01$).

4.4.2 Adipocyte area of mice fed varying quantity of dietary n-3 PUFA

H&E staining of the gonadal fat pads revealed smaller adipocytes in male mice fed the high *n*-3 PUFA diet compared to other groups (Fig. 4.1A, B and C); however, no noticeable difference was observed among the three dietary groups in the female mice (data not shown). Imaging and statistical analyses of the stained sections showed a significant interaction between sex and diet on adipocyte area ($P<0.001$; Fig. 4.1D). The female mice in the medium and low *n*-3 PUFA groups had significantly smaller adipocytes ($P<0.001$; Fig. 4.1D) compared to their male counterparts. Male mice on the high *n*-3 PUFA diet had significantly smaller adipocytes compared to male mice on both medium and low *n*-3 PUFA diet ($P<0.001$; Fig. 4.1D); no difference was observed in the male mice between the medium and low *n*-3 PUFA groups. There was no difference in the size of adipocytes in female mice among the three dietary groups.

Table 4.2. Gonadal TG fatty acid composition of male and female mice fed diets varying in the quantity of *n*-3 polyunsaturated fatty acids

Fatty Acids (%)	Male			Female			Main effect		
	Low *n*-3	Medium *n*-3	High *n*-3	Low *n*-3	Medium *n*-3	High *n*-3	Diet	Sex	Diet*Sex
C14:0	0.82 ± 0.04^{c}	1.1 ± 0.07^{b}	1.71 ± 0.25^{a}	0.83 ± 0.13^{z}	0.99 ± 0.08^{y}	1.66 ± 0.19^{x}	P<0.0001	NS	NS
C16:0	13.8 ± 0.45^{b}	14.2 ± 0.77^{b}	15.7 ± 1.47^{a}	11.22 ± 1.35^{y}	12.76 ± 1.15^{xy}	13.8 ± 1.5^{x}	0.0002	P<0.0001	NS
C18:0	2.78 ± 0.36^{ab}	2.45 ± 0.36^{b}	3.35 ± 0.84^{a}	1.91 ± 0.26^{y}	2.16 ± 0.18^{y}	2.68 ± 0.46^{x}	0.0007	0.0002	NS
∑ SFA	17.4 ± 0.69^{b}	17.7 ± 0.95^{b}	20.8 ± 1.71^{a}	14.0 ± 1.66^{z}	15.8 ± 1.25^{y}	18.15 ± 1.71^{x}	P<0.0001	P<0.0001	NS
C16:1*n*7	4.03 ± 0.75	4.53 ± 0.57	4.56 ± 0.37	7.4 ± 1.28	6.25 ± 1.28	5.68 ± 0.64	NS	P<0.0001	NS
C18:1*n*9/18:1*n*7	50.26 ± 3.2^{c}	45.87 ± 4.5^{b}	35.18 ± 1.65^{a}	49.7 ± 5.71^{z}	44.0 ± 3.35^{y}	34.8 ± 1.81^{x}	P<0.0001	NS	NS
C20:1*n*9	0.69 ± 0.08	0.56 ± 0.3	0.6 ± 0.04	0.95 ± 0.45^{y}	0.67 ± 0.11^{y}	0.41 ± 0.13^{x}	0.008	NS	0.049
∑ MUFA	55.0 ± 2.95^{b}	51.0 ± 4.62^{b}	40.3 ± 1.68^{a}	58.0 ± 6.0^{z}	51.0 ± 4.12^{y}	40.9 ± 2.4^{x}	P<0.0001	NS	NS
C18:2*n*6	27.2 ± 3.23^{b}	30.7 ± 4.5^{b}	36.8 ± 2.18^{a}	26.9 ± 7.25^{y}	31.9 ± 3.22^{xy}	38.0 ± 2.90^{x}	P<0.0001	NS	NS
C20:4*n*6	0.22 ± 0.03^{b}	0.13 ± 0.04^{a}	0.15 ± 0.02^{a}	0.2 ± 0.07	0.16 ± 0.06	0.11 ± 0.06	0.009	NS	NS
∑ Omega-6	27.4 ± 3.26^{b}	30.8 ± 4.52^{b}	37.1 ± 2.21^{a}	27.1 ± 7.24^{y}	32.1 ± 3.27^{y}	38.9 ± 2.15^{x}	P<0.0001	NS	NS
C18:3*n*3	0.17 ± 0.04	0.14 ± 0.1	0.19 ± 0.03	0.12 ± 0.1	0.19 ± 0.11	0.18 ± 0.11	NS	NS	NS
C18:4*n*3	0.06 ± 0.06	0.08 ± 0.05	0.14 ± 0.05	0.33 ± 0.33	0.15 ± 0.11	0.33 ± 0.33	NS	NS	NS
C20:4*n*3	ND	ND	0.1 ± 0.03	0.17 ± 0.09	0.13 ± 0.09	0.08 ± 0.06	0.08		
C20:5*n*3	ND	0.18 ± 0.07^{b}	0.48 ± 0.14^{a}	0.29 ± 0.09^{y}	0.24 ± 0.15^{y}	0.5 ± 0.16^{x}	P<0.0001	0.001	P<0.0001
C22:5*n*3	ND	ND	0.3 ± 0.05	ND	0.11 ± 0.1^{y}	0.28 ± 0.03^{x}	P<0.0001		
C22:6*n*3	ND	0.12 ± 0.1^{b}	0.58 ± 0.08^{a}	0.11 ± 0.08^{y}	0.18 ± 0.08^{y}	0.49 ± 0.07^{x}	P<0.0001	0.004	NS
∑ Omega-3	0.3 ± 0.18^{c}	0.52 ± 0.2^{b}	1.76 ± 0.28^{a}	0.9 ± 0.4^{y}	1 ± 0.19^{y}	2.1 ± 0.79^{x}	P<0.0001	P<0.0001	NS

**Data are expressed as weight percentage of the total extracted fatty acids. Values are expressed as mean ± SD, n = 6. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Letters (a,b,c) were used to denote significant differences between dietary groups in males, and letters (x,y,z) represent significant differences between dietary groups in females. Σ SFA= sum of saturated fatty acids; Σ MUFA= sum of monounsaturated fatty acids; Σ PUFA= sum of polyunsaturated fatty acids; Σ Omega-6= sum of omega-6 polyunsaturated fatty acids; Σ Omega-3= sum of omega-3 polyunsaturated fatty acids, ND = Not detected; NS = Not significant*

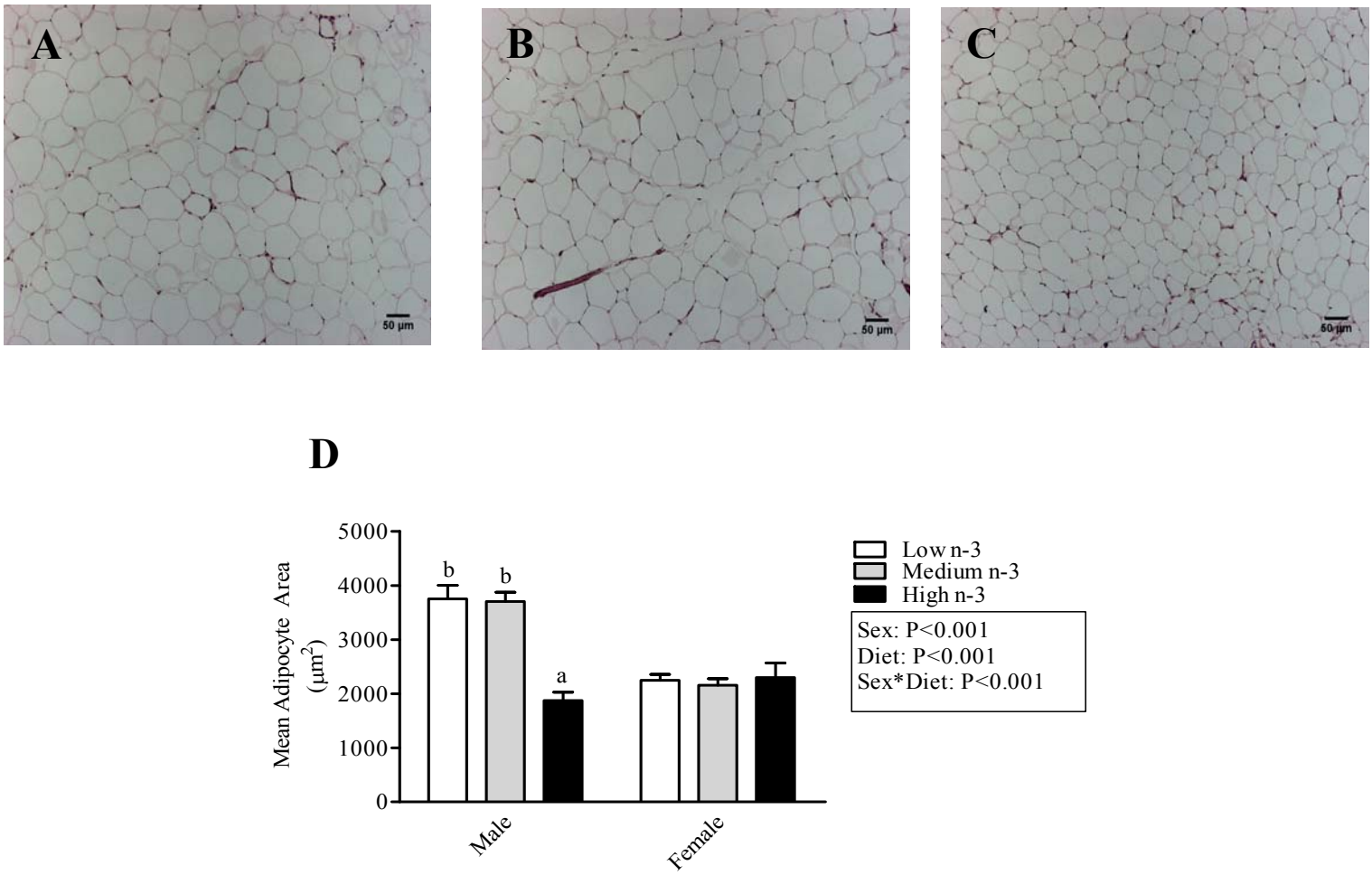


Figure 4.1. Effect of diets varying in the quantity of n-3 PUFA on gonadal adipocyte size: *Haematoxylin and eosin stained histological sections of gonadal fat of male mice fed: (A) low n-3 PUFA diet; (B), medium n-3 PUFA diet; and (C), high n-3 PUFA diet, at image capture of 10X magnification. Figure D represents the mean surface area of gonadal adipocytes in male and female mice; bar represents 50µm. Values are expressed as means ± SD, n=5 per dietary group; 800 cells were measured per group. Data were assessed using two way ANOVA to determine the main effects and interactions of diets and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed significant difference. Letters (a, b) represent significant differences between dietary groups in male mice where P<0.05 was considered significant.*

4.4.3 Gonadal mRNA expressions of FABP4, DGAT2, leptin, and PPARγ of mice fed varying quantity of dietary n-3 PUFA

The mRNA expressions of key genes involved in adipocyte hypertrophy were measured using real time qPCR. No significant interaction was observed between sex and diet in the mRNA expression levels of FABP4 and leptin (Fig. 4.2A and B). Interestingly, similar to the adipocyte data, the female mice fed the low and medium *n*-3 PUFA diets showed lower mRNA expression levels of FABP4 and leptin compared to the male counterparts ($P<0.05$; Fig.4.2A and B). An independent effect of diet was observed, where the high *n*-3 PUFA diet revealed significantly lower mRNA expression of FABP4 and leptin compared to low *n*-3 PUFA diet ($P<0.05$; Fig. 4.2A and B). There was an interaction between sex and diet on the mRNA expression of DGAT2 and PPARγ ($P<0.05$; Fig 4.2C and D), where female mice showed a lower expression. The high *n*-3 PUFA group had significantly lower mRNA expression of DGAT2 and PPARγ compared to the low *n*-3 PUFA groups in male mice ($P<0.05$; Fig. 4.2C and D); however, no significant differences were observed among the three dietary groups in female mice.

4.4.4 Plasma biochemical parameters of mice fed varying quantity of dietary n-3 PUFA

Obesity could result in dyslipidaemia, characterised by an increase in plasma concentration of lipids and lipoproteins; thus we measured these parameters in mice fed the high, medium and low levels of *n*-3 PUFA (**Table 4.3**). There was no independent effect of sex on plasma concentration of NEFA; there was also no interaction between sex and diet. However, both male and female mice fed the low *n*-3 PUFA diet had a significantly higher concentration of NEFA

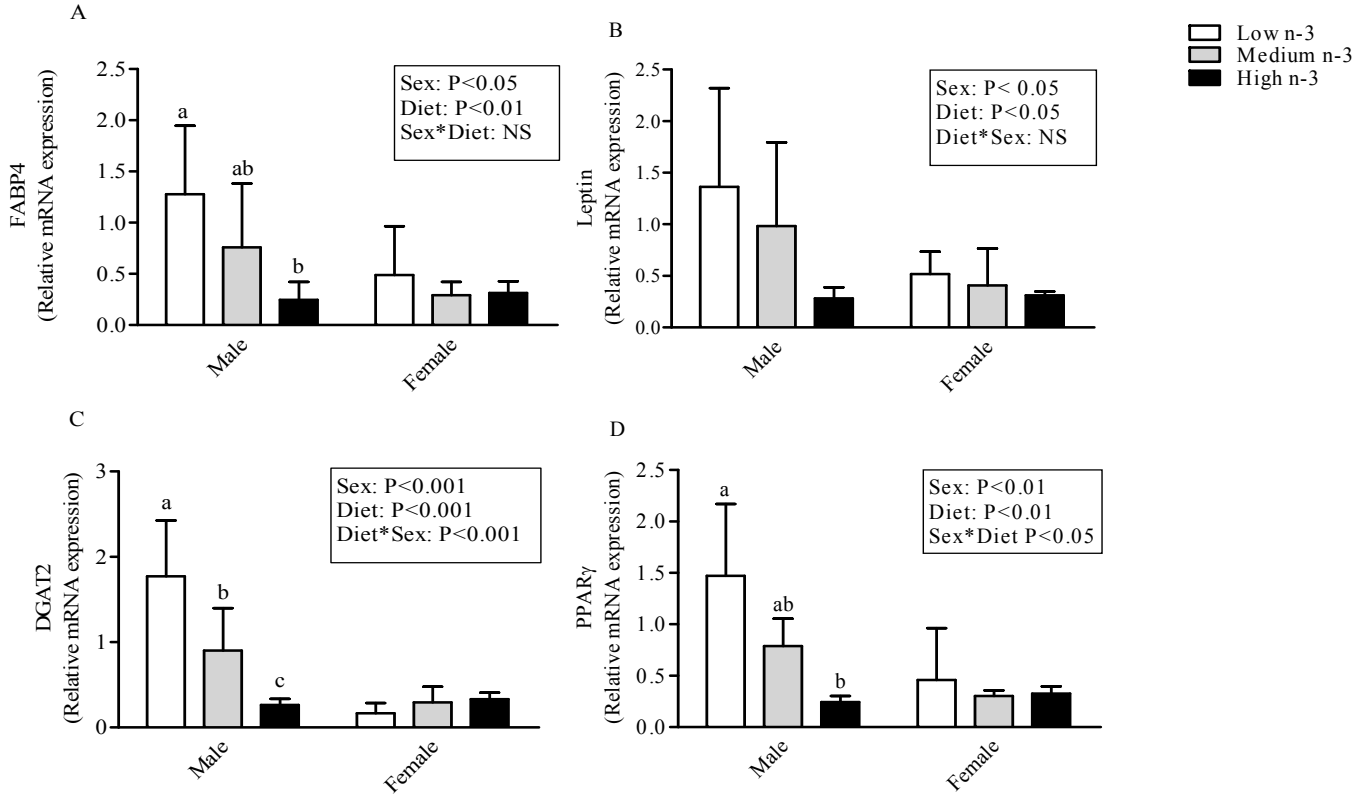


Figure 4.2. Effect of diets varying in the quantity of n-3 PUFA on gonadal mRNA expression: *The data represent male and female gonadal mRNA expression of: A) fatty acid binding protein-4 (FABP4); B) leptin, C) Acyl CoA:diacylglycerol acyltranferase-2 (DGAT2), and D) peroxisome proliferator activator receptor protein-gamma (PPAR-γ) normalized with RPLPO as the house-keeping gene. Values are expressed as means ± SD, n=6 per dietary group. Data were assessed using two way ANOVA to determine the main effects and interactions of diets and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was an observed significant difference. Letters (a, b, c) represent significant differences between dietary groups in males, where P<0.05 was considered significant. NS, not significant*

compared to the medium and high *n*-3 PUFA groups ($P<0.01$). However, there was no significant difference in plasma NEFA concentration between the high and medium *n*-3 PUFA groups in both males and females. Similar to the adipocyte area data, there was a sex-dependent effect of diet on plasma concentration of TG ($P<0.001$). The female mice in the low and medium *n*-3 PUFA groups had a significantly lower plasma TG concentration compared to their male counterparts ($P<0.01$). The concentration of TG was significantly lower in the high *n*-3 PUFA groups compared to the medium and low *n*-3 PUFA groups in male offspring ($P<0.001$); however, no difference was observed between the low and medium *n*-3 PUFA groups. Interestingly, female mice showed no significant effect of the diet in any of the dietary groups. Plasma TC concentration was lower in female mice compared to male mice ($P<0.01$). The high *n*-3 PUFA group had a significantly lower concentration of TC compared to the medium and low *n*-3 PUFA groups in both male and female mice ($P<0.01$). There was no effect of sex on plasma LDL-c levels; however, similar to TC data, LDL-c was lower in the high *n*-3 PUFA group compared to the medium and low *n*-3 PUFA groups in both male and female mice, and no difference was observed between medium and low *n*-3 PUFA groups ($P<0.01$). Diet and sex had no effect on the plasma concentration of HDL-c in both male and female mice.

Table 4.3 Plasma biochemical parameters of mice fed diets varying in the quantity of *n*-3 PUFA*

Lipids (mmol/L)	Male			Female			Main effect		
	Low *n*-3	Medium *n*-3	High *n*-3	Low *n*-3	Medium *n*-3	High *n*-3	Diet	Sex	Diet*Sex
NEFA	0.49 ± 0.19^{a}	0.32 ± 0.07^{b}	0.27 ± 0.08^{b}	0.41 ± 0.12^{x}	0.3 ± 0.08^{y}	0.24 ± 0.1^{y}	P<0.0001	NS	NS
TG	0.41 ± 0.08^{a}	0.46 ± 0.1^{a}	0.23 ± 0.08^{b}	0.28 ± 0.05	0.28 ± 0.08	0.28 ± 0.08	P<0.0001	P<0.0001	P<0.0001
TC	1.78 ± 0.23^{a}	1.86 ± 0.16^{a}	1.25 ± 0.15^{b}	1.63 ± 0.15^{x}	1.54 ± 0.24^{x}	1.1 ± 0.18^{y}	P<0.0001	P<0.0001	NS
LDL	1.22 ± 0.24^{a}	1.34 ± 0.2^{a}	0.77 ± 0.16^{b}	1.15 ± 0.17^{x}	1.13 ± 0.23^{x}	0.71 ± 0.16^{y}	P<0.0001	NS	NS
HDL	0.38 ± 0.07	0.32 ± 0.1	0.41 ± 0.15	0.36 ± 0.13	0.28 ± 0.09	0.31 ± 0.08	NS	NS	NS

**Values are expressed as means ± SD, n =8. Data were assessed using two way ANOVA to determine the main effects and interactions of diets and sex; pairwise comparison using Bonferroni correction was used to determine differences when there was a significant difference. Letters (a,b) were used to denote significant differences between dietary groups in males, and letters (x,y) represent significant differences between dietary groups in females. $P < 0.05$ was considered significant. Abbreviation: NEFA, non-esterified fatty acids; TG, triglycerides; TC, total cholesterol; LDL, low-density lipoprotein, HDL, high-density lipoprotein. NS, not significant*

4.5 Discussion

The underlying mechanism connecting obesity with the pathogenesis of T2D and CVD is poorly understood, however, the role of adipose tissue has been implicated (Ferroni et al., 2004). Obesity can be defined at the cellular level as the enlargement of adipose tissue mass as a result of hyperplasia and/or hypertrophy. In the current study, the sex dependent effect of diets varying in the quantity of *n*-3 PUFA was investigated on adipocyte hypertrophy, and the mRNA expression of key genes such as DGAT2, FABP4, leptin, and PPARγ involved in adipose tissue enlargement. We report a novel effect of *n*-3 PUFA on the cellular events that lead to obesity by preventing adipocyte hypertrophy through the downregulation of the mRNA expression of DGAT2 and FABP4. Our findings also suggest a sex dependent anti-obesity effect of a diet high in *n*-3 PUFA.

Adipose tissue composition of essential fatty acids is mainly determined by dietary intake (Hodson et al., 2008); however, factors such as sex also play a major role in tissue accretion of these fatty acids (Childs et al., 2008). It was thus imperative to assess the accretion of dietary *n*-3 PUFA in adipose tissue TG. As expected, there was a dose-dependent accretion of EPA, DHA, and total *n*-3 PUFA in adipose tissue. The high *n*-3 PUFA group had the highest accretion of EPA, DHA, and total *n*-3 PUFA compared to the two other groups; these findings are in line with previously reported data (Hodson et al., 2008). Interestingly, female mice had a higher accretion of gonadal fat TG DHA and total *n*-3 PUFA compared to the male mice. Clinical studies have indicated that females have a higher concentration of DHA than their male counterparts in the serum and plasma (Crowe et al., 2008, Garneau et al., 2012). This has been suggested to be due to higher and more efficient conversion of ALA to EPA and DHA (Pawlosky et al., 2001, Burdge and Wootton, 2002), and also a decreased retroconversion of

DHA to EPA and DPA in females (Brossard et al., 1996). This is evident in our data, with no difference in TG ALA composition between male and female mice; however, female mice had more accretion of EPA compared to male mice. Human studies have also reported a higher incorporation of DHA in female adipose tissue compared to male (Walker et al., 2014), further confirming our observations. Another interesting observation was the percentage of different fatty acids present in the gonadal TG; there was higher accretion of medium chain and saturated fatty acids compared to longer chain and highly unsaturated fatty acids in the high *n*-3 PUFA group. Studies have shown that the mobilization of fatty acids from adipose tissue is a function of their structure and not their composition in adipose tissue (Conner et al., 1996). SFA was reported to be mobilized the least, followed by MUFA, and PUFA was mobilized the most, especially the 20 carbon PUFAs such as EPA and AA because they serve as precursors to eicosanoids production (Conner et al., 1996). Thus, lower levels of EPA and AA in our study could be due to rapid mobilization of these fatty acids for physiological functions. The high *n*-3 PUFA group, however, showed a lower concentration of AA and higher concentration of EPA in the gonadal TG. AA is metabolised to pro-inflammatory, pro-aggregatory, and vasoconstrictive eicosanoids, which support the development of CVD (Farooqui et al., 2007). Conversely, EPA derived eicosanoids are anti-inflammatory and prevent the development CVD (Adkins and Kelley, 2010). Higher amounts of *n*-3 PUFA in mice fed with a high *n*-3 PUFA diet suggests that these fatty acids and their bioactive metabolic products could be responsible for regulating key genes involved in adipocyte metabolism, thereby preventing obesity and associated complications.

An increase in the mass of the adipose tissue is characterised by an increase in size and/or number of adipocytes (Hausman et al., 2001). Mature adipocytes are postmitotic and can only

undergo hypertrophy; as a result, new adipocytes arise from a population of preadipocytes (Gray and Vidal-Puig, 2007). The progression of obesity does not affect the death or production of adipocyte, however there is enlargement of adipose tissue mass primarily by adipocyte hypertrophy (Bjorntorp, 1974). Our data show a reduction of adipocyte hypertrophy in response to high dietary *n*-3 PUFA in male mice; however, this response was not observed in female mice. Furthermore, there was no effect of any of the diets on body weight of male or female mice. Only very few rodent studies have reported that *n*-3 PUFA could reduce obesity by reducing the size of an adipocyte without affecting the adipocyte number and the body weight (Parrish et al., 1990). Given the gender disparity in the development of obesity and CVD, no study has investigated the sex-specific effect of *n*-3 PUFA on adipocyte hypertrophy. We are the first to report sex specific effects of diets varying in the amount of *n*-3 PUFA on adipocyte hypertrophy. The mechanism for the observed sex specific differences in adipocyte hypertrophy between male and female is unclear; our speculation is that the differences are mediated by sex hormones that needs to be investigated in the future. Body composition differs between male and females, and fat deposition is driven by testosterone and oestrogen differently in both genders (Guo et al., 1998). The female sex hormone oestrogen has been shown to drive adipose tissue proliferation (Roncari and Van, 1978). Furthermore, the metabolism of *n*-3 PUFA is affected by oestrogen (Childs et al., 2008); thus there could be a possible interplay in the metabolism of *n*-3 PUFA, sex hormones, and adipose tissue hypertrophy.

Adipocyte hypertrophy is mainly caused by excess storage of TG in the adipose tissue; therefore, regulating the storage of TG in adipose tissue could be a potentially viable mechanism for preventing obesity. DGAT catalyses the final step of TG synthesis (Chen et al., 2002), which makes it an important enzyme in TG accumulation in adipose tissue. Our study demonstrates that

high *n*-3 PUFA diet reduced the mRNA expression of DGAT2 in a sex-dependent fashion; with smaller adipocytes showing the lowest mRNA expression of DGAT2. Two isoforms of DGAT have been identified (Cases et al., 1998, Lardizabal et al., 2001); the genes that code for DGAT1 and DGAT2 belong to different families, however, they have comparable substrate specificity (Cases et al., 2001). Increased expression of DGAT2 is associated with the formation of large lipid droplets, on the contrary overexpression of DGAT1 produces small lipid droplets (Stone et al., 2004), emphasizing the importance of DGAT2 in TG synthesis. Furthermore, DGAT2 can compensate for the function of DGAT1, however, DGAT1 cannot compensate for DGAT2. *In-vitro* downregulation of DGAT2 results in reduction of TG synthesis, and inhibition of DGAT2 alleviates markers of hyperlipidaemia, obesity and CVD by inhibiting the enzymes involved in lipogenesis (Yu et al., 2005). DGAT2 null mice are lipopenic with a 93% reduction in total carcass TG (Stone et al., 2004). Our finding showing inhibition of DGAT2 mRNA expression by a high *n*-3 PUFA diet is novel, and to the best of our knowledge, no study has linked adipocyte hypertrophy to the regulation of DGAT2 by *n*-3 PUFA. There is a paucity of data on the anti-obesity effect of DGAT2 in adults as DGAT2 knockout mice are lipopenic and die after birth (Stone et al., 2004). Dietary *n*-3 PUFA could serve as a promising therapy for downregulating the expression of DGAT2 and consequently reducing obesity.

DGAT2 requires the presence of free fatty acids to catalyse the covalent binding of acyl CoA to DG, and larger adipocytes are known to release more fatty acids into circulation compared to smaller adipocytes (Zimmermann et al., 2004, Holm et al., 1975). FABP are proteins found in the cytoplasm that binds strongly with a variety of fatty acids, and are evolutionarily conserved between species and are involved in the transport of fatty acids for utilization by enzymes and tissues (Zimmerman and Veerkamp, 2002). Under normal conditions, FABP4 is responsible for

regulating the release and transport of fatty acids (Baar et al., 2005), and is largely expressed in adipocytes and macrophages (Boord et al., 2002, Pelton et al., 1999). It has been reported that the production and secretion of FABP4 in the adipose tissue result in lipogenesis and excess secretion of free fatty acids and TG, which could lead to ectopic lipid accumulation and consequent development of metabolic syndrome (Rasouli et al., 2007). We report for the first time, a reduction in the mRNA expression of FABP4 in response to high *n*-3 PUFA diet and adipocyte hypertrophy; however, this effect was sex-specific, where female mice showed a lower mRNA expression of FABP4 compared to male mice. The role of FABP4 in obesity is not well studied; however, it has been suggested that FABP4 could be a novel adipokine just like leptin and adiponectin whose expression levels correlate with the size of the adipocyte (Kralisch and Fasshauer, 2013). Obese individuals have been shown to have higher expression of FABP4 (Xu et al., 2006, Reinehr et al., 2007) and individuals who lost weight were reported to have a reduced expression of FABP4. FABP4 is transcriptionally regulated (Chmurzynska, 2006); it has been suggested that FABP4 has a peroxisome proliferator receptor element (PPRE) in its promoter region (Schachtrup et al., 2004). PPARγ is highly expressed in the adipose tissue and FABP4 may act closely with PPARγ to elicit its biological function (Tan et al., 2002). Upon interacting with PPARγ ligand, cytosolic FABP4 translocates to the nucleus where it interacts with PPARγ thereby stimulating its transcriptional activity (Tan et al., 2002). Interestingly, similar to our findings with downregulation of FABP4 mRNA expression by a high *n*-3 PUFA diet, the mRNA expression of PPARγ was also significantly lower in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group. Furthermore, the effect was sex specific with female mice showing a lower expression of PPARγ compared to male mice. Targeting the expression of

FABP4 by *n*-3 PUFA could be a potential mechanism to prevent adipocyte hypertrophy and diet induced obesity.

The function of the adipose tissue is not only limited to the storage of TG, it also functions as an endocrine organ, and release a number of adipokines such as leptin into circulation (Kershaw and Flier, 2004). The mRNA expression of leptin has been shown to be reduced by *n*-3 PUFA in both *in-vitro* and *in-vivo* studies (Reseland et al., 2001). We found a sex-dependent reduction in the mRNA expression of leptin in response to high *n*-3 PUFA diet, with the female mice showing a lower expression compared to male mice that correspond with the size of the adipocytes. Leptin is mostly expressed following the differentiation of pre-adipocytes to adipocytes which marks the onset of lipid accumulation in the cell (Mitchell et al., 1997), and the expression is increased postweaning in rodents (Rousseau et al., 1997). As the adipose tissue grows, the secretion of leptin also increases (Considine et al., 1996). A strong association has been reported between leptin secretion and adipocyte size (Zhang et al., 2002) which confirms our observations. We are however, reporting for the first time that the effect of *n*-3 PUFA on leptin mRNA expression is sex specific. There is a direct correlation between the mRNA expression of leptin and PPARγ; a reduction in leptin mRNA expression also leads to a reduction in the mRNA expression of PPARγ (Reseland et al., 2001). Our findings demonstrate a reduction in leptin and PPARγ mRNA expression on a high *n*-3 PUFA diet, further supporting the regulation of leptin by PPARγ. It has also been suggested that leptin controls circulating lipid concentration and regulates the size of an adipocyte by influencing the expression of DGAT through the central nervous system thereby controlling the level of TG synthesis (Suzuki et al., 2005), supporting our observations on similar changes in leptin, DGAT and adipocyte hypertrophy.

Obesity is closely associated with dyslipidaemia which is a disorder of lipid and lipoprotein metabolism, involving hypercholesterolaemia, hypertriglyceridaemia, and elevated LDL-cholesterol concentrations (Misra et al., 2005, Misra et al., 2006). One of the major causes of dyslipidaemia is the alteration of fatty acid metabolism in the adipose tissue (Smith et al., 2006, Sniderman et al., 1998). It has been reported that the production and secretion of FABP4 in the adipose tissue result in lipogenesis and excess secretion of free fatty acids and TG, which could lead to ectopic lipid accumulation and consequent development of metabolic syndrome (Rasouli et al., 2007). The plasma biochemical parameters in our study showed that the high *n*-3 PUFA diet had reduced plasma concentration of TG and NEFA in the male offspring; however, the female mice showed no difference in plasma TG concentration, which is similar to the trend we observed in the adipocyte cell size. Furthermore, *n*-3 PUFAs have been shown to reduce TG by stimulating β-oxidation of NEFA among other hypotriglyceridaemic effects of *n*-3 PUFA (Pegorier et al., 2004). An increase in the release of NEFA from adipocytes leads to the accelerated uptake of these fatty acids by the liver and channelled towards the production of TG, cholesterol and LDL-c (Julius, 2003). This was confirmed in our study, as the low *n*-3 PUFA group had higher plasma LDL-c and NEFA concentration compared to the low *n*-3 PUFA groups.

A higher concentration of TC was also observed in low and medium *n*-3 PUFA groups compared to the high *n*-3 PUFA group in male mice. It has been reported that the higher concentration of cholesterol observed in obese individuals leads to the production of more LDL-c resulting in the downregulation of the expression of LDL- r and reduced clearance of LDL-c (Cox et al., 1995, Katan and Beynen, 1987). It was interesting however, to notice that the plasma NEFA and TC cholesterol concentrations were lower in the high *n*-3 PUFA group

compared to the low *n*-3 PUFA group in female mice, despite no significant difference in adipocyte size, suggesting a sex-specific effect on lipid and lipoprotein metabolism. The sex-specific effects of high *n*-3 PUFA diet on plasma lipids and lipoprotein concentration (Balogun et al., 2014) is likely due to a clear distinction in hormonal regulation between male and female (McNamara et al., 2009).

Taken together, my data suggest a novel mechanism by which *n*-3 PUFA affects the cellular events that may lead to obesity, and I am the first to report a sex-specific effect of *n*-3 PUFA on adipocyte hypertrophy. I am proposing that the downregulation of DGAT2, FABP4 and leptin by *n*-3 PUFA is facilitated by PPARγ. Moreover, an association between leptin and DGAT2 has been previously reported (Suzuki et al., 2005) that supports our data. My findings show that feeding high dietary *n*-3 PUFA could prevent obesity by reducing the mRNA expression of critical genes involved in adipose hypertrophy. My findings also demonstrate that although female mice had lower expression of the key genes involved in adipocyte hypertrophy, lower levels of lipids and lipoproteins, and smaller adipocytes compared to male mice, however males appeared to be more responsive to high *n*-3 PUFA diet. Thus, my findings emphasize that sex differences should be incorporated in the dietary recommendation of *n*-3 PUFA for the prevention of obesity and associated complications.

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CHAPTER FIVE

The dose and age specific effects of n-3 PUFA on the accretion of docosahexaenoic acid in the brain and the expression of neurotrophins

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5.1 Abstract

N-3 PUFA and neurotrophins are pivotal to the proper functioning of the central nervous system. We investigated the effects of perinatal and postweaning *n*-3 PUFA diets on cerebral cortical phospholipid fatty acid composition, and the expression of neurotrophins at weaning and 16 weeks postweaning of the male offspring of C57BL/6 mice. Female C57BL/6 mice were fed semi-purified diets (20% w/w fat) containing 10% (high) and 2% (low) *n*-3 PUFA before mating, during pregnancy, and until weaning. Offspring were studied at weaning and 16 weeks postweaning on their mother's designated diet. Cerebral cortical phospholipid fatty acids and mRNA expressions of BDNF, NGF, CREB, and TrkB were measured. The protein concentration of phosphorylated CREB (pCREB) was determined by ELISA. DHA and total *n*-3 PUFA were significantly higher in cortical phospholipids of the high *n*-3 PUFA group compared to the low *n*-3 PUFA group ($P<0.0001$), and increased significantly from weaning to 16 weeks ($P<0.0001$). There was a significant effect of diet ($P<0.05$) and age ($P<0.05$) on the mRNA expression of NGF. The mRNA expression of BDNF increased significantly ($P<0.01$) in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group. The mRNA expression of TrKB was significantly higher ($P<0.0001$) at 16 weeks in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group; however, no difference was observed at weaning. PCREB/Total CREB was higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group ($P<0.05$). Our findings demonstrate that perinatal and postweaning diets high in *n*-3 PUFA lead to accretion of *n*-3 PUFA in brain cortex. We further found that *n*-3 PUFA upregulates the expression of neurotrophins and their target receptors in an age-dependent fashion.

5.2 Introduction

There is an alarming rise in the prevalence of mental and neurological disorders, with a global burden surpassing cancer and CVD (Collins et al., 2011). According to a United Nations report published in 2007, nearly 1 in 6 people of the global population suffer from neurological disorders (UN, 2007). The causes of most neurological disorders are poorly understood, and are defined by numerous factors in which genetics and environment play vital roles. Nutrition as an environmental factor is important in brain development and approximately 60% of the brain's structural component is lipid (Crawford, 1992). During development, there is accumulation of PUFA in the brain, and this represents about 15-30% dry weight of the brain (Hallahan and Garland, 2005). The brain is highly enriched with PUFA of the *n*-6 class such as AA and *n*-3 class such as DHA; 22:6*n*-3 (Kitajka et al., 2004). Both *n*-3 and *n*-6 PUFA are essential fatty acids as the body is unable to synthesize these fatty acids *de novo,* thus the developing foetus depends on the mother's supply (Crawford et al., 1976). Over the past generations, there has been a decline in the consumption of *n*-3 PUFA in the Western diet (Bazan et al., 2011), and a concurrent increase in the burden of neurological disorders (Collins et al., 2011). Furthermore, there is inadequate consumption of *n*-3 PUFA by pregnant women in Canada and Europe (Loosemore et al., 2004, Denomme et al., 2005), which could partly be responsible for the increasing incidence of neurological disorders.

During development, the human brain is formed in the late period of pregnancy and early postnatal periods (Oppenheim, 1991). This is the period during which DHA accumulates in the brain, where it functions as an important component of neuronal membrane (Clandinin et al., 1980a). A decrease in neuronal membrane DHA positively correlates with an increased predisposition to neurodegenerative disorders (Yehuda et al., 2002). DHA has been shown to be

involved in neurocognition (Fedorova and Salem, 2006); studies have also revealed the preventive roles of DHA in diseases such as Alzheimer's disease and multiple sclerosis (Georgieff, 2007). Furthermore, both rodent and human studies support the roles of DHA in brain development and neurocognition (McNamara and Carlson, 2006, Luchtman and Song, 2013). Fully differentiated neurons are incapable of mitotic division, and the final composition and number of neurons are determined early in development (Oppenheim, 1991). Any alteration at this point could be deleterious, and may be carried throughout lifespan. Altered brain development during perinatal period is consequently a high risk factor for the development of neurodegenerative disorders (Miller and O'Callaghan, 2008, Barlow et al., 2007, Palubinsky et al., 2012). Some secretory trophic factors are involved in neurogenesis and growth, and the most studied of these trophic factors are neurotrophins (Barde, 1990). Neurotrophins are pivotal to the proper functioning of the central nervous system (Chao et al., 2006), and are central to the regulation of neuronal survival, function, and synaptic plasticity (Bramham, 2008, Cohen and Greenberg, 2008, Reichardt, 2006). The upregulation of neurotrophin is considered therapeutic for the alleviation of the symptoms of neurological disorders (Huang and Reichardt, 2001). BDNF and NGF are the best characterised neurotrophins, and are synthesized as their precursor proteins (pro-neurotrophins), which are further cleaved to release the mature neurotrophins (Chao et al., 2006). BDNF is abundant in the brain and elicit its biological functions by binding to its high affinity receptor, TrKB, which signals the downstream activation of the transcription factor CREB (Bhatia et al., 2011). Activation of CREB turns on the expression of genes involved in neuronal survival and plasticity. Abnormalities in neurotrophin expression in the brain underlie the pathology of most neurological disorders (Chao et al., 2006, Dawbarn and Allen, 2003).

Neurotrophins have been suggested to affect brain functions differently at different stages of life. Studies have shown that the mRNA and protein expressions of BDNF and NGF fluctuate at different stages of development, emphasizing the different regulatory roles of BDNF and NGF at different developmental stages (Maisonpierre et al., 1990). BDNF is involved in neuronal development and survival during development by preventing the death of key peripheral, sensory, cholinergic, dopaminergic, and motor neurons (Ernfors et al., 1994). In adulthood, BDNF plays an important role in modulating synaptic plasticity, thereby enhancing long term potentiation (Hu et al., 2011, Korte et al., 1995); learning and memory (Egan et al., 2003), and preventing brain insults (Lindvall et al., 1994). Studies have shown that the expression of BDNF and NGF decreases with age (Maisonpierre et al., 1990, Deogracias et al., 2004). However, the effects of exposure to perinatal and sustained post-weaning diets varying in *n*-3 PUFA quantity on the expression of BDNF, NGF, TrKB, and CREB at different stages of life are not known.

In the present study, we hypothesized that a perinatal and sustained postweaning diet high in *n*-3 PUFA will cause an accretion of DHA in the brain of the offspring, and consequently increase the mRNA expressions of BDNF, NGF, TrKB, and CREB in an age dependent fashion. The specific objectives were: 1) to investigate the effects of perinatal and sustained postweaning diets high in *n*-3 PUFA on cerebral cortical phospholipid fatty acid composition of the offspring of C57BL/6 mice at different stages of life (weaning and 16 weeks); 2) to investigate the effects of perinatal and sustained postweaning diets high in *n*-3 PUFA on the regulation of mRNA expression of BDNF, NGF, TrKB, and CREB at different stages of life; and 3) to investigate the effects of perinatal and sustained postweaning diets high in *n*-3 PUFA on the phosphorylation of CREB. Our findings show for the first time that perinatal and postweaning diets high in *n*-3 PUFA cause accretion of *n*-3 PUFA in the brain cortex, with a concomitant increase in the

expression of neurotrophins and their target receptors in an age dependent fashion. Our data also suggest that *n*-3 PUFA regulates the expression of CREB at the post-translational level by phosphorylation at serine-133.

5.3 Materials and Methods

5.3.1. Diets

The diets were prepared as described in section 2.3.1.The fatty acid composition of the experimental diet is given in **Table 2.2.**

5.3.2. Animals and experimental design

The experimental animals were treated as described in section 2.3.2. After the period of acclimatization, female mice were randomly divided into two groups. Each group was fed one of the two experimental diets that differed in their *n*-3 PUFA composition, and designated as "High *n*-3" and "Low *n*-3" diets, for two weeks before mating. Female mice were continued on the experimental diets throughout gestation, lactation, and until weaning. Half of the male offspring (n=6 per treatment group) were studied at weaning, while the rest of the male offspring (n=6 per treatment group) were continued on their mothers' designated diet for 16 weeks postweaning. Mice were sacrificed after an overnight fast either at weaning or after 16 weeks postweaning on the specified diets using isoflurane. Tissues were collected and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80ºC until further analyses. Throughout the experimental period, animals were provided with water and fresh food *ad-libitum*, every other day. All the experimental procedures were done in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee

5.3.3. Fatty acid analyses of cortical phospholipids

Total lipids were extracted from cerebral cortex using the method of Folch *et al.* (Folch et al., 1957) as per our previous publication (Chechi et al., 2010). Cortical phospholipids were separated on thin layer chromatography (TLC) plates using hexane: ethyl ether: acetic acid (70:30:2 v/v) (Keenan MHJ, 1982, Cheema and Clandinin, 2001). Fatty acid composition of total phospholipids was determined using GLC as previously described in section 2.3.4.

5.3.4 RNA extraction and real-time qPCR

Total RNA was extracted from cortical tissues using Trizol method (Chomczynski and Sacchi, 1987). Contaminating genomic DNA was removed by treating with DNAse enzyme (Promega, USA). RNA concentration was measured using Nano Drop 2000 (Thermo Scientific, USA). RNA integrity was assessed using the Agilent RNA 6000 kit (Agilent Technologies, USA) according to the manufacturer's instructions. The chip was run using an Agilent bioanalyzer (Agilent Technologies, USA) and samples with RNA integrity number (RIN) of 9.5 and above were used for real-time qPCR. All primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and obtained from IDT technologies (IA, USA); primer sequences are given in Table 5.1. Amplification was performed using iQ SYBER Green Supermix (Biorad, USA). The reactions were run in a reaction volume of 25 µl and 10 ng cDNA per reaction. The delta Ct was recorded for each of the gene of interest, and normalized with β-actin as the house keeping gene. The expression levels between the two groups were compared using the Livak method (Livak and Schmittgen, 2001).

Table 5.1. Sequences of primers used for qPCR*

Gene	Primers
NGF (S)	gcagtgaggtgcatagcgta
NGF (AS)	tctccttctgggacattgct
BDNF (S)	tacttcggttgcatgaaggcg
BDNF (AS)	gtcagacctctcgaacctgcc
TrKB (S)	cggcacataaatttcacacg
TrKB (AS)	ttacccgtcaggatcaggtc
CREB (S)	acaatggtacggatggggta
CREB (AS)	ctgctgtccatcagtggtc

**All primers were designed using NCBI primer blast, and obtained from IDT technologies. Abbreviation: S, sense primer; AS, anti-sense primer; NGF, nerve growth factor; BDNF, brain derived neurotropic factor; TrKB, tropomyosin receptor kinase; CREB, cAMP response element binding protein*

5.3.5. Measurement of CREB protein concentration

Cortex samples were homogenized in an extraction buffer containing: 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, and protease inhibitor cocktail (Sigma, CA). Total protein concentration of the lysate was measured using the bicinchoninic acids BCA protein assay (Bradford, 1976), with bovine serum albumin as standards. Total CREB and pCREB) protein concentrations were measured using ELISA kits (Invitrogen, USA) according to the manufacturer's instructions. The intensity of the colored

product was measured at 450 nm using PowerWave XS microplate reader (Biotek, USA). One unit of standard is equivalent to the amount of pCREB derived from 80 pg of CREB that was phosphorylated by protein kinase A. Values of pCREB (Units/mL) were normalized for total CREB (ng/mL) content, and results are presented as ratios of pCREB to total CREB (pCREB/Total CREB).

5.3.6. Statistical Analysis

Data were analysed using IBM SPSS Statistics (version 20.0). Means were compared using two way analysis of variance (ANOVA) to determine main effects of diet and age. Pairwise comparisons were performed using Bonferrroni correction to determine differences among the four groups when there was an observed interaction between diet and age. Results are expressed as mean ± standard deviation (SD). Pearson's correlation was used to compare the relationship between cortical phospholipid DHA composition and gene or protein expression levels. Cortical phospholipid fatty acid compositions were expressed as weight percentage of the total extracted fatty acids; fatty acid composition data were then arcsine transformed before subjecting to statistical analysis. Differences were considered to be statistically significant if the associated *P* value was < 0.05.

5.4 Results

5.4.1. Effects of a diet high in n-3 PUFA on cortical phospholipid fatty acid composition of the offspring at weaning and 16 weeks postweaning

The cortical phospholipid fatty acid composition of the male offspring at weaning and 16 weeks are presented in **Table 5.2.** Diet had no significant effect on individual and total SFA; however, cortical myristic acid (C14:0), palmitic acid (C16:0), and total SFA were significantly

reduced from weaning to 16 weeks (P<0.001). There was no independent effect of diet on total MUFA at weaning or 16 weeks between the two diet groups; however there was a significant interaction between diet and age (P<0.01), with age causing an increase from weaning to 16 weeks (P<0.0001). Oleic acid (C18:1n9) also revealed a significant interaction between diet and age similar to total MUFA (P<0.01), and showed an increase from weaning to 16 weeks (P<0.0001).

There was a significant interaction between diet and age for LA (P<0.05). Furthermore, the high *n*-3 PUFA group showed a significant decrease in cortical phospholipid LA (P<0.01) compared to the low *n*-3 PUFA group at 16 weeks. There was a significant independent effect of diet (P<0.01), and age (P<0.01) on cortical AA, adrenic acid (C22:4n6; ADA), and total *n*-6 PUFA. There was also a significant reduction of AA (P<0.001), ADA (P<0.01), and total *n*-6 PUFA (P<0.001) from weaning to 16 weeks. Moreover, the high *n*-3 PUFA group showed a significant decrease in cortical AA (P<0.01), ADA (P<0.0001), and total *n*-6 PUFA (P<0.0001) compared to the low *n*-3 PUFA group.

There was a significant independent effect of diet and age on cortical phospholipid individual and total *n*-3 PUFA (P<0.05). DHA and total *n*-3 PUFA significantly increased from weaning to 16 weeks (P<0.0001). Exposure to high perinatal and postweaning *n*-3 PUFA caused an accretion of DHA in the offspring cortical phospholipids (P<0.0001). There was also a significant increase in total cortical phospholipid DHA in the high *n*-3 PUFA group compared to the low *n*-3 PUFA (P<0.0001). Interestingly, EPA was not detected in the offspring cortex in both dietary groups at weaning, however, the offspring in the high *n*-3 PUFA group had a significantly higher concentration of EPA (P<0.01), and docosapentaenoic acid (22:5n3; DPA) (P<0.01) at 16 weeks, compared to the low *n*-3 PUFA group.

Table 5.2. Effects of a diet high or low in n-3 PUFA on the cortical phospholipids fatty acid composition of male offspring at weaning and 16 weeks postweaning*

Fatty Acids	Weaning		I6 Weeks		Main effect		
	High *n*-3	Low *n*-3	High *n*-3	Low *n*-3	Diet	Age	Diet*Age
C14:0	0.54±0.09	0.44±0.06	0.20±0.023	0.24±0.09	NS	P<0.0001	P<0.01
C16:0	25.49±1.26	24.5±0.82	22.77±1.47	22.77±0.61	NS	P<0.0001	NS
C18:0	23.5±0.76	24.2±0.46	24.05±0.85	24.82±1.27	NS	NS	NS
∑ SFA	49.54±0.99	49.05±0.84	47.02±2.09	47.84±1.29	NS	P<0.001	NS
C16:1*n*7	0.56±0.09	0.51±0.04	0.5±0.03	0.50±0.05	NS	NS	NS
C18:1*n*9	11.82±0.44	12.34±0.57	15.79±1.48[a]	14.26±0.69[b]	NS	P<0.0001	P<0.01
C18:1*n*7	2.58±0.15	2.76±0.16	2.91±1.8	3.04±0.16	P<0.01	P<0.0001	NS
C20:1*n*9	0.38±0.09	0.47±0.19	1.14±0.48	1.01±0.2	NS	P<0.0001	NS
∑ MUFA	15.34±0.57	16.08±0.86	20.34±1.98	18.81±0.93	NS	P<0.0001	P<0.01
C18:2*n*6	1.18±0.15	1.18±0.05	0.84±0.23[a]	1.18±0.2[b]	P<0.05	P<0.05	P<0.05
C20:4*n*6	12.69±0.76	13.80±0.76	9.29±1.27	10.58±0.1	P<0.01	P<0.0001	NS
C22:4*n*6	2.61±0.17	3.42±0.22	2.28±0.27	3.10±0.38	P<0.0001	P<0.01	NS
∑ Omega-6	16.48±0.81	18.4±0.95	12.41±1.56	14.86±1.13	P<0.0001	P<0.0001	NS
C18:4*n*3	ND	ND	0.08±0.04	0.06±0.04			
C20:5*n*3	ND	ND	0.11±0.07[a]	0.06±0.05[b]			
C22:5*n*3	0.78±0.51	0.54±0.34	0.51±0.11	0.10±0.06	P<0.05	P<0.05	NS
C22:6*n*3	17.87±1.1	15.98±0.56	19.52±0.83	18.28±0.1	P<0.0001	P<0.0001	NS
∑ Omega-3	18.65±0.93	16.51±0.55	20.22±0.91	18.5±1.02	P<0.0001	P<0.0001	NS

**Data are expressed as weight percentage of the total extracted fatty acids. Values are expressed as mean ± SD, n = 6. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Within a row, statistically significant values are marked with unlike superscripts when a significant interaction was observed (P < 0.05). Σ SFA= sum of saturated fatty acids; Σ MUFA= sum of monounsaturated fatty acids; Σ PUFA= sum of polyunsaturated fatty acids; Σ Omega-6= sum of omega-6 polyunsaturated fatty acids; Σ Omega-3= sum of omega-3 polyunsaturated fatty acids, ND = Not detected*

5.4.2 Effects of a diet high or low in n-3 PUFA on cortical mRNA expressions of BDNF, TrKB and NGF of the offspring at weaning and 16 weeks postweaning

The effects of perinatal and sustained postweaning diets high in *n*-3 PUFA on cortical mRNA expressions of NGF, BDNF and TrKB were assessed using real time qPCR analyses. There was an independent significant effect of diet ($P<0.05$) and age ($P<0.01$) on the mRNA expression of NGF. The mRNA expression of NGF reduced significantly from weaning to 16 weeks ($P<0.01$; Figure 5.1A). On the other hand, a diet high in *n*-3 PUFA significantly increased the mRNA expression of NGF ($P<0.05$; Figure 5.1A) compared to the low *n*-3 PUFA diet. Furthermore, there was a significant positive correlation between NGF mRNA expression and cortical phospholipid DHA at weaning (r=0.95; $P<0.0001$; Figure 5.1B); however, no significant correlation was observed at 16 weeks (Figure 5.1C).

BDNF mRNA expression was not altered with age (Figure 5.2A); however there was a significant effect of diet ($P<0.01$; Figure 5.2A). Diet high in *n*-3 PUFA significantly increased the mRNA expression of BDNF compared to the low *n*-3 PUFA diet ($P<0.01$; Figure 5.2A). Furthermore, there was a positive correlation between BDNF mRNA expression and cortical phospholipids DHA at 16 weeks (r=0.83, $P=0.0009$; Figure 5.2C) and at weaning (r=0.83, $P=0.0017$; Figure 5.2B).

A significant interaction was observed between diet and age on the mRNA expression of TrKB ($P<0.01$; Figure 5.3A). There was a significant increase in the mRNA expression of TrKB from weaning to 16 weeks in the high *n*-3 PUFA group ($P<0.01$; Figure 5.3A). However, diet had no effect on the mRNA expression of TrKB at weaning (Figure 5.3A), whereas TrKB mRNA expression was significantly higher in the high *n*-3 PUFA group at 16 weeks compared to the low *n*-3 PUFA group ($P<0.001$; Figure 5.3A).

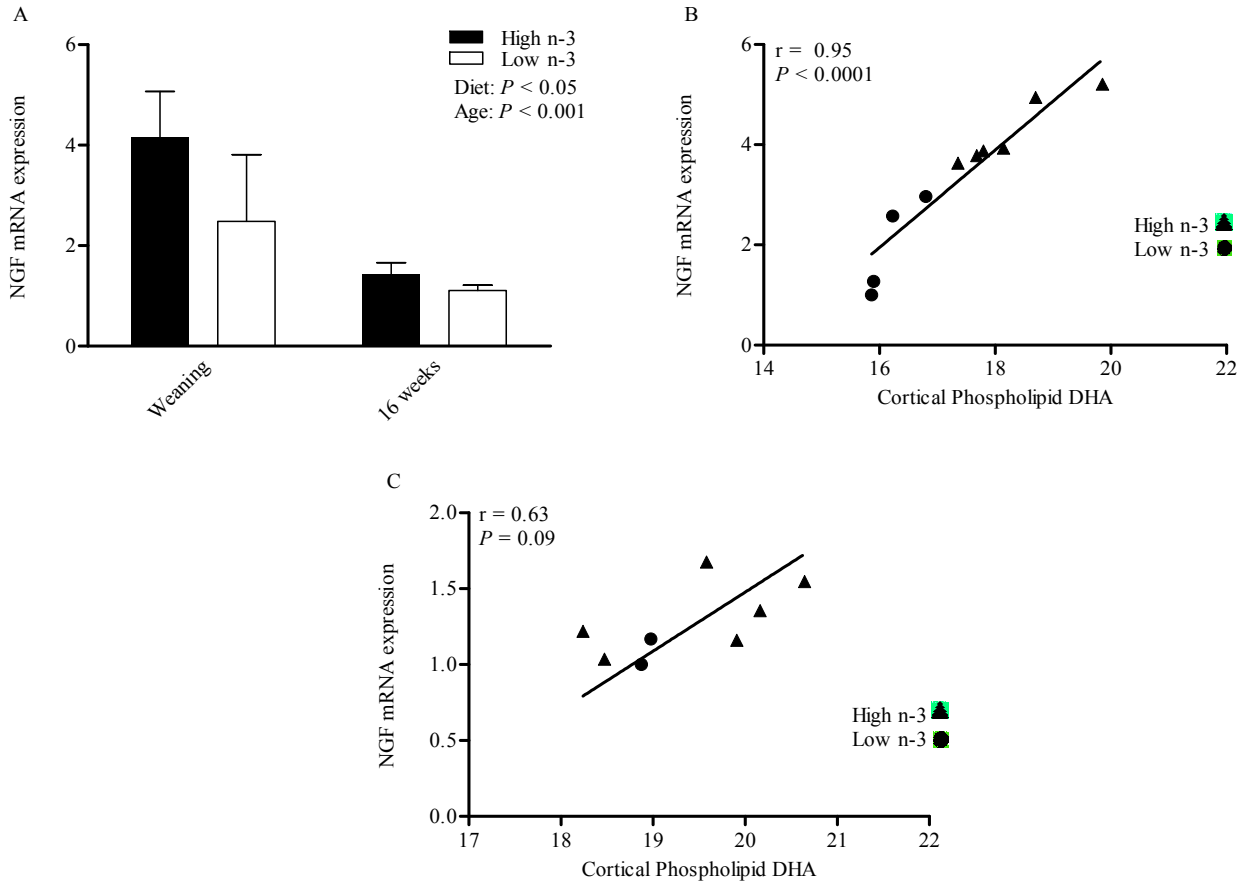

Figure 5.1. Effects of a diet high or low in n-3 PUFA on the mRNA expression of nerve growth factor (NGF) in male offspring at weaning and 16 weeks postweaning: *The data represent male cortical mRNA expression of NGF at weaning and 16 weeks (A) normalized with β-actin as the house-keeping gene, and Pearson's correlation analyses between the mRNA expression of NGF and cortical phospholipid DHA at weaning (B) and 16 weeks postweaning (C). Main effects and interactions were determined by two-way ANOVA. There was an independent effect of diet (P < 0.05) and age (P < 0.001). Data are presented as mean (n=6) ± SD.*

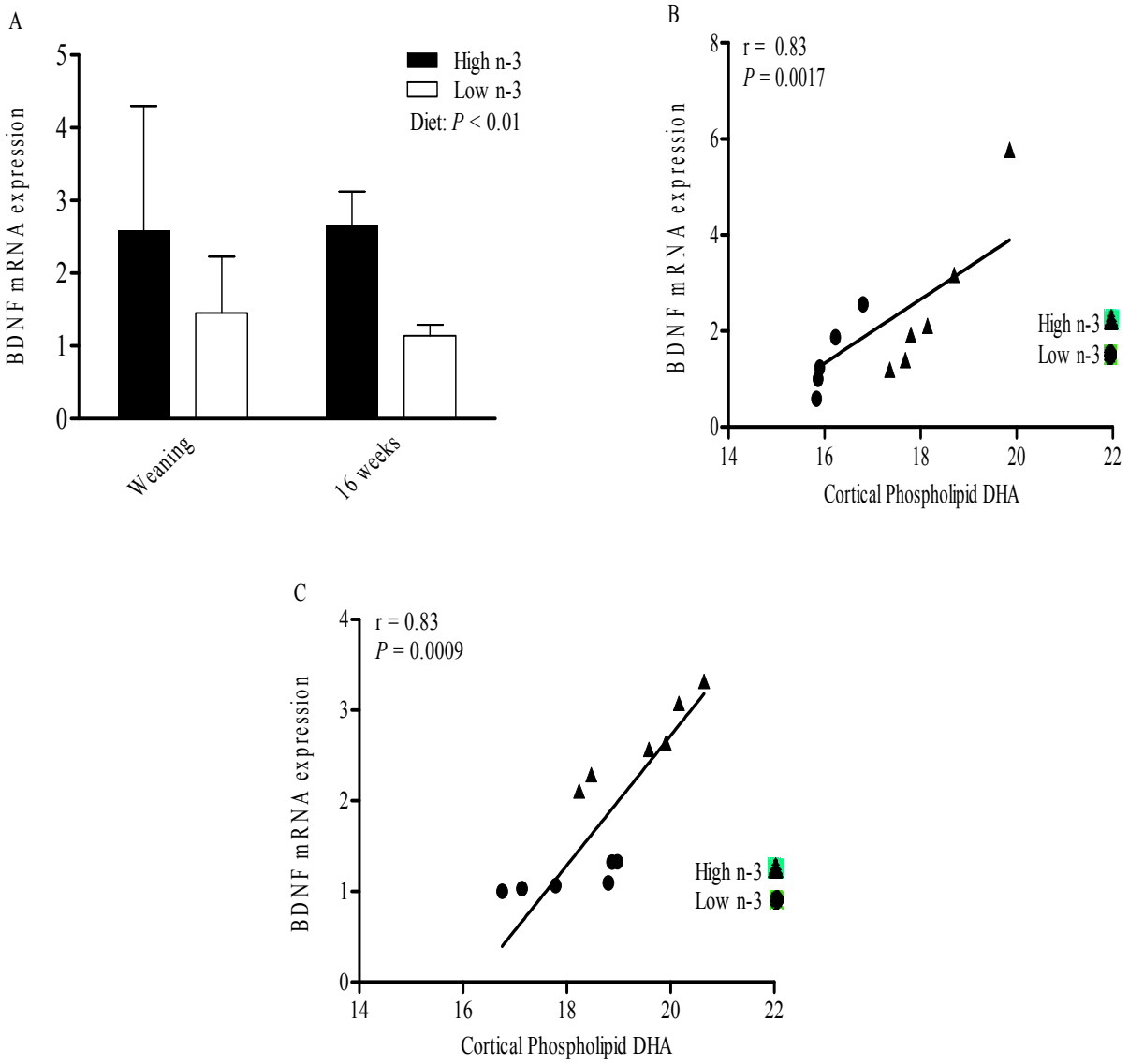

Figure 5.2. Effects of a diet high or low in n-3 PUFA on the mRNA expression of brain derived neurotropic factor (BDNF) in male offspring at weaning and 16 weeks postweaning: *The data represent male cortical mRNA expression of BDNF at weaning and 16 weeks (A) normalized with β-actin as the house-keeping gene, and Pearson's correlation analyses between the mRNA expression of BDNF and cortical phospholipid DHA at weaning (B) and 16 weeks postweaning (C). Main effects and interactions were determined by two-way ANOVA. There was an independent effect of diet (P < 0.01). Data are presented as mean (n=6) ± SD.*

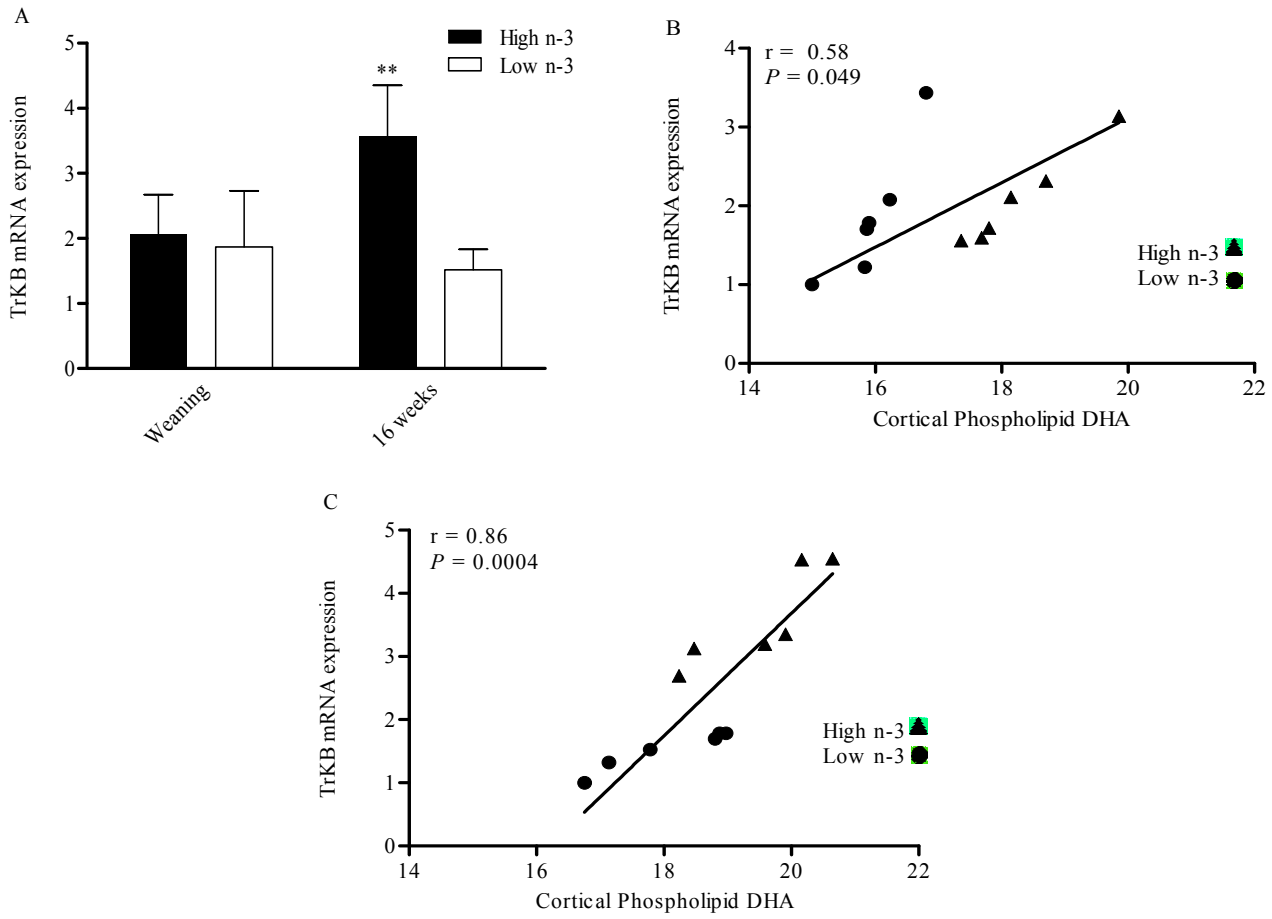

Figure 5.3. Effects of a diet high or low in n-3 PUFA on the mRNA expression of tropomyosin receptor kinase (TrKB) in male offspring at weaning and 16 weeks postweaning: *The data represent male cortical mRNA expression of TrKB at weaning and 16 weeks postweaning (A) normalized with β-actin as the house-keeping gene; and Pearson's correlation analyses between the mRNA expression of TrKB and cortical phospholipid DHA at weaning (B) and 16 weeks (C). Main effects and interactions were determined by two-way ANOVA. Pairwise comparisons were performed using Bonferrroni correction to determine differences among the four groups; data are presented as mean (n=6) ± SD **P<0.05.*

There was a positive correlation between TrKB mRNA expression and cortical phospholipids DHA at weaning (r=0.58, *P*=0.049; Figure 5.3B), and 16 weeks (r=0.86, *P*=0.0004; Figure 5.3C).

5.4.3. Effects of a diet high or low in n-3 PUFA on cortical CREB in offspring at weaning and 16 weeks postweaning

CREB has been shown to be activated by phosphorylation, thus the protein expressions of total CREB and CREB phosphorylated at Ser-133 (pCREB) were measured to investigate the effects of *n*-3 PUFA. There was a significant effect of diet on the relative expression of pCREB to total CREB (pCREB/Total CREB) ($P<0.01$; Figure 5.4B); however, age had no effect. Phosphorylated CREB/Total CREB was significantly higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA ($P<0.05$; Figure 5.4B). There was also a positive correlation between pCREB/Total CREB and cortical phospholipids DHA at weaning (r=0.88, *P*=0.0001; Figure 5.4C) and 16 weeks (r=0.83, *P*=0.0013; Figure 5.4D).

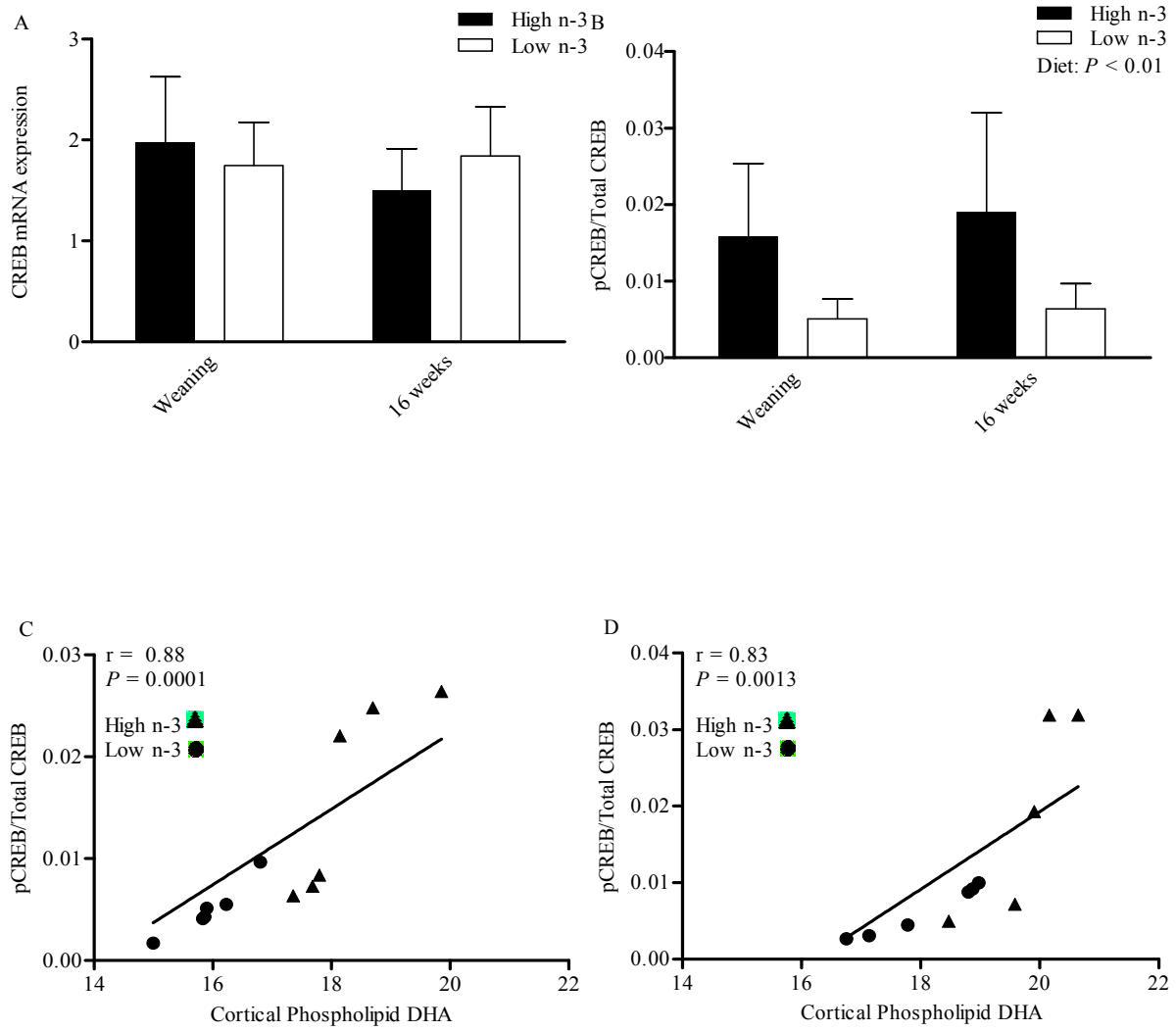

Figure 5.4. Effects of a diet high or low in n-3 PUFA on the expression of cAMP response element binding protein (CREB) in male offspring at weaning and 16 weeks postweaning: *The data represent male cortical mRNA expression of CREB normalized with β-actin as the house-keeping gene at weaning and 16 weeks (A), male phosphorylated CREB (pCREB) protein concentration normalized for total CREB (pCREB/Total CREB) at weaning and 16 weeks (B), and Pearson's correlation analyses between the pCREB/Total CREB and cortical phospholipid DHA at weaning (C) and 16 weeks (D). Main effects and interactions were determined by two-way ANOVA. There was an independent effect of diet (P < 0.01) on pCREB/Total CREB. Data are presented as mean (n=6) ± SD.*

5.5 Discussion

The effects of *n*-3 PUFA on the regulation of neurotrophins during early and later stages of life are not clear. During development, there is rapid incorporation of *n*-3 PUFA into brain phospholipids where they play important roles in neurodevelopment and cognition (Clandinin et al., 1980b, Hoffman et al., 1993). We investigated the effects of perinatal and sustained postweaning diets high in *n*-3 PUFA on accretion of DHA in the brain of the offspring, and the regulation of mRNA expressions of BDNF, NGF, TrKB, and CREB. We report for the first time an age- and diet-dependent accretion of DHA in the brain. Our findings also show an age and diet dependent regulation of neurotrophins. The brain's architecture is built on lipids, and approximately 35% of the brain's structural component is PUFA primarily AA and DHA (Haag, 2003, Wainwright, 2002). There is high foetal accretion of AA and DHA during the third trimester and early postnatal period, which represents the stages of rapid brain development (Arbuckle and Innis, 1992, Wainwright, 2002). We observed no effect of diet on total SFA and MUFA at weaning and 16 weeks; however there was an age-dependent decrease in SFA, while MUFA increased with age from weaning to 16 weeks. This is an interesting finding being reported for the first time. Our observations suggest an increase in desaturation and elongation pathways with age to convert SFA into MUFA; however the rationale for this is not clear and needs further investigation.

There was an independent effect of diet and age on cortical phospholipid *n*-6 PUFA. The high *n*-3 PUFA treatment caused a decrease in total *n*-6 PUFA compared to the low *n*-3 PUFA group. Amongst individual *n*-6 PUFA, cortical phospholipid LA composition was not different at weaning between high and low *n*-3 PUFA dietary groups. Moreover, there was no change in the levels of LA from weaning to 16 weeks in animals fed a low *n*-3 PUFA diet. Suganuma *et al*

(Suganuma et al., 2010) also reported similar observations where neonatal rats in the control and *n*-3 PUFA groups showed no statistical significant difference in brain phospholipid LA composition. The high *n*-3 PUFA diet however caused a decrease in AA and ADA in high *n*-3 PUFA diet group compared to the low *n*-3 PUFA diet group. Desaturation of LA to AA has been shown to be greatly reduced during ageing process in mouse brain (Horrobin, 1981, Bourre et al., 1990, Cook, 1991). Studies have also shown that in adult rat, the desaturation and elongation of LA to AA is greatly reduced (DeMar et al., 2006). Reduced incorporation of AA into brain phospholipids has also been suggested to be due to an increase in the expression of phospholipase A_2 (Terracina et al., 1992). Our findings to show a decrease in AA and no changes in LA suggest that there is a decrease in elongation and desaturation process, or an increase in the expression of phospholipase A_2 with ageing.

A decrease in brain *n*-6 PUFA has been shown to be compensated by an increase in brain DHA (Wainwright et al., 1991). DHA is important in brain development and accumulate during perinatal period, which is around the last three days of gestation in rodents such as rats, and the third trimester in humans (Clandinin et al., 1980a, Clandinin et al., 1980b, Green and Yavin, 1996). Accretion of DHA in brain phospholipids plays a vital role in enhancing neuronal membrane fluidity, and an abnormal deficiency of neuronal membrane DHA could lead to irregularities in cellular signalling (Marteinsdottir et al., 1998, Zimmer et al., 2000). The high *n*-3 PUFA treatment caused an increase in total *n*-3 PUFA compared to the low *n*-3 PUFA group. Amongst *n*-3 PUFA, there was a higher accretion of DHA in the high *n*-3 PUFA group. Similar observations were reported by Bhatia *et al* (Bhatia et al., 2011) after feeding rat pups for 15 weeks on high *n*-3 PUFA diet, and Suganuma *et al*, in neonatal rat pups (Suganuma et al., 2010). Our findings to show an increase in DHA, and a concomitant decrease in AA on a high *n*-3

PUFA diet supports that an increase in brain DHA is compensated by a decrease in brain AA (Wainwright et al., 1991). The concentration of EPA was not detectable at weaning in both dietary groups; however, the high-3 PUFA group had a significantly higher cortical EPA concentration compared to the low *n*-3 PUFA group at 16 weeks. Compared to other PUFA, the cortical concentration of EPA was low, which is similar to the findings, reported by others (Brenna and Diau, 2007, Diau et al., 2005). A plausible explanation for lower levels of EPA is rapid conversion of EPA to longer chain DHA, or a quick metabolism through β-oxidation in the brain (Chen et al., 2013).

The incorporation of *n*-3 and *n*-6 PUFA, such as DHA and AA, are important in neuronal growth and function (Yehuda et al., 2002), and play a critical role in neuronal signalling pathways (Yehuda et al., 2002) regulated by neurotrophins. The high *n*-3 PUFA treatment caused an increase in the mRNA expression of NGF compared to the low *n*-3 PUFA group. Interestingly, there was a decrease in the mRNA expression of NGF from weaning to 16 weeks in mice. There is only one other report to date that show a decrease in the protein expression of NGF with age (Katoh-Semba et al., 1998). NGF promotes survival and prevents neuronal apoptosis (Chen et al., 1997), and is also important in the functioning of cholinergic neurons vital for memory functions (Chen et al., 1997). Consistent with the health benefits of *n*-3 PUFA, NGF has been shown to alleviate the symptoms of neurodegenerative disorders such as Alzheimer's disease (Chao et al., 2006), and an improvement of the impairment of memory function associated with the pathology of Alzheimer's diseases. Our NGF data reaffirm the neuroprotective benefits of *n*-3 PUFA, and offers a platform for further investigative studies on the mechanisms involved in the regulation of the expression of NGF by *n*-3 PUFA at different developmental stages.

BDNF plays a major role in the regulation of synaptic plasticity, neuronal survival, and differentiation in the nervous system (Lewin and Barde, 1996, Huang and Reichardt, 2001). Abnormalities in the synthesis and release of BDNF are potential culprits for development of neurological disorders (Rehn and Rees, 2005). Interestingly, the cortical mRNA expression of BDNF was higher in the high *n*-3 PUFA group compared to the low *n*-3 PUFA; however, age had no effect on the mRNA expression of BDNF. Bhatia *et al* reported a similar observation where a high *n*-3 PUFA diet caused an increase in the protein expression of BDNF in the hypothalamus and hippocampus when male rat offspring of dams fed high *n*-3 PUFA diet during gestation and lactation were weaned on their mother's diet for 15 weeks postweaning (Bhatia et al., 2011). However, Bhatia *et al* did not measure BDNF expression of the offspring at weaning; thus our findings compared for the first time the effect of age on the mRNA expression of BNDF. The expression of BDNF has been shown to be both basal and activity dependent (Bramham and Messaoudi, 2005, Poo, 2001, Thoenen, 1995), which control the permissive and instructive roles of BDNF respectively. Furthermore, BDNF functions differently during development and adulthood. During development, BDNF is involved in axonal path-finding (Hu et al., 2005), neuronal survival (Ernfors et al., 1994), and the growth of inhibitory synapses (Hong et al., 2008). On the other hand, in adulthood, BDNF plays critical role in synaptic plasticity (Hu et al., 2011), memory (Egan et al., 2003), and neuroprotection (Saylor and McGinty, 2008). The exhibition of different functionality by BDNF at different stages of life could very much call for a different regulatory control at different stages of life. There was a positive correlation between cortical mRNA expression of BDNF and cortical phospholipid DHA composition reinforcing the importance of DHA in regulating BDNF.

BDNF exerts its biological function by binding to its high affinity receptor TrKB (Huang and Reichardt, 2001). The interaction between BDNF and TrKB leads to the phosphorylation of TrKB, and a consequent stimulation of downstream signalling cascades vital to the activities of BDNF (Hashimoto et al., 2005, Numakawa et al., 2010). We report for the first time an interaction between high *n*-3 PUFA diet and age in the mRNA expression of TrKB. The mRNA expression of TrKB increased significantly with sustained dietary exposure to high *n*-3 PUFA from weaning to 16 weeks. There was no difference in cortical mRNA expression of TrKB between the two dietary groups at weaning; however, there was a significant increase in the cortical mRNA expression of TrKB in the high *n*-3 PUFA group compared to the low *n*-3 PUFA group at 16 weeks. This is consistent with the mRNA expression of BDNF, indicating a regulatory relationship between BDNF and TrKB. It has been shown that BDNF rapidly upregulates the surface expression of TrKB in hippocampal neurons (Haapasalo et al., 2002), which supports our observation of a concomitant increase in BDNF and TrKB. Bhatia *et al* also found that feeding a high *n*-3 PUFA diet to rats for 15 weeks postweaning increased the protein expression of TrKB in the brain (Bhatia et al., 2011). However, these authors did not measure the regulation of TrKB at different stages of life. We found a positive relationship between the mRNA expression of TrKB and cortical phospholipid DHA both at weaning and at 16 weeks, reinforcing the role of DHA in regulating TrKB.

The BDNF/TrKB complex stimulates the activation of downstream transcription factor CREB (Bhatia et al., 2011), which is vital to the nervous system (Lonze and Ginty, 2002). CREB's target genes includes BDNF (Nibuya et al., 1996, Finkbeiner et al., 1997), and other key genes involved in neuronal survival (Bonni et al., 1999), neuroprotection (Deak et al., 1998), addiction (McClung and Nestler, 2003), learning and memory (Alberini, 2009), circadian rhythm

(Kornhauser et al., 1996), and many others. We found no effects of diet and age on the mRNA expression of CREB between the two diet groups. CREB protein is activated by post-translational modification by phosphorylation at serine-133 (Gonzalez and Montminy, 1989); and this activation is needed to activate its downstream targets including BDNF (Finkbeiner et al., 1997). We found a significant effect of diet on the phosphorylation of CREB, with the high *n*-3 PUFA group showing an increase in pCREB/Total CREB compared to the low *n*-3 PUFA group. However, age had no effect on the phosphorylation of CREB, which is consistent with our BDNF data. This is a novel finding suggesting the possible regulatory effects of CREB by *n*-3 PUFA at the post-translational level. Although we found no effect of diet on the mRNA expression of CREB, there was a significant increase in pCREB/Total CREB suggesting that *n*-3 PUFA regulates CREB at the post-translational level. It is possible that *n*-3 PUFA directly regulate one or more of the kinases responsible for the phosphorylation of CREB. Furthermore, the expression of TrkB in neurons has been shown to be regulated by CREB (Deogracias et al., 2004), and CREB is activated by downstream signalling of TrKB (Kaplan and Miller, 2000). The activation of CREB by increased expression of TrKB could be a potential mechanism by which *n*-3 PUFA regulates neurotrophin signalling. The potential mechanisms by which DHA regulates neurotrophin signalling are depicted in Figure 5.

In conclusion, we report for the first time that the metabolism of *n*-3 and *n*-6 PUFA in the brain is influenced by age and diets varying in *n*-3 PUFA quantity. Furthermore, our findings demonstrate an age and dietary *n*-3 PUFA dependent regulation of BDNF, TrKB, and NGF, and suggest that *n*-3 PUFA regulates the expression of CREB at post-translational level. Our findings also established that longer exposure to *n*-3 PUFA is important in regulating neurotrophin levels. Neurotrophins are important during foetal development and in adulthood

(Huang and Reichardt, 2001), and a deficiency in their secretion have been implicated in the pathogenesis of most neurological disorders (Chao et al., 2006). Maintaining the levels of neurotrophins throughout life span will be important to prevent neurological disorders, thereby highlighting the importance of dietary *n*-3 PUFA.

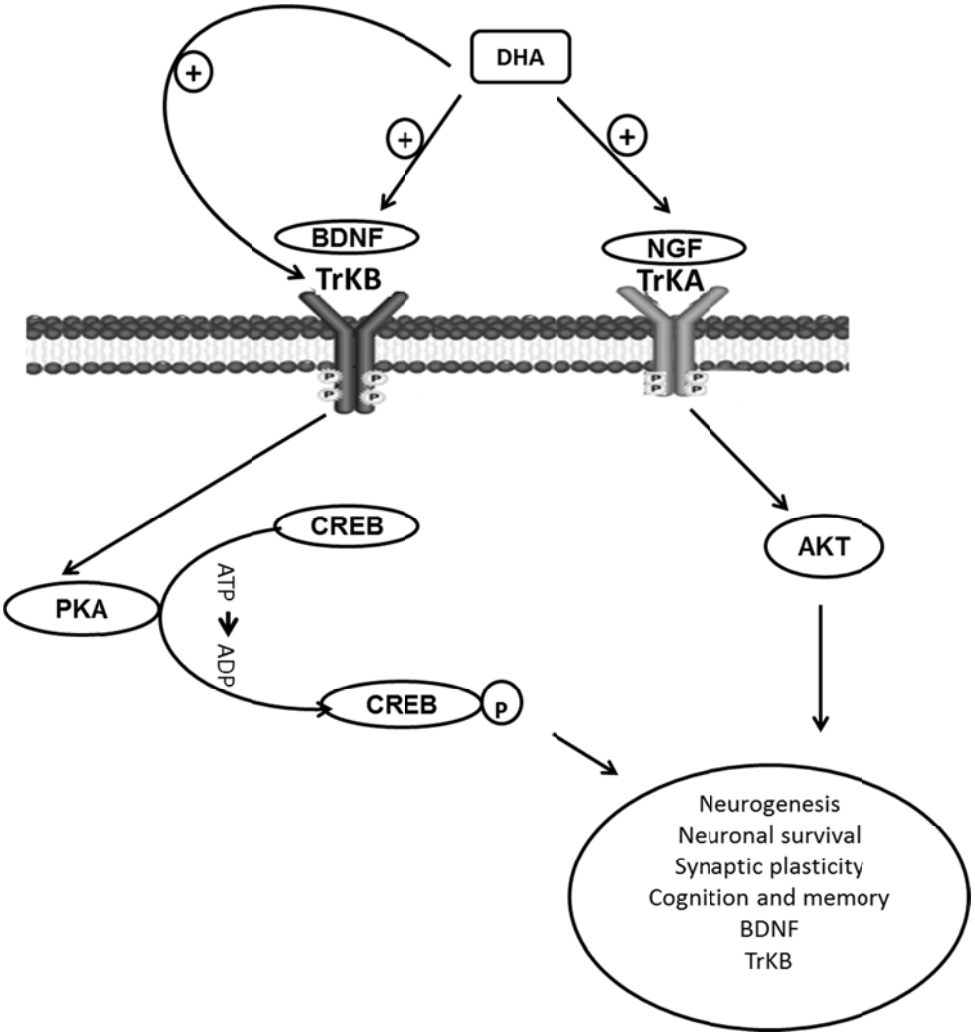

Figure 5.5. Proposed schematic representation of the regulation of neurotrophin signalling by DHA: *DHA upregulates the expression of BDNF and TrKB, which leads to an increased phosphorylation of CREB. Phosphorylated CREB consequently regulates the expression of BDNF, TrKB, and other genes involved in neurogenesis, neuronal survival, synaptic plasticity, cognition, and memory. BDNF: brain-derived neurotropic factor; CREB: cAMP response element binding protein; NGF: nerve-growth factor; PKA: protein kinase A; TrKB: tropomyosin receptor kinase B.*

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CHAPTER SIX

Summary and Conclusions

6.1 Summary and conclusions

CVD is ranked as the number one cause of death worldwide and accounts for 50% of all mortality in North America (Holub, 2009). The causes of CVD are defined by the interplay between genetics and environmental factors; this interplay contributes to the complexity of the pathophysiology of CVD. The risk of development of CVD increases with age (Gupta et al., 2009), and research evidence suggests that sex also play a vital role in the development of CVD. It has been reported that men and women differ significantly in their circulating blood lipids (Johnson et al., 2004). In spite of the advancement in medical science and drug discovery, the prevalence of CVD is still on the rise. The most important environmental factor in the pathogenesis of CVD is nutrition, especially the role of dietary fats. There are numerous reports supporting the cardioprotective effects of *n*-3 PUFA; however, there are also controversial reports. Given the complexity and physiological variation of the human population, it is pertinent to consider the various factors that could potentially affect the metabolism and hence the health benefits of *n*-3 PUFA before a strong recommendation can be made.

The main objective of the current thesis was to investigate the effects of *n*-3 PUFA on the risk factors of CVD such as dyslipidaemia and obesity with particular focus on how sex, age and dose of *n*-3 PUFA affect lipid and lipoprotein metabolism. The thesis investigated the mechanisms by which *n*-3 PUFA could potentially prevent obesity by reducing adipocyte hypertrophy and how sex influences this process, in addition to how the sex-, age- and dose-dependent effects of *n*-3 PUFA affect plasma lipid and lipoprotein concentrations. Furthermore, the effects of *n*-3 PUFA on the accretion of DHA in the brain and the signalling of neurotrophins during development and adult stages were studied. The findings from the current thesis demonstrate a sex-specific effect of *n*-3 PUFA on the risk factors of CVD, and further suggest

novel regulatory pathways by which *n*-3 PUFA could reduce dyslipidaemia and obesity. Furthermore, results from this thesis demonstrate the importance of long term exposure to high *n*-3 PUFA on the accretion of DHA levels in the brain, and the regulation of neurotrophins. Finally, the findings provide clues for the propensity of *n*-3 PUFA to be neuroprotective and cardioprotective through a common neurotrophin signalling pathway.

6.1.1 Key observations

1. Perinatal exposure to high dietary *n*-3 PUFA had no significant effect on the offspring's plasma NEFA and TG at weaning, however, after sustained exposure of the offspring to high *n*-3 PUFA diet for 16 weeks postweaning, there was a significant reduction in plasma NEFA and TG in both male and female offspring. Based on this observation, it was concluded that in addition to high amount of *n*-3 PUFA, a longer exposure to postnatal diet high in *n*-3 PUFA is required to observe a significant lipid lowering effect (Chapter 2).
2. A longer exposure to postnatal diet high in *n*-3 PUFA increased plasma concentration of LDL-c in both male and female offspring. Interestingly, the medium *n*-3 PUFA diet showed the lowest levels of plasma LDL-c concentration in the female offspring, demonstrating a sex-dependent effect of *n*-3 PUFA on LDL-c metabolism. It was also observed that high *n*-3 PUFA diet reduced the circulating concentration of atherogenic very small LDL particles in both male and females. As the effect of *n*-3 PUFA on plasma LDL-c remains controversial, our findings show that a longer exposure to high *n*-3 PUFA increased the plasma concentration of LDL-c; however, this increase was towards a less atherogenic phenotype of LDL particles (Chapter 2).

3. No pronounced effect of high dietary *n*-3 PUFA was observed on the plasma concentration of HDL-c; however, plasma samples from mice fed the high *n*-3 PUFA diet caused a higher cholesterol efflux in the male offspring compared to the low *n*-3 PUFA group. This effect was not observed in female mice, suggesting an effect of sex in *n*-3 PUFA mediated cholesterol efflux. It was thus concluded that *n*-3 PUFA influences reverse cholesterol transport not by increasing HDL concentration, but by enhancing the functionality of HDL, and that the effect is sex specific (Chapter 2).
4. A diet high in *n*-3 PUFA altered the plasma and liver lipidomic profile. *N*-3 PUFA was preferentially incorporated into PC and LPC; despite the changes in lipidomic profile, the total concentrations of these lipids were not altered. Additionally, dietary *n*-3 PUFA was capable of remodelling the fatty acyl moieties of PC, LPC, and CE, which may have important physiological implications. It was thus concluded that the health benefits of *n*-3 PUFA are due to alterations in the fatty acid composition of bioactive lipids, without necessarily changing the total concentration of specific lipid classes (Chapter 3).
5. A diet high in *n*-3 PUFA reduced adipocyte area in males, with no effect observed in the females. The females had smaller adipocyte area compared to male mice in response to high *n*-3 PUFA, suggestive of an effect of sex. High *n*-3 PUFA diet caused downregulation of the mRNA expression of DGAT2, FABP4, PPARγ and leptin in males, however no effect was observed in females, which was similar to the data for the adipocyte area. These observations show that feeding high dietary *n*-3 PUFA could prevent obesity by reducing the mRNA expression of critical genes involved in adipocyte hypertrophy. (Chapter 4).

6. Perinatal diet high in *n*-3 PUFA caused an accretion of DHA in the brain cortex of the male offspring at weaning, and continuous exposure to high *n*-3 PUFA further increased the accretion of DHA into the brain cortex. Furthermore, it was observed that dietary *n*-3 PUFA-dependent regulation of BDNF, TrKB, and NGF was age-dependent, and *n*-3 PUFA regulates the expression of CREB at post-translational level. It was thus concluded that *n*-3 PUFA regulate neurotrophin signalling by increasing the phosphorylation of CREB (Chapter 5).

6.2 Implications and future directions

The series of studies presented in the current thesis support the protective effects of highly unsaturated *n*-3 PUFA in the prevention of dyslipidaemia, a well-known risk factor of CVD; however, the effects are sex-dependent. Judging by the counterintuitive effect of *n*-3 PUFA on plasma TC and LDL-c in female mice presented in chapter 2, it is obvious that the effect of *n*-3 PUFA on cholesterol metabolism is counteracted by a sex specific component which is likely oestrogen. It is believed that endogenous oestrogen protects females from cardiovascular diseases (Mendelsohn and Karas, 1999). This finding has been corroborated by the fact that women are more prone to develop CVD after menopause or surgical removal of the ovaries (Colditz et al., 1987). Clinical and laboratory findings have shown that there is a regulatory relationship between PPARα and oestrogen receptor (ER), and this relationship is influenced by *n*-3 PUFA and oestrogen (Souidi et al., 1999, Wang and Kilgore, 2002). Before a solid conclusion can be made on the effect of *n*-3 PUFA on circulating concentration of cholesterol, it will be important to control for the effect of oestrogen. Future studies could look more closely to characterise in detail, the crosstalk between *n*-3 PUFA and oestrogen with regards to lipid and lipoprotein metabolism. Regardless of the sex-specific effect of *n*-3 PUFA on the plasma

concentration of LDL-c, the concentration of atherogenic very small LDL particle was reduced in response to high dietary *n*-3 PUFA in both male and female mice.

Increasing the concentration of HDL appears to be one of the most attractive pharmacotherapeutic options of preventing CVD because of its role in reverse cholesterol transport. However, the HDL concentration hypothesis in the alleviation of CVD has been greatly challenged with conflicting data, and attempts to increase HDL concentration have not always yielded positive cardioprotective effects (Tardif et al., 2014, Degoma and Rader, 2011). It is thus imperative to look to new mechanisms to enhance the functionality of HDL. Interestingly, high *n*-3 PUFA diet did not increase the concentration of plasma HDL-c in both male and female mice in our study. We however observed an increase in macrophage cholesterol efflux in response to plasma samples from the high *n*-3 PUFA groups in male mice; no effect on cholesterol efflux was observed in females. I can thus safely speculate that *n*-3 PUFA increases cholesterol efflux; however, I cannot say unequivocally that this increase in cholesterol efflux was due to the improvement of the functionality of the HDL, as I used plasma samples from the different dietary groups as our acceptor, which are known to contain other apolipoproteins and proteins capable of influencing cholesterol efflux. Future studies could investigate the cholesterol efflux capacity of HDL isolated from plasma after *n*-3 PUFA treatment. This will give direct information on the influence of *n*-3 PUFA on the functionality of HDL. The size of HDL particles and concentration of APO-A1 could also influence cholesterol efflux capacity; however we found no effect of high dietary *n*-3 PUFA on the particle size of HDL and plasma concentration of APO-A1. Furthermore, proteins such as ABCA-1, LCAT, SR-B1 involved in reverse cholesterol transport can influence cholesterol efflux; however, I found no effect of high

n-3 PUFA diet on the hepatic mRNA expression of these proteins. It is thus imperative to explore other possible mechanisms by which *n*-3 PUFA could influence cholesterol efflux.

LPC has been shown to promote cholesterol efflux from macrophages (Hara et al., 1997). Although the mechanism involved is unclear, it has been suggested that LPC promote cholesterol efflux through its association with HDL-associated paraoxonase 1(PON1) (Rosenblat et al., 2006). An increase in HDL-LPC production increases the binding of HDL to macrophages thereby stimulating cholesterol efflux. My results in chapter 3 show that high *n*-3 PUFA diet is capable of remodelling the fatty acid structure of LPC to contain more *n*-3 PUFA in plasma and liver. I speculate that LPC containing DHA or EPA is atheroprotective by stimulating an increase in cholesterol efflux. Figure 6.1 summarizes the potential of *n*-3 PUFA enriched LPC to act as an anti-atherogenic molecule. Overall, there is plenty of evidence that enrichment of membrane *n*-3 PUFA content has the ability to modify metabolic and physiological functions, thereby inducing cardioprotective effects. Future studies could explore the effect of *n*-3 PUFA on the fatty acid composition of HDL associated phospholipids and the effect on cholesterol efflux. Furthermore, *in vitro* mechanistic studies could be designed to investigate the effect of LPC with different fatty acid moieties on macrophage cholesterol efflux, and LPC fatty acid composition could then be used as a potential biomarker of CVD risk.

Obesity is central to the development of CVD. My study reports a sex-specific effect of *n*-3 PUFA on the cellular events that lead to obesity, by preventing adipocyte hypertrophy through the downregulation of DGAT2 and FABP4 in male mice. My findings suggest that targeting the expression of FABP4 by *n*-3 PUFA may be a mechanism to prevent adipocyte hypertrophy and

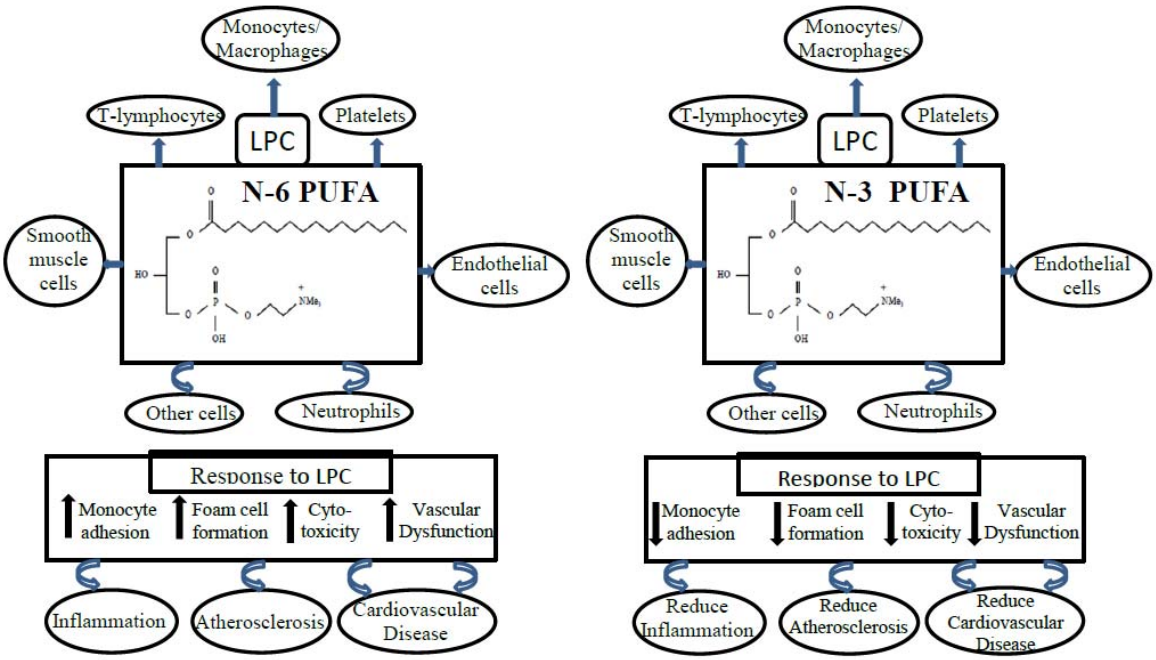

Figure 6.1: Potential atheroprotective mechanisms of n-3 PUFA enriched lysophosphatidylcholine (LPC)

diet-induced obesity. Although I did not measure markers of T2D, ectopic fat deposition and adipose tissue dysfunction are common in obese individuals; I can infer from the plasma lipid and liproprotein data in chapter 4, that the dyslipidaemia associated with obesity is reduced by the administration of high *n*-3 PUFA diet. Body composition differs between male and females, and fat deposition is driven by testosterone and oestrogen differently in both sexes (Guo et al., 1998); the female sex hormone oestrogen has been shown to drive adipose tissue proliferation (Roncari and Van, 1978). Furthermore, the metabolism of *n*-3 PUFA is affected by oestrogen (Childs et al., 2008); thus, there could be a possible interplay in the metabolism of *n*-3 PUFA, sex hormones, and adipose tissue hypertrophy. Furthermore, the accumulation of fat differs between males and females, but I only used visceral fat pad in our study. Future studies could compare the effects of dietary *n*-3 PUFA on the different major adipose tissue depot (subcutaneous and visceral) and adipocyte hypertrophy; this will give vital information on the depot specific effect of *n*-3 PUFA on adipose tissue and how it relates to obesity. Furthermore, exploring the effect of *n*-3 PUFA on mouse model of obesity will provide information on whether *n*-3 PUFA can reverse the obesity phenotype and ameliorate the complications associated with obesity.

The neuroprotective effects of *n*-3 PUFA are well documented; however, it is not known whether longer exposure to *n*-3 PUFA causes accretion of cortical DHA, and regulates the expression of neurotrophins. I investigated the age-dependent effect of *n*-3 PUFA on the expression of proteins involved in neurotrophin signalling in males, acknowledging that the effects of *n*-3 PUFA and neurotrophins change with age. I observed an increase in the cortical mRNA expression of BDNF and TrKB in response to a high *n*-3 PUFA diet as presented in chapter 5. My data suggest that the increased expression of BDNF was due to increased

phosphorylation of CREB by *n*-3 PUFA. This finding is novel; however, there are a number of pathways that lead to the phosphorylation of CREB. I have not investigated the effect of *n*-3 PUFA on the activation of pathways that subsequently lead to the downstream activation of CREB.

I speculate that the upregulation of neurotrophin signalling by *n*-3 PUFA might be the central mechanism by which *n*-3 PUFA prevent both cardiovascular and neurological disease given that neurotrophins have been identified to possess both cardioprotective and neuroprotective potentials. My speculation is based on the correlation analysis between the cortical mRNA expression of BDNF and plasma concentration of TG and NEFA in response to dietary *n*-3 PUFA; those data shows a negative correlation (*Appendix VI*) suggesting that the effects of *n*-3 PUFA are likely through a common pathway. To appreciate the biological significance of the effect of *n*-3 PUFA on neurotrophin signalling, it will be important to investigate how the changes in neurotrophin signalling in response to high *n*-3 PUFA influence behavioural analysis in mice. The effect of neurotrophin signalling can also be explored in mouse model of neurodegenerative diseases; this will provide an insight into the therapeutic potential of altering the expression of neurotrophins in neurodegenerative disease state. Furthermore, my observation of the upregulation of neurotrophin signalling by *n*-3 PUFA was made using male mice; it will be interesting to see whether there is a sex-specific effect of *n*-3 PUFA on neurotrophin signalling using female mice.

Finally, we have made speculations and identified novel targets on the cardioprotective and neuroprotective potentials of *n*-3 PUFA; however, we acknowledge that both cardiovascular and neurodegenerative disease are chronic in nature, and age is a significant risk factor for both conditions. We studied our animals at 16 weeks postweaning which in human years is relatively

young to develop these conditions spontaneously. We however decided to use this age so as to eliminate the metabolic disturbances and other confounding factors that come with ageing. C57BL/6 mice can develop obesity, atherosclerosis, and hyperglycaemia if fed a high fat diet over a long period of time; therefore, to investigate the effect of *n*-3 PUFA on end-stage disease states, longer time period is required.

In conclusion, the results from the current thesis demonstrate that males are more responsive to the cardioprotective effects of high dietary *n*-3 PUFA compared to females. The results show that high *n*-3 PUFA markedly prevented adipocyte hypertrophy and increased cholesterol efflux in males but had no effect in females. Furthermore, the findings from the current thesis show that a high dietary dose of *n*-3 PUFA is required to elicit the cardioprotective effects of *n*-3 PUFA. However, it appears that the medium *n*-3 PUFA diet is better at reducing LDL-c in females; nonetheless, this conclusion cannot be made emphatically without controlling for the effect of oestrogen.

In light of the observed sex, age, and dose differences on the effect of *n*-3 PUFA on the risk factors of CVD, it is pertinent to consider these factors in the recommendation of *n*-3 PUFA as a therapeutic agent. Furthermore, the current thesis presents novel understanding of the mechanism of action of *n*-3 PUFA. Our findings report that *n*-3 PUFA reduces the concentration of atherogenic lipoprotein and that *n*-3 PUFA sex dependently enhances the functionality of HDL by stimulating cholesterol efflux. As the understanding of the cardioprotective mechanisms of *n*-3 PUFA continues to grow, my data also report that *n*-3 PUFA remodelled the plasma and hepatic lipidomic profile to produce bioactive lipids with *n*-3 PUFA moieties and suspected cardioprotective benefits. I am also the first to report that *n*-3 PUFA could sex specifically prevent obesity by downregulating the mRNA expression of DGAT2 and FABP4 thereby

preventing adipocyte hypertrophy. A thorough understanding of the mechanism involved will provide valuable information on the therapeutic potentials of DGAT2 and FABP4 as principal anti-obesity targets. Finally, my findings demonstrate that perinatal and postweaning diets high in *n*-3 PUFA lead to accretion of *n*-3 PUFA in brain cortex, and I also found that *n*-3 PUFA upregulates the expression of neurotrophins and their target receptors in an age dependent fashion. Furthermore, there was a positive correlation between the cortical mRNA expression of BDNF and plasma concentrations of triglycerides and non-esterified fatty acids, suggesting a relationship between neurotrophins and regulation of lipid metabolism.

Overall, dietary intervention remains the safest strategy to prevent the development of CVD; however, with the physiological variation in human population, it is impossible to make a robust general recommendation without considering human variability in response to dietary regimen. The cardioprotective properties of *n*-3 PUFAs have been studied over the years, with more supportive than confuting findings. This represents a very promising therapeutic option for the prevention and treatment of CVD; however more studies need to be undertaken to understand the detailed mechanism of action of *n*-3 PUFA in light of the aforementioned effects of sex and age. Furthermore, it will be important to gain new insights into the neurological origin of CVD and the implication of *n*-3 PUFA in facilitating a central therapeutic option for the prevention and treatment of both neuropsychiatric and cardiovascular diseases.

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Appendix I

RNA Isolation

RNA was isolated from tissues using the Trizol method (according to the information provided by the manufacturer). Briefly, 50-100 mg of tissue samples were homogenized is 1ml volume of Trizol reagent. The homogenate was incubated at room temperature for 5 minutes to allow for total dissociation of nucleoprotein complexes. The homogenate was centrifuged at 12,000 rpm for 10 mins at 4°C to remove all cell debris. The supernatant was transferred to a new tube, and treated with 0.2 ml chloroform per 1 ml of Trizol reagent to facilitate phase separation of nucleic acids. The sample was vortexed and centrifuged at 12,000rpm for 15 mins at 4°C. The upper aqueous phase containing the RNA was carefully transferred into a new tube; and RNA was precipitated by adding 0.5 ml isopropanol per 1 ml of Trizol reagent used at the beginning of the homogenization. The sample was incubated at room temperature for 10 minutes, and then centrifuged at 12,000rpm for 15 mins at 4°C. RNA was washed by the addition of 1 ml 75% ethanol and air-dried. Isolated RNA was dissolved in DEPC-treated water and the concentration of RNA was determined using nanodrop.

Appendix II

Body weight at sacrifice and average weekly food intake of male mice fed diets varying perinatal and postweaning (16 weeks) concentration of *n*-3 PUFA (Chapter 2)

	High *n*-3	Medium *n*-3	Low *n*-3
Body Weight (g)	29.77± 2.33	29.59±3.06	28.18 ± 3.60
Food Intake (g/week)	22.94 ± 0.70	21.93±1.18	22.27 ± 0.98

Values are expressed as means ± SD, *n*=8. Data were analyzed using one-way analysis of variance. PUFA= Polyunsaturated fatty acids

Body weight at sacrifice and average weekly food intake of female mice fed diets varying perinatal and postweaning (16 weeks) concentration of *n*-3 PUFA (Chapter 2)

	High *n*-3	Medium *n*-3	Low *n*-3
Body Weight (g)	22.94± 2.12	24.76±3.84	23.17 ± 3.70
Food Intake (g/week)	21.35 ± 0.66	24.41±1.16	22.96 ± 1.13

Appendix III

Correlation analyses between experimental diets and breast milk fatty acid composition

Breast Milk	**Diet**					
	High *n*-3		**Medium *n*-3**		**Low *n*-3**	
	r	*P*	*r*	*P*	*r*	*P*
High *n*-3	1	< 0.05	0.96	< 0.05	0.94	< 0.05
Medium *n*-3	0.87	< 0.05	0.87	< 0.05	0.87	< 0.05
Low *n*-3	0.84	< 0.05	0.87	< 0.05	0.87	< 0.05

Pearson's correlation coefficients of individual and total fatty acids of maternal diet and breast milk. Breast milk fatty acids of each dietary group are significantly correlated with the maternal diet.

Appendix IV

Total concentrations of plasma and liver phospholipids of mice fed high and low *n*-3 PUFA diets

	PLASMA (nmol/µl)		LIVER (nmol/mg)	
	High *n*-3	**Low *n*-3**	**High *n*-3**	**Low *n*-3**
PC	1.6 ± 0.51	1.7 ± 0.18	28.9 ± 3.00	29.6 ± 3.82
LPC	0.3 ± 0.05	0.3 ± 0.06	4.3 ± 0.92	4.3 ± 1.11
PE	0.3 ± 0.22	0.3 ± 0.08	7. 6 ± 2.19	7.6 ± 2.30
SM	0.01 ±0 .005	0.01 ± 0.002	0.12 ± 0.01	0.12 ± 0.03
CER*	2.7 ± 0.90	2.7 ± 1.56	0.6 ± 0.15	0.7 ± 0.15

Total concentrations of phospholipids were quantified using ESI-MS. Values are expressed as means ± SD, *n*=6. Data were analyzed using unpaired t-test. PC (phosphatidylcholine), LPC (lysophosphatidylcholine), PE (phosphatidylethanolamine), SM (sphingomyelin), CER (ceramide), PUFA (Polyunsaturated fatty acids).* Plasma ceramide concentration is pmol/µl

Appendix V

Body weight at sacrifice and average weekly food intake of male mice fed diets varying perinatal and postweaning (16 weeks) concentration of *n*-3 PUFA (Chapter 4)

	High *n*-3	Medium *n*-3	Low *n*-3
Body Weight (g)	30.45± 2.75	35.9.±4.09	31.85 ± 4.78
Food Intake (g/week)	23.44 ± 0.92	22.73±1.26	23.27 ± 1.12
Body Fat (g)	2.13±0.87	2.46±0.57	2.02±0.73

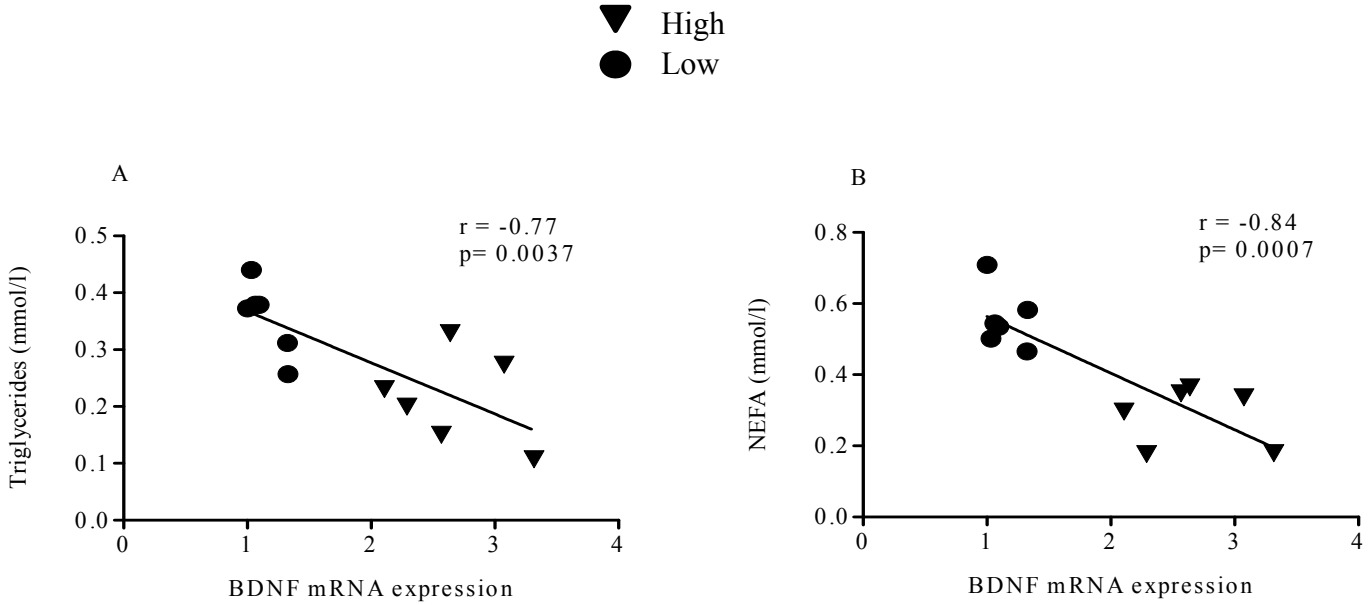
Values are expressed as means ± SD, *n*=8. Data were analyzed using one-way analysis of variance. PUFA= Polyunsaturated fatty acids

Body weight at sacrifice and average weekly food intake of female mice fed diets varying perinatal and postweaning (16 weeks) concentration of *n*-3 PUFA

	High *n*-3	Medium *n*-3	Low *n*-3
Body Weight (g)	25.51± 3.79	23.47±3.24	25.52 ± 1.34
Food Intake (g/week)	22.64 ± 0.73	23.21±1.45	22.68 ± 1.14
Body Fat (g)	1.26±0.82	0.84±0.48	0.66±0.40

Values are expressed as means ± SD, *n*=8. Data were analyzed using one-way analysis of variance. PUFA= Polyunsaturated fatty acids

Appendix VI



The effect of dietary *n*-3 PUFA diet on cortical BDNF mRNA expression and plasma lipids in male offspring at 16 weeks postweaning. Pearson's correlation analyses between cortical BDNF and plasma triglycerides (A) and plasma NEFA (B).