EFFECT OF MATERNAL DIETARY OMEGA-6 TO OMEGA-3 POLYUNSATURATED FATTY ACID RATIOS ON LIPID AND LIPOPROTEIN METABOLISM OF THE OFFSPRING OF C57BL/6 MICE

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Effect of Maternal Dietary Omega-6 to Omega-3 Polyunsaturated Fatty Acid Ratios on Lipid and Lipoprotein Metabolism of the Offspring of C57BL/6 Mice

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ABSTRACT

Dietary fats are a major component of our diet; the quantity and the quality of dietary fats have been associated with the risk of cardiovascular disease (CVD). Epidemiological and laboratory studies have shown that increased consumption of saturated fatty acids (SFA) are associated with an increased risk of CVD, whereas consumption of polyunsaturated fatty acids (PUFA) are associated with reducing the risk of CVD. The current recommendations to replace SFA with PUFA have led to an increased consumption of omega-6 (n-6) PUFA, shifting n-6 to omega-3 (n-3) PUFA ratio from the 2-3:1 in the diets of early hunter-gatherers to 25:1 in the Western diet, which may raise the risk of CVD. One of the objectives of this thesis was to investigate the effect of various dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism using C57BL/6 mice as an animal model. The findings showed significant alterations in biochemical parameters of C57BL/6 mice fed diets varying in n-6 to n-3 PUFA ratios, which formed the basis for the maternal nutrition study. It is now apparent that maternal diet during gestation and lactation may predispose the offspring to CVD in later life. According to the 'developmental origins of health and disease' hypothesis, foetus responds to the nutritional environment by undergoing a series of irreversible adaptations that predisposes the offspring to metabolic disorders in later life. Given the health benefits of maintaining a proper ratio of dietary n-6 to n-3 PUFA, it was of interest to understand the role of altered maternal dietary n-6 to n-3 PUFA ratio on the regulation of lipid and lipoprotein metabolism in the offspring of C57BL/6 mice at weaning. Offspring at weaning were selected to isolate the effects of pre-weaning diet,

excluding the post weaning diet, on the offspring's lipid and lipoprotein metabolism. The C57BL/6 mice were selected for the current study as these have been already established as an animal model in our laboratory to study 'in utero' programming of lipid and lipoprotein metabolism in the offspring.

In the one month feeding study, female C57BL/6 mice were fed a diet containing 20% w/w fat with n-6 to n-3 PUFA ratio of either 5:1, 15:1 or 30:1 to establish the effect on the regulation of lipid and lipoprotein metabolism. Mice were sacrificed after one month and various metabolic parameters were measured. Feeding diets with varying n-6 to n-3 PUFA ratios to C57BL/6 mice led to the incorporation of dietary fatty acids in red blood cell (RBC) phospholipids (PL), and also altered the regulation of lipid and lipoprotein metabolism. In the maternal nutrition study, 8 week old female C57BL/6 mice were fed a diet containing 20% w/w fat with n-6 to n-3 PUFA ratios of either 5:1, 15:1 or 30:1 for two weeks before mating, during gestation and lactation. Both male and female offspring from each dietary group (n =10/group) were sacrificed at weaning and various metabolic parameters were measured. A higher n-6 to n-3 PUFA ratio in the maternal diet of C57BL/6 mice led to higher plasma lipid and lipoprotein concentrations compared to a lower ratio in the offspring at weaning. Moreover the effect of maternal diet was gender specific. In conclusion, a maternal diet high in n-6 to n-3 PUFA ratio resulted in higher levels of lipid and lipoproteins in the offspring at weaning, which may be associated with an increase risk of CVD in later life.

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ABBREVIATIONS

AA	arachidonic acid
ACAT	acyl-Co A cholesterol acyltransferase
ALA	alpha linolenic acid
C	cholesterol
CE	cholesterol ester
CETP	cholesterol ester transport protein
CPT-1	carnitine palmitoyltramsferase 1
CRP	C reactive protein
CVD	cardiovascular disease
DGAT	diglyceride acyltransferase
DHA	docosahexaenoic acid
EFA	essential fatty acid
EPA	eicosapentaenoic acid
FABP	intracellular fatty acid binding proteins
FABPpm	plasma membrane fatty acid binding protein
FAS	fatty acid synthase
FAT	fatty acid translocase
FATP	fatty acid transporter proteins
FC	free cholesterol
FFA	free fatty acid
GLC	Gas liquid chromatography
HDL	high density lipoprotein
IL-1	Inetrleukin-1

IL-6	Interleukin-6
IP	Intraperitoneal
LA	linoleic acid
LCAT	lecithin cholesterol acyltransferase
LC-PUFA	long chain polyunsaturated fatty acid
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
LPL	lipoprotein lipase
MUFA	monounsaturated fatty acid
n-3 PUFA	omega-3 polyunsaturated fatty acid
n-6 PUFA	omega-6 polyunsaturated fatty acid
PL	phospholipids
PUFA	polyunsaturated fatty acid
RBC	red blood cell
RCT	reverse cholesterol transport
SFA	saturated fatty acid
SR-B1	scavenger receptor 1
SREBP-1	sterol regulatory element binding protein-1
TC	total-cholesterol
TG	triglyceride
VLDL	very low density lipoprotein
WHO	world health organization

Chapter 1: Introduction

1.1 Cardiovascular Disease

1.1.1 Global burden of cardiovascular disease

Cardiovascular disease (CVD) is one of the leading causes of death in North America and globally. It involves diseases of the heart and blood vessels and is the most common end stage of a number of distinct diseases and therefore is multifactorial in nature (Riediger *et al.*, 2008). CVD is the largest single contributor to global mortality (WHO, 2009). The World Health Organization claims that 30% of global deaths in 1999 occurred due to CVD (WHO, 2005). It is estimated that a 55% rise will occur in the proportion of deaths due to CVD, between 1990 and 2020 in the developing countries (Murray and Lopez, 1996). According to Statistics Canada (2008), 29% (69,648) of deaths were due to CVD; there was one death due to CVD in every 7 minutes. The costs associated with CVD in Canada may exceed $20.9 billion annually (Statistics- Heart and Stroke Foundation of Canada, 2008). Thus there is an urgent need to design dietary based therapeutic strategies for the treatment and prevention of CVD.

1.1.2. Risk factors associated with cardiovascular disease

Risk factors of CVD are of two types: genetic and environmental (Tymchuk *et al.*, 2006). Since a rapid progression of incidence of CVD within a generation has been observed during the last few decades, some researchers suggest that the causes for CVD are mostly environmental rather than genetic (Symonds & Gardner, 2006). In contrast, population based studies illustrate that the pathogenesis of CVD evolves over decades and has a genetic component and begins as early as childhood (Gillman, 2005).

1.1.3. Dietary fats as a risk factor of cardiovascular disease

Among environmental factors, diet and nutrition have long been identified as major risk factors for CVD. The current research claims that increased incidence of CVD within past few decades is due to sedentary lifestyle and consumption of a high calorie, high fat diet (Drewnowski & Popkin, 1997; Popkin, 2006). A high fat diet alters the regulation of lipid and lipoprotein metabolism which may predispose the individual to develop CVD (Guo & Jen, 1995; Chechi & Cheema, 2006; Magdeldin *et al.*, 2009).

1.2. Regulation of lipid and lipoprotein metabolism

Dietary lipids are a complex group of biomolecules which act as building blocks of cellular membranes (German, 2011). They act as substrates for metabolic energy and provide a precursor pool for a diverse range of metabolic signalling molecules (German, 2011). The recommended fat intake for adults is between 20-35% of total calories (Dietary Guidelines of Americans, 2005). The lipids usually introduced with diet are triglycerides (TG), phospholipids (PL) and cholesterol esters (CE) (Rader & Daugherty,

2008). Once dietary fats and cholesterol are absorbed, they are transported via the lymphatic system as chylomicrons and enter into the blood circulation. Chylomicrons transport fatty acids to peripheral tissues such as adipose and muscle tissue. Lipoprotein lipase (LPL) acts on chylomicrons releasing glycerol and free fatty acids (FFA) which are taken up by the muscle and adipose tissues.

Liver is the main organ involved in the regulation of lipid and lipoprotein metabolism. The liver is capable of *de novo* fatty acid synthesis and secretes very-low-density lipoproteins (VLDL) which, upon the action of LPL, are converted to low-density lipoprotein (LDL). LDL is cleared from the circulation mainly through liver LDL receptors. In addition, high-density lipoprotein (HDL) is generated by the intestine and the liver, and is mainly involved in the reverse cholesterol transport (RCT) process. HDL scavenges cholesterol from peripheral tissues and macrophages through the actions of the transporter ABCA-1, forming nascent HDLs, promoting the efflux of cholesterol from tissues. The free cholesterol (FC) in nascent HDL is esterified to CE by the enzyme lecithin cholesterol acyltransferase (LCAT) which creates mature HDLs, to deliver cholesterol directly to the liver through the receptor SR-B1 and indirectly by cholesterol ester transfer protein (CETP) (Rader & Daugherty, 2008; Flock *et al.*, 2011). Alterations in the regulation of lipid and lipoprotein metabolism is associated with an increased risk of CVD(Rader & Daugherty, 2008).

1.2.1 Pathogenesis of Cardiovascular Disease: Role of lipids and lipoproteins

Research on pathogenesis of CVD has identified several independent factors associated with the morbidities of CVD, i.e. oxidative stress (Ceriello, 2002), high LDL-cholesterol (Carmena *et al.*, 2004; St-Pierre *et al.*, 2005) hyperglycemia (Wahab *et al.*, 2002; Eguchi *et al.*, 2007), hyperinsulinemia (Ingelson *et al*, 2005), and elevated markers of inflammation such as C-reactive protein (CRP) and interleukin-6 (IL-6) (Pearson *et al*, 2003). Amongst these factors, high LDL-cholesterol levels play a significant role in the pathogenesis of CVD (Carmena *et al.*, 2004). Oxidative modifications of LDL have been shown to result in numerous changes in its biologic properties that could have pathogenic importance in atherosclerosis (Steinbrecher *et al.*, 1990). These oxidized LDL particles trigger a series of inflammatory responses resulting an increased expression of adhesion molecules in the endothelial cells (Kita *et al.*, 2001). This leads to the recruitment of macrophages which take up the oxidized LDL, transforming them into foam cells. The foam cells continue to grow and then rupture leading to a huge deposit of cholesterol on the endothelial wall, recruiting more macrophages and the cycle continues (Kita *et al.*, 2001).

It has been reported that 1% increase in LDL-cholesterol is associated with a 2% increase in CVD, while a 1% decrease in HDL-cholesterol was associated with a 3-4% increase in CVD (Wilson, 1990). Although, the role of TG levels as an independent risk factor of CVD remains uncertain, increased plasma TG levels were also known to be associated with increased risk of CVD (Shaikh *et al.*, 1991; Bergeron & Havel, 1997). An increased plasma TG concentration has been shown to be associated with the formation of

small CE rich chylomicron remnants, which mediate cholesterol influx into the endothelial wall along with LDL (Shaikh *et al.*, 1991). Therefore, factors related to altered plasma lipid and lipoprotein levels are highly associated with CVD risk, and dietary fats have been identified as one of the major causes leading to altered lipid and lipoprotein parameters (Mattson & Grundy, 1985a).

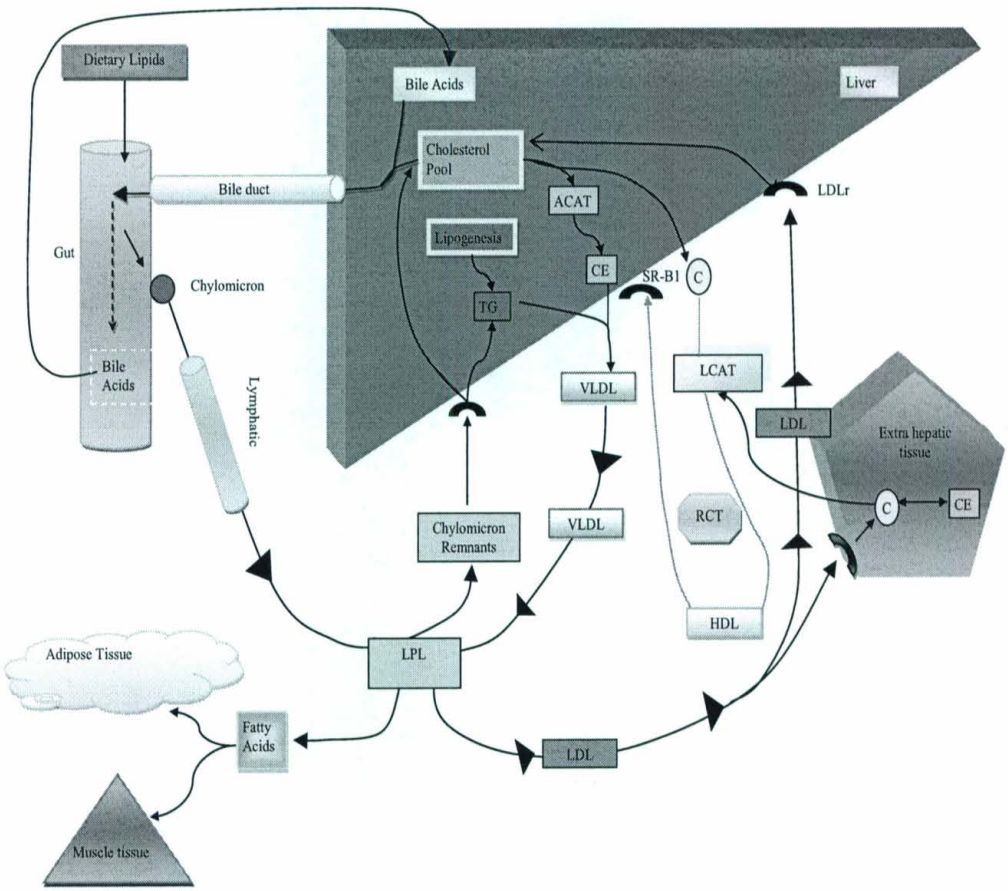


Figure 1.1 Lipid and lipoprotein metabolism

Modified from Flock *et al.*, 2011. CETP and cholesterol ester exchange between lipoproteins and HDL was removed from this figure as mice lack CETP.

TG = Triglycerides, ACAT = Acyl-Co A cholesterol acyltransferase, CE = cholesterol ester, SR-B1 = scavenger receptor-1, C = cholesterol, VLDL = very low-density lipoprotein, LDL = low-density lipoprotein, RCT = Reverse cholesterol transport, HDL = high-density lipoprotein , LDLr = low-density lipoprotein receptor

1.3. Quality of dietary fats and cardiovascular disease

1.3.1. Dietary saturated and polyunsaturated fatty acids in cardiovascular disease

Previous research has shown that the quality of dietary fats play a major role in altering plasma lipid and lipoprotein levels (Mattson & Grundy, 1985b; Mensink & Katan, 1989). Based on their saturation levels, dietary fats are mainly of three classes; saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). An increased consumption of saturated fatty acids (SFA) has been associated with an increased prevalence of CVD (Hu *et al.*, 2001). Saturated fatty acids increase serum total cholesterol (TC), LDL-cholesterol and TG levels in human subjects, which are known to be CVD risk factors (Hegsted *et al.*, 1993). A prospective epidemiological study of 1001 middle aged men reported that those who died from CVD had high intake of SFA and a low consumption of PUFA, 20 years prior to their death (Kushi *et al.*, 1985). Similarly, high TC and LDL-cholesterol levels were reported in humans who consumed a diet rich in SFA compared to a diet rich in PUFA (Mattson & Grundy, 1985b). On the other hand, a meta-analysis of 60 selected trials concluded that the intake of PUFA, especially the intake of linoleic acid (LA), has been associated with reduced serum TG levels and TC levels compared to the diets rich in SFA (Mensink *et al.*, 2003). Linoleic acid is also associated with lowering LDL-cholesterol when replacing dietary SFA (Hayes, 2000). Thus, increased consumption of PUFA has been suggested to be associated with decreased risk of CVD (Russo, 2009). In contrast, dietary intake of SFA may increase the risk of CVD.

1.3.1.1 Effects of dietary omega-6 polyunsaturated fatty acids in cardiovascular disease

Dietary PUFA consist of omega-6 (n-6 PUFA) and omega-3 PUFA (n-3 PUFA), which are essential fatty acids for humans due to lack of desaturase enzymes responsible for introduction of double bonds beyond Δ9 position for *de novo* fatty acid synthesis. Linoleic acid is the primary dietary n-6 PUFA, which gets converted to arachidonic acid (AA) after elongation and desaturation (Harris *et al.*, 2009). On the other hand, parent n-3 PUFA, α- linolenic acid (ALA), gets converted to longer chain polyunsaturated fatty acid (LC-PUFA), essentially docosahexaenoic acid (DHA) and eicosapentaneoic acid (EPA) after elongation and desaturation, but this conversion is poor in the presence of n-6 PUFA.

Previously it was reported that higher dietary PUFA levels are associated with a reduced ratio of total to HDL-lipoprotein cholesterol (Siguel, 1996). On the other hand, diets high in LA have been shown to increase the susceptibility of LDL-cholesterol oxidation promoting vascular inflammation (Steinberg *et al.*, 1989; Tsimikas *et al.*, 1999). An increased risk of acute myocardial infarction was reported with high dietary AA intake (Kark *et al.*, 2003). Omega-6 PUFA are also involved in increasing the oxidation susceptibility of LDL and VLDL and thereby exerting deleterious effects on development of CVD (Louheranta *et al.*, 1996). Collectively, these data indicate that the beneficial effects of n-6 PUFA on the onset of CVD are controversial. Thus, it appears that the cardio protective effect of PUFA may be highly attributed to n-3 PUFA

1.3.1.2. Effects of omega-3 polyunsaturated fatty acids in cardiovascular disease

Among PUFA, n-3 PUFA are known to exert cardioprotective effects and are thus increasingly being used in the prevention and management of several CVD risk factors such as hypertension, dyslipidemia and metabolic syndrome (Yashodhara *et al.*, 2009). Cardiovascular benefits from n-3 PUFA are mediated through several aspects, i.e. modifications of the lipoprotein profile, essentially by reducing TG levels, TC levels and by increasing the LDL particle size (Sanders *et al.*, 1997; Kelley *et al.*, 2007). Furthermore, n-3 PUFA have been shown to reduce tumour necrosis factor-alpha (TNF-alpha) and interleukin-1 levels, which are known inflammatory cytokines involved in atherosclerosis (Caughey *et al.*, 1996). In addition, n-3 PUFA are associated with reduced platelet aggregation (von Schacky, 2000), anti-arrhythmic effects (Kang & Leaf, 2000) and improved endothelial dysfunction (Goodfellow *et al.*, 2000), exerting cardioprotective effects.

1.3.2. Effects of omega-3 polyunsaturated acids on lipid and lipoprotein metabolism

Hypertriglyceridemia is a known risk factor for atherosclerosis (Groot *et al.*, 1991; Austin, 1998); a number of studies have confirmed that n-3 PUFA can reduce plasma TG. Dietary supplementation of n-3 PUFA to humans has been shown to reduce plasma VLDL and TG levels compared to dietary supplementation of safflower oil rich in n-6 PUFA (Fisher *et al.*, 1998; Chan *et al.*, 2002). Similarly, a 24% reduction in fasting TG levels and 92% reduction in large VLDL particles were reported in a double blind

randomized study after consuming 7.5 g of DHA oil/day for 45 days in hypertriglyceridemic men (Kelley *et al.*, 2007).

Animal studies have also demonstrated the TG lowering effects of n-3 PUFA; by increasing beta oxidation in the liver (Yamazaki *et al.*, 1987; Halminski *et al.*, 1991) and by decreasing the delivery of non-esterified fatty acids to the liver (Otto *et al.*, 1992). These mechanisms decrease plasma TG levels by reducing the substrate availability for TG synthesis. Moreover, n-3 PUFA have also been shown to lower TG synthesis by reducing the diglyceride acyltransferase (DGAT) activity which is the rate limiting enzyme of TG synthesis (Geelen *et al.*, 1995).

Increased LDL-cholesterol levels are well known to be associated with an increased risk of CVD (Mensink, 2011). However, cardioprotective effects of n-3 PUFA are not associated with decreasing the plasma LDL levels, but by increasing the LDL particle size as smaller LDL particles are more atherogenic and larger particles are less atherogenic. Previous studies have reported an increase in the diameter of LDL-cholesterol particles after consumption of n-3 PUFA in humans with hyperlipidemia (Contacos *et al.*, 1993; Kelley *et al.*, 2007). A 0.25 nm increase was demonstrated in the LDL particle size in hyperlipidemic men after consuming 4 g of purified DHA for 6 weeks, although an 8% increase in the LDL cholesterol levels had also been observed in hyperlipidemic men (Mori *et al.*, 2000). In addition, it was suggested that larger amounts of fish oil could lower LDL-cholesterol concentrations (Harris *et al.*, 1983).

Another known cardioprotective mechanism by which n-3 PUFA could lower cholesterol synthesis is by down regulating sterol regulatory element-binding protein-1 (SREBP-1), a transcription factor that plays a vital role in cholesterol, fatty acid, and TG metabolism. A study in LDL-r knockout mice reported that dietary n-3 PUFA, essentially EPA and DHA, markedly decreased plasma lipid levels by suppressing the activity of hepatic fatty acid synthesis; this was due to reduction in the mRNA level of fatty acid synthase (FAS) enzyme that was mediated via the reduction in SREBP-1 expression (Vasandani *et al.*, 2002). Recent studies, however, suggest that a proper n-6 to n-3 PUFA ratio is more beneficial in terms of reducing CVD risk than the higher intake of n-3 PUFA alone (Simopoulos, 2002; Simopoulos, 2008).

1.3.3 Significance of the omega-6 to omega-3 polyunsaturated fatty acid ratio in cardiovascular disease

Mammals cannot convert n-6 PUFA to n-3 PUFA as they lack the converting enzyme n-3 desaturase (Harris *et al.*, 2008). Since these two classes of essential fatty acids (EFA) are not inter-convertible and are metabolically and functionally different, they often exert opposing physiological functions. Thus, it is important to have both of these EFAs in the diet. Elongation and desaturation of LA and ALA result in their biologically active forms of longer chain derivatives such as AA, EPA and DHA. Since these EFAs compete for the same enzyme system for their conversion, an optimum balance is required for proper physiological functioning (Wijendran & Hayes, 2004; Harris *et al.*, 2008)

Both n-3 and n-6 PUFA act as primary substrates for eicosanoids, which are signalling molecules, derived from the oxidation of fatty acids and further production of prostaglandins, thromboxanes, and leukotrienes (Youdim *et al.*, 2000; Jordan, 2010). Biologically active eicosanoids from AA, an n-6 PUFA, are pro-inflammatory, prothrombotic, and generally promote atherosclerosis, while eicosanoids derived from EPA and DHA, the n-3 PUFA, have been shown to be anti-inflammatory anti-thrombotic, and have protective effects against atherosclerosis (Greenberg *et al.*, 2008; Ott *et al.*, 2011). It has been stated that EPA and DHA are produced more efficiently than AA, under higher levels of dietary ALA (Cetin *et al.*, 2009). Thus, higher dietary intake of n-3 PUFA are recommended for many pathological conditions such as CVD and autoimmune diseases in order to reduce the production of prostanoids derived from AA (Cetin *et al.*, 2009). Although it has been shown that the desaturation and elongation processes favour n-3 PUFA over n-6 PUFA (Hagve & Christophersen, 1984), a higher consumption of n-6 PUFA diets shifts the pathway in favour of the production of more n-6 PUFA derivatives (Jordan, 2010). Thus, it has been recommended that a lower n-6 to n-3 PUFA ratio is beneficial in lowering the risk of CVD (Sanders *et al.*, 1997; Simopoulos, 2008).

Many human studies have reported beneficial effects of a lower dietary n-6 to n-3 PUFA ratio on lipid and lipoprotein metabolism. A decrease in both fasting and postprandial plasma TG levels were reported in older men and women fed a lower n-6 to n-3 PUFA ratio compared to a higher n-6 to n-3 PUFA ratio (Moore *et al.*, 2006; Sanders *et al.*, 2006). Similarly, reduction in plasma TG levels and favourable changes in LDL

size were associated with the lower n-6 to n-3 PUFA ratio where subjects were fed n-6 to n-3 ratio of 3:1, 5:1 and 10:1 (Griffin *et al.*, 2006). In addition, an in vivo study confirmed that platelet aggregation was decreased, which is a known risk factor for atherosclerosis, as the dietary n-6 to n-3 PUFA ratio was decreased (Freese *et al.*, 1994). It has been shown that the potential CVD benefit of ALA was achieved only when the dietary LA is reduced concurrently rather than when fed higher LA levels in male pigs (Ghosh *et al.*, 2007). Similarly, a study in apoE-/- LDLr-/- double knockout mice reported a significant reduction in plasma LDL-cholesterol levels after feeding a low dietary n-6 to n-3 PUFA ratio (0.29) compared to a higher dietary ratio between 1.43-8 (Yamashita *et al.*, 2005). It is therefore apparent that the n-6 to n-3 PUFA ratio plays an important role in development of CVD in adult life.

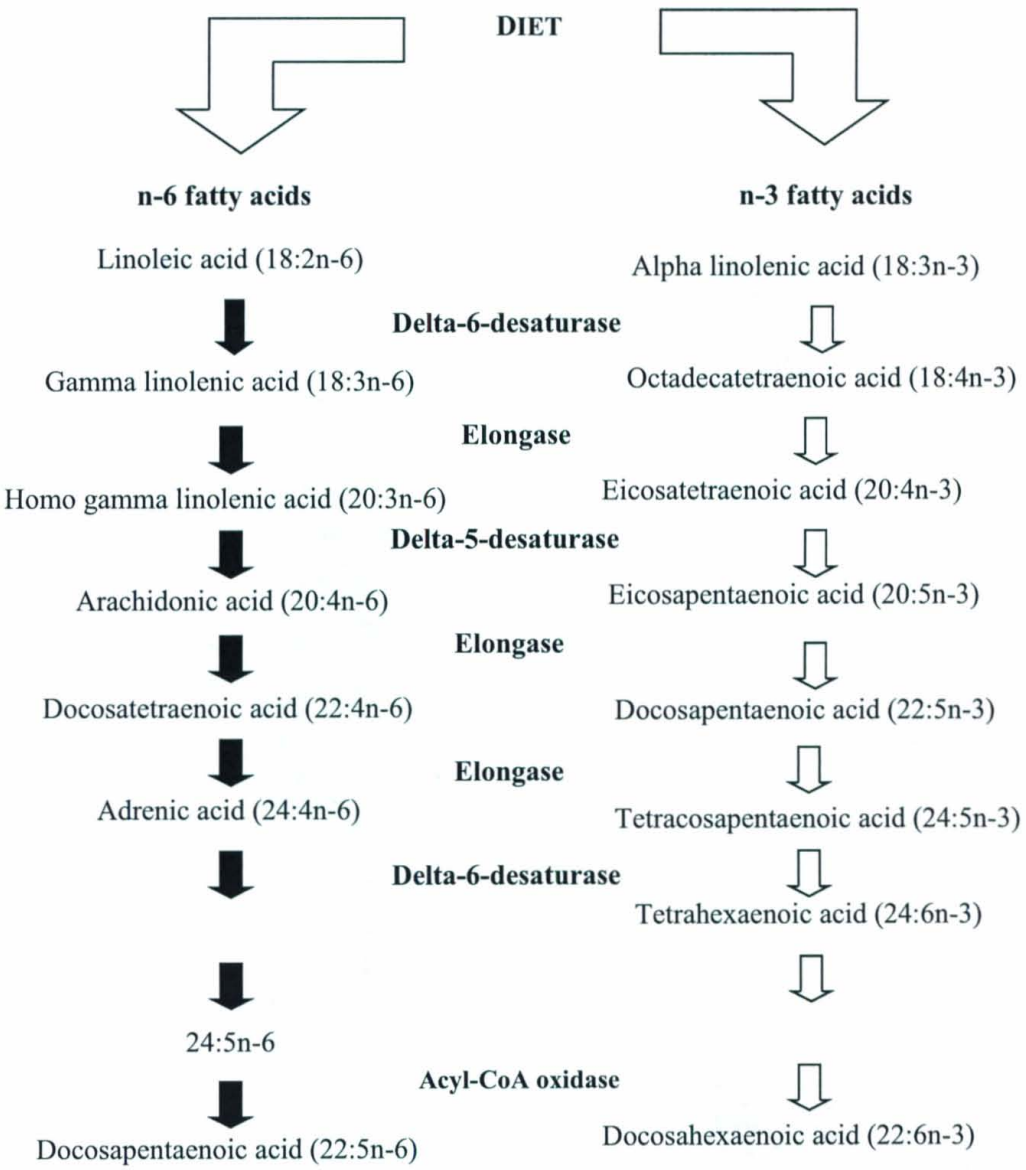


Figure 1.2: Elongation and desaturation of omega-6 and omega-3 polyunsaturated fatty acid

Modified from (Gao *et al.*, 2011)

1.4. Foetal Origins Hypothesis

The foetal origins hypothesis states that the conditions *in-utero* have a programming effect on the foetal physiology and metabolism (Barker *et al.*, 1990; Barker, 1997). This was originally proposed by Barker and according to his theory, if a foetus is deprived of adequate nutrient supply during the gestation period, it will be irrevocably programmed in order to survive in the adverse conditions, predisposing the offspring to develop diseases in later life. Preliminary epidemiological studies carried out by Barker provided the initial evidence supporting the foetal origins hypothesis. These studies showed a relationship between maternal under-nutrition, low birth weight and development of adult diseases such as dyslipidemia (Barker *et al.*, 1993), hypertension (Barker *et al.*, 1990) and insulin resistance (Phillips *et al.*, 1994). All these studies highlighted the importance of maternal nutrition on the onset of CVD in offspring's later life. Thus, it has been suggested that low birth weight was a result of maternal under-nutrition during the critical period of the development of the foetus. Subsequent studies from Australia and South India on poor nutrition have further confirmed the strong relationship between maternal under-nutrition and high risk of developing CVD in the offspring's later life (Fall *et al.*, 1998; Hoy *et al.*, 1999).

It is suggested that animals are capable of developing a variety of ways of adapting to the environment. Small size and slow metabolism facilitate an animal's ability to thrive in adverse circumstances, whereas large size and rapid metabolism facilitate reproductive success when the resources are more abundant (Hales & Barker, 2001; Rajaleid *et al.*, 2011; Wells, 2011). Often, these characteristics are induced in early life,

depending on the environment where the mother is being exposed. However, once an individual is adapted to one environment, it may be at risk when exposed to a different environment; this phenomenon is called developmental plasticity (Bateson *et al.*, 2004).

Observations in some human studies, where no relationship was reported between maternal under-nutrition and development of CVD in offspring's adult life (Stanner *et al.*, 1997; Huxley *et al.*, 2002), have been explained on the basis of the "thrifty genotype hypothesis". This hypothesis explains the selection of a "thrifty gene" and an increase in the body's capacity to store fat during periods of food shortage. This predisposes the individual to an increased risk of developing insulin resistance when food becomes abundant. This adaptation could be deleterious if there is a difference between *in-utero* and postnatal nutrition, which predisposes the individual to diseases in later life (Neel, 1962). Nonetheless, foetal programming is not an expression of pathological progression; rather it is an adjustment made during foetal development to ensure adaptation to postnatal life and sustenance of good health (Barker, 1990; Gluckman & Hanson, 2007; Gluckman *et al.*, 2008). Considering these theories as the base, the effect of maternal under-nutrition on development of CVD in the offspring's later life has been extensively studied over the last few decades (Garofano *et al.*, 1997; Vickers *et al.*, 2000). However, in recent years, the attention has also been focused on excess maternal consumption of dietary fats, which is one of the major causes behind the risk for CVD.

1.5. Role of placenta in intrauterine fatty acid transfer

According to the foetal origins hypothesis, there are critical periods during gestation where nutrition plays a vital role in the development of the foetus leading to persistent alterations in adulthood. The foetus solely depends on the nutrients provided by the mother for its growth and development during intrauterine life. Among nutrients, PUFA are mainly provided to the foetal circulation by placental transport and the quality and quantity of PUFA reaching the placenta depend on the maternal diet and metabolism (Wittmaack *et al.*, 1995; Hanebutt *et al.*, 2008). During pregnancy, maternal metabolism is altered in order to support the fatty acid requirement of the foeto-placental unit. In addition, the placenta also plays a major role in determining the quality and quantity of PUFA levels in the foetal circulation through its transfer ability and metabolism (Hendrickse *et al.*, 1985; Berghaus *et al.*, 1998; Haggarty, 2002).

Previously it was reported that the fatty acid profile is different in the foetal circulation compared with that of the maternal, where a higher proportion of LC-PUFA and a lower percentage of their precursors, LA and ALA were observed in the umbilical artery (Crawford *et al.*, 1976; Benassayag *et al.*, 1999). This observation suggested the ability of the placenta to increase LC-PUFA percentages in foetal blood in order to support rapid foetal growth and central nervous system development. However, stable isotope experiments *in vitro* and *in vivo* showed that the higher accretion of AA and DHA in the foetus is due to the placental ability to preferentially transfer DHA and AA over ALA and LA into the foetal circulation (Ruyle *et al.*, 1990; Haggarty *et al.*, 1999; Larque *et al.*, 2003).

Recent studies have shown that specific transport proteins in the placenta lead to this preferential transfer of LC-PUFA to the foetus. These proteins are plasma membrane fatty acid binding proteins (FABPpm), fatty acid translocase protein (FAT), fatty acid transporter proteins (FATP) and intracellular fatty acid binding proteins (FABP) located in microvillus and endothelial membranes of foetal capillaries (Campbell *et al.*, 1998a; Campbell *et al.*, 1998b). The lack of specificity for a particular type of fatty acid and the location of FAT and FATP on both sides of trophoblast cells in the placenta allows fatty acids to transport bidirectionally. However, for its exclusive location on the maternal side and its preference for LC-PUFA, FABPpm seems to be implicated in their sequestration in the placenta (Dutta-Roy, 2000). Besides, fatty acids are also transported via simple diffusion across the concentration gradient to a certain extent (Hanebutt *et al.*, 2008).

Once in the placenta, part of the fatty acids are oxidized in mitochondria to produce energy and the rest are incorporated into phospholipids. Also a certain proportion of longer chain n-6 and n-3 PUFA are converted to prostaglandins by cyclooxygenases. The residual fatty acids are elongated, desaturated and then released into the foetal circulation (Coleman & Haynes, 1987; Thorburn, 1991). This placental metabolism of fatty acids is also a reason for increased LC-PUFA levels in the foetal circulation compared with that of the maternal circulation. All of these data suggest that adequate LC-PUFA transfer to the foetal side is ensured via placental fatty acid transport proteins in order to facilitate foetal growth and development. Since, foetal fatty acids are correlated to the maternal levels, it is important to maintain proper LC-PUFA status in the mother through diet in order to ensure optimal health of the offspring.

1.5.1. Role of maternal diet on fatty acid composition of milk

The maternal dietary fatty acid composition is one of the major factors which determines not only the quality of fatty acids transferred across the placenta but also the quality of fatty acids secreted in the breast milk. The fatty acids in milk mainly originate from one of three sources: mobilization of endogenous stores of fatty acids, synthesis of fatty acids by the liver or breast tissue, and derivation from the diet (Insull *et al.*, 1959). It has been shown that high maternal SFA and PUFA intake were reflected in breast milk fatty acids (Mellies *et al.*, 1979; Finley *et al.*, 1985; Helland *et al.*, 2001). A high DHA supplementation to women during the lactation period also showed an elevated DHA content in milk and in plasma of the infant (Jensen *et al.*, 2000). Similarly, a number of animal studies have demonstrated that there was a significant correlation between the maternal diet and the breast milk fatty acid composition where animals were fed different n-3 to n-6 PUFA ratios in rats (Jen *et al.*, 2009) and in mice (Kagohashi *et al.*, 2010). Furthermore, it has been reported that n-6 to n-3 PUFA ratios in breast milk and erythrocyte (RBC) composition was nearly the same as that of the maternal diet. All of these studies have demonstrated that breast milk fatty acid composition is greatly altered by the maternal diet which plays a significant role in determining the health of the offspring in later life (Innis, 2005).

1.6. Maternal dietary fatty acids and foetal programming

Studies on the evolutionary aspect of the human diet indicate that major changes had taken place in the quality and quantity of dietary fats during last 200 years with a significant increase in total and SFA intake (Simopoulos, 1991; Simopoulos, 2006). Therefore, a high consumption of dietary SFA and a low PUFA intake may have increased the prevalence of CVD. It is therefore, important to focus on the role of dietary fats in developmental origins of CVD.

1.6.1. Effect of maternal dietary saturated and polyunsaturated fatty acids on lipid and lipoprotein metabolism of the offspring

Previous studies carried out in our laboratory using C57BL/6 mice have shown that, feeding a high fat diet (20% W/W) rich in SFA during the perinatal period was deleterious compared to a diet rich in n-6 PUFA (Chechi & Cheema, 2006). Elevated total- and LDL-cholesterol levels were observed in 11 week old offspring from dams fed SFA during the perinatal period, while high HDL-cholesterol levels were observed in offspring of dams fed n-6 PUFA (Chechi & Cheema, 2006). In addition, it was reported that higher LDL-cholesterol levels were associated with reduced LDL-r expression in female offspring obtained from mothers fed a diet rich in SFA (Chechi *et al.*, 2009). Therefore, maternal dietary exposure to SFA may be associated with an increased risk of CVD in the offspring by inducing dyslipidemia.

Other studies have also confirmed deleterious effects of maternal consumption of SFA, and favourable effects of maternal consumption of PUFA on offspring's lipid

metabolism. Khan *et al.*, (2003) reported higher plasma TC levels in male offspring and TG levels in female offspring at 360 days of age, when dams were fed a high fat diet rich in SFA during the perinatal period compared to offspring of dams fed a control diet in rats. Both male and female offspring were reported to have low levels of HDL-cholesterol suggesting an adverse effect of perinatal SFA exposure (Khan *et al.*, 2003). Similarly, higher TC levels and lower HDL-cholesterol levels were observed in 160 day old rat offspring obtained from dams fed a high fat diet rich in SFA compared to offspring from dams fed a control diet (Ghosh *et al.*, 2001). Contrary to maternal exposure of a high fat diet rich in SFA, a diet rich in EPA and DHA, resulted in lower TC level and TG levels in macrosomic Wistar rats at 2 and 3 months compared to the offspring obtained from dams fed a control diet (Yessoufou *et al.*, 2006). These studies on foetal programming suggest that the quality of dietary fatty acids in maternal diet plays a significant role in predisposing the offspring to develop CVD in later life.

Docosahexaenoic acid and AA are rapidly incorporated in the retina and nervous tissue during last trimester of pregnancy and lactation period (Clandinin *et al.*, 1980a; Martinez, 1992) and it has been estimated that the human foetus needs 50 mg/kg/day of n-3 PUFA and 400 mg/kg/day of n-6 PUFA (Clandinin *et al.*, 1980b). Therefore, many studies on foetal programming have investigated the developmental effects of n-3 PUFA on neurocognitive and visual functions of the offspring. Perinatal n-3 PUFA supplementation showed beneficial effects on visual acuity (Birch *et al.*, 1992) and increased IQ levels (Helland *et al.*, 2001) of the human offspring. However, to date, there is little evidence of

developmental effects of maternal n-3 PUFA on offspring's lipid and lipoprotein metabolism.

1.6.2. Effect of maternal omega-3 polyunsaturated fatty acid on the lipid and lipoprotein metabolism of the offspring

As discussed in the above section (1.3.2), the cardioprotective effects of n-3 PUFA in adults are firmly established. A handful of studies have investigated the role of maternal n-3 PUFA supplementation on the regulation of lipid and lipoprotein metabolism of the offspring. Low plasma TG levels and high HDL-cholesterol levels were reported in 11 week old rat offspring of dams who were supplemented with DHA compared to dams fed a control diet (Gong *et al.*, 2009). Similarly, low VLDL and LDL levels were reported in rat offspring where diabetic dams were fed an EPA/DHA diet compared to diabetic dams fed a diet rich in vegetable oil (Soulimane-Mokhtari *et al.*, 2008). Another study in diabetic rats demonstrated that supplementation of EPA/DHA during pregnancy decreased serum TG and cholesterol levels in macrosomic pups in adulthood, at day 60 and day 90 (Soulimane-Mokhtari *et al.*, 2005). Thus, maternal supplementation of EPA and DHA assisted in improving lipid anomalies in the offspring. Suggested plausible mechanisms of improvement of TG levels in the offspring of dams fed n-3 PUFA included reducing the substrates for TG synthesis through enhancement of mitochondrial beta oxidation, which was supported by an increase in carnitine palmitoyltransferase I (CPT-1) mRNA expression in the rat offspring of dams supplemented with DHA (Gong *et al.*, 2009). CPT-I is the rate-limiting step of the carnitine palmitoyltransferase system, catalyzing the transfer of the acyl group from

coenzyme A to carnitine to form acylcarnitine, which is an essential step in the beta-oxidation of fatty acids.

Although the above data support a beneficial effect of maternal n-3 PUFA supplementation on offspring, detrimental effects of dietary n-3 PUFA supplementation also have been reported. Recently it was shown that excessively rich or deficient levels of n-3 PUFA in the maternal diet led to shortened life span of the offspring (Church *et al.*, 2010), which was suggested to be due to adverse effects of excessive n-3 PUFA during the perinatal period causing "nutritional toxicity". Although the mechanisms of these programming effects are still unexplained, above data draw the conclusion that any change, essentially inadequate or excess fatty acid supply during critical periods of development can affect cell growth and differentiation leading to health issues in the offspring in later life (Georgieff & Innis, 2005). Therefore, it may be important to consider the ratio of n-6 to n-3 PUFA in the maternal diet as opposed to simply increasing the intake of n-3 PUFA.

1.6.3. Effect of maternal omega-6 to omega-3 polyunsaturated fatty acid ratio in body weight and body length of the offspring

According to the developmental origins of diseases hypothesis, adverse intrauterine conditions lead to disproportionate foetal growth or low birth weight (Barker, 1995). Some studies have suggested that the perinatal dietary n-6 to n-3 PUFA ratio is significant in determining health outcomes compared to the individual supplementation of these fatty acids (Simopoulos, 2002; Simopoulos, 2008).

Growth and behavioural retardation has been reported in the offspring of dams fed a diet with a n-6 to n-3 PUFA ratio of (0.32) compared to offspring from dams fed a n-6 to n-3 PUFA ratio of four (Wainwright *et al.*, 1999). The authors reported a 12% decrease in body weight at weaning suggesting that an imbalance of the dietary n-6 to n-3 PUFA ratio in the maternal diet deleteriously affected the growth rate of the offspring. Others have also reported a shorter body length in the offspring of dams fed either an extremely low or a very high n-6 to n-3 PUFA ratio (Korotkova *et al.*, 2002; Korotkova *et al.*, 2004; Santillan *et al.*, 2010). Some of the programming effects are not reversible as significantly lower DHA levels were detected in the hypothalamus of offspring from dams fed a high n-6 to n-3 PUFA ratio after switching to a diet rich in ALA during the postnatal period (Kodas *et al.*, 2002; Li *et al.*, 2006). The above studies collectively suggest that maternal exposure to extreme n-6 to n-3 PUFA ratios decreased growth rate of the offspring thereby demonstrating the importance of maintaining proper dietary n-6 to n-3 PUFA ratio during pregnancy.

Low birth weight was found to be associated with an increased risk of CVD (Barker *et al.*, 1989; Frankel *et al.*, 1996; Rich-Edwards *et al.*, 1997). Several studies have shown the impact of maternal n-6 to n-3 PUFA ratio on the body weight and body length of the offspring, thus a balance between the n-6 to n-3 PUFA in the maternal diet may be an important factor in the outcome of CVD in the offspring. To date, there are no studies investigating the impact of maternal n-6 to n-3 PUFA ratio on the regulation of the offspring's lipid and lipoprotein metabolism which may be related to the future development of CVD.

1.7. Evolutionary aspects of dietary fatty acids

Today's diet has undergone nutritional transition, which may be a major factor for the increased incidence of CVD. The dietary shift from increased complex carbohydrate and high fiber content to an energy-dense diet containing high levels of SFA, was a major factor contributing to the dramatic increase in CVD (Drewnowski & Popkin, 1997). Recent recommendations are to replace atherogenic SFA diet with PUFA (Mozaffarian *et al.*, 2010; Martikainen *et al.*, 2011), which has led to an increased consumption of n-6 PUFA in the Western world (Simopoulos, 2006; Simopoulos, 2008). Although considerable amounts of plant derived ALA are consumed through walnuts, flax seeds, canola oil, spinach and Brussels sprouts in a typical Western diet, bioconversion of ALA to EPA and DHA is low (Jordan, 2010). In contrast, preformed DHA and EPA are consumed to a lower extent in a Western diet (Kris-Etherton *et al.*, 2002; Innis, 2011). Thus, dietary shift from SFA to n-6 PUFA may not facilitate the reduction of the incidence of CVD; instead, it may aid in the development of chronic diseases due to inadequate n-3 PUFA consumption and lead to an imbalance in the dietary n-6 to n-3 PUFA ratio. It has been estimated that the PUFA ratio of n-6 to n-3 in the present Western diet is approximately 20-30:1 (Simopoulos, 2008; Wan *et al.*, 2010; Gomez Candela *et al.*, 2011) whereas humans evolved on a ratio of 1:1 (Kris-Etherton *et al.*, 2000; Simopoulos, 2008; Wan *et al.*, 2010; Gomez Candela *et al.*, 2011). It has been suggested that, the appropriate ratio of dietary n-6 to n-3 PUFA for optimal body function is around 3-5:1 (Kris-Etherton *et al.*, 2000).

The Food and Agricultural Organization reported that consumption of vegetable oil has increased from 13 g person^{-1}day^{-1} to 30 g person^{-1}day^{-1} in developed countries over the period of 1961-1963 to 2001-2003 (Wolmarans, 2009). Accordingly, epidemiological studies conducted in Canada and Europe have confirmed that the majority of pregnant women do not consume adequate amounts of n-3 PUFA, especially DHA (Loosemore *et al.*, 2004; Denomme *et al.*, 2005). Given that the dietary n-6 to n-3 PUFA ratio in the current North American diet is 25-30:1, it is most likely that this is the n-6 to n-3 PUFA ratio consumed by women of child bearing age and during pregnancy and lactation in Western society.

1.8. Rationale of the study

N-3 and n-6 PUFA have independent effects on metabolic pathways, thus an optimum balance is required between these classes of PUFA to maintain whole body homeostasis. The present study was designed to investigate whether altering n-6 to n-3 PUFA ratios in the maternal diet has programming effects on the regulation of lipid and lipoprotein metabolism of the offspring as altered lipid metabolism is a risk factor for the development of CVD. C57BL/6 mice are highly susceptible to diet-induced hyperlipidemia and atherosclerosis (Schreyer *et al.*, 1998). Our laboratory has established C57BL/6 mice as an animal model to study maternal dietary fat-mediated programming of lipid and lipoprotein metabolism in the offspring (Chechi & Cheema, 2006; Chechi *et al.*, 2009; Chechi *et al.*, 2010). Thus, C57BL/6 mice were used as an animal model in the current study to investigate the effects of various maternal dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism of the offspring at weaning. Dietary

ratios of n-6 to n-3 PUFA were 5:1, 15:1 and 30:1; the 30:1 ratio represents the current ratio in a Western diet, 5:1 ratio represents the recommended , while 15:1 ratio is in the middle for a dose response effect of maternal dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism of the offspring at weaning. Our previous studies have shown gender specific regulation of lipid and lipoprotein metabolism in C57BL/6 mice (Chechi & Cheema, 2006; Chechi *et al.*, 2009), thus both male and female offspring were used to identify gender specific effects of maternal diets varying in n-6 to n-3 PUFA ratios. The contribution of post-natal diet on development of CVD can be controlled by altering the dietary habits of an individual. However, it is important to determine whether maternal diets during gestation and lactation will affect the regulation of lipid and lipoprotein metabolism of the offspring at weaning. The findings from this study design may assist in designing proper dietary regimen for pregnant and lactating mothers (pre-weaning diets) to prevent the onset of metabolic diseases in the offspring in later life. The focus of the current study was thus to investigate the effects of maternal diet on the lipid and lipoprotein metabolism of the offspring at weaning thereby eliminating the effect of post-weaning diets.

The mother is the sole provider of nutrients for the developing foetus, while breast milk is a nutrient source after birth. Determination of total milk fatty acid composition of dams fed various n-6 to n-3 PUFA ratios would indicate the effect of breast milk fat composition on changes in the lipid and lipoprotein metabolism of the offspring. The PL fatty acid composition of RBCs are known to be correlated with dietary fatty acid compositions (Witte *et al.*, 2010) and is commonly used as a biomarker for n-3 PUFA

status in the body (Harris *et al.*, 2009). Therefore, analyses of the offspring RBC PL fatty acids will assist in understanding the degree of incorporation of maternal dietary fatty acids intake in the body. Total milk fatty acid analysis and RBC PL analysis of various dietary groups were therefore determined in the current study. The current study was designed to address the above research questions with the specific aims and underlying hypotheses as stated below.

1.9. Objectives and hypotheses

Aim 1: (one month feeding study) - To investigate whether feeding a high fat diet with varying n-6 to n-3 PUFA ratios will alter the regulation of metabolic parameters in C57BL/6 female mice after 4 weeks of feeding.

Hypothesis: A diet high in n-3 PUFA is known to reduce plasma TG levels compared to a diet high in n-6 PUFA. It was hypothesized that a lower dietary n-6 to n-3 PUFA ratio will lower plasma lipid levels of females compared to a higher dietary n-6 to n-3 PUFA ratio after 4 weeks of feeding.

Aim 2: (Maternal nutrition study) – To investigate the effects of altered maternal dietary n-6 to n-3 PUFA ratios on breast milk fatty acid composition.

Hypothesis: Varying the maternal n-6 to n-3 PUFA ratios will alter breast milk fatty acid composition to reflect the dietary composition of the mothers.

Aim 3: To investigate the effects of altered maternal dietary n-6 to n-3 PUFA ratios on the RBC PL fatty acid composition of the offspring.

Hypothesis: Varying the maternal n-6 to n-3 PUFA ratios will alter RBC PL fatty acid composition to reflect the dietary composition of the mothers.

Aim 4: To investigate the effects of a high fat maternal diet with varying ratios of n-6 to n-3 PUFA on the lipid and lipoprotein metabolism of the offspring at weaning.

Hypothesis: A lower maternal dietary n-6 to n-3 PUFA ratios will lower plasma lipid levels of the offspring at weaning compared to a higher maternal dietary n-6 to n-3 PUFA ratio.

Findings from the current study will establish whether a lower maternal n-6 to n-3 PUFA dietary ratio will lower plasma lipid levels of the offspring at weaning.

Chapter 2: Methodology

2.1 Diets and Animals

2.1.1 Diets

A base semi-synthetic diet designed specifically to permit the control of fat level at 20% w/w was obtained in powdered form with the fat source omitted (MP Biomedicals, OH, USA). The macronutrient composition of the semi-synthetic diet is given in Table 2.1. Fish oil (Menhaden) was obtained from Sigma-Aldrich (USA), whereas lard, safflower oil and extra-virgin olive oil were obtained from a local supermarket to prepare three different oil mixtures with n-6 to n-3 PUFA ratios of 5:1, 15:1, and 30:1. The amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and total polyunsaturated fatty acids (PUFA) was kept constant. Gas-liquid chromatography (GLC) was utilized to determine the fatty acid composition of the oil mixtures and to confirm the proper n-6 to n-3 PUFA ratios in all experimental diets, which is given in Table 2.2. The high-fat diets with different n-6 to n-3 PUFA ratios were prepared by mixing semi-synthetic powdered diets with the oil mixtures at 20% w/w and diets were kept frozen at -20°C under nitrogen.

2.1.2 Animals:

2.1.2.1 Feeding diets with varying omega-6 to omega-3 polyunsaturated fatty acid ratios to C57BL/6 female mice

Seven week old, female C57BL/6 mice were purchased from Charles River Laboratories (MA, USA). Animals were housed under controlled temperature (21 ± 1^{0}C) and humidity (35 ± 5%) in a single room with a 12-hour light/12-hour dark (7am–7pm,

light; 7pm-7am, dark) period cycle. Mice were fed commercial rodent chow pellets during one week of acclimatization. Mice were randomly divided into three groups and fed experimental diets (n-6 to n-3 PUFA ratios of 5:1, 15:1 and 30:1) for one month (n =8 per diet treatment). Animals were provided with deionized water and fresh food ad-libitum, every other day. Body weights were recorded once a week while food intake was recorded every other day.

After one month (30 days) of feeding, mice fasted overnight and were euthanized the next morning using isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4) and centrifuged immediately at 3000g, 4°C for 15 minutes to separate plasma. All biochemical analyses were performed within one week using fresh plasma stored on ice at 4°C. Various tissues and organs were also removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80°C until further analyses. 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.

2.1.2.2 Feeding diets with varying omega-6 to omega-3 polyunsaturated fatty acid ratios to female C57BL/6 mice during gestation and lactation to study their effect on the offspring metabolism

Seven week old C57BL/6 male and female mice were purchased from Charles River Laboratories (MA, USA). Female and male mice were housed in separate cages under controlled temperature (21± 1°C) humidity (35 ± 5%) conditions in a single room

with a 12-hour light/12-hour dark (7am–7pm, light; 7pm-7am, dark) period cycle. Mice were fed commercial rodent chow pellets during a one week acclimatization period. After this period, female mice were randomly divided into three groups and fed experimental diets that differed in n-6 to n-3 PUFA ratios (5:1, 15:1 and 30:1) for two weeks. One male was then introduced into each female cage for mating and was removed after 14-days. Pregnancy was confirmed by vaginal plug formation. Pregnant female mice were then housed in individual clean new cages until pups were born. After 2 weeks, cages were examined daily for the presence of litters. Once the litters were born, they were counted on postnatal day 1, after which the mothers and pups were not disturbed in order to prevent cannibalism that was previously noted in our laboratory with high fat diets. Mothers were continued on the experimental diets throughout the gestation and lactation periods. Fresh deionized water and food was provided ad-libitum daily. Body weight and food intake of dams were recorded each week during gestation and lactation. 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.

At the time of weaning (3 weeks after birth), pups were fasted over night. Body weight and body length of the pups were measured; pups were then euthanized using isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4) and centrifuged immediately at 3000g, 4°C for 15 minutes to separate plasma. Plasma was stored on ice at 4°C and all biochemical analyses were performed within one week. The remaining plasma was stored at -80°C for further analyses. Various tissues and

organs were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80°C until further analyses. At weaning, dams were milked after anesthetizing with pentobarbital (35mg/kg/ intraperitoneal (IP) / (0.1ml/100g) and stimulating milk secretion using oxytocin (4 IU/kg IP). Dams were then euthanized; blood and tissues were collected as described above.

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

Ingredients	Semi -synthetic diets (g/kg)
Casein	200
DL -methionine	3
Sucrose	305
Corn starch	190
Alphacel non-nutritive bulk	50
Vitamin mix$^{\Psi}$	11
Mineral mix*	40
Fat	200

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

$^{\Psi}$**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 Acid	5:1 Diet	15:1 Diet	30:1 Diet
		%w/w	
C14:0	1.26	0.39	0.11
C16:0	8.71	7.43	6.32
C18:0	2.67	4.53	5.35
∑ SFA	12.64	12.35	11.77
C16:1n7	2.41	0.09	0.36
C18:1n9 + C18:1n7	25.14	25.81	27.82
C20:1n9	0.61	0.54	ND
∑ MUFA	28.16	26.43	28.18
C18:2n6	47.86	57.03	57.73
C20:4n6	0.23	0.14	0.11
C18:3n6	0.10	0.04	0.04
C22:4n6	0.54	ND	0.09
∑ Omega-6	48.90	57.18	57.92
C18:3n3	0.78	0.64	0.55
C20:5n3	3.64	1.37	0.31
C22:6n3	3.19	1.16	0.39
C18:4n3	0.87	0.20	0.15
C22:5n3	0.63	0.32	0.46
C20:4n3	0.66	0.12	0.08
∑Omega-3	9.76	3.81	1.93
∑ PUFA	59.38	61.05	60.00
∑Omega-6/Omega-3	5.00	15.00	30.01

Lipids were extracted from various diets and the fatty acid composition was determined by gas chromatography. Abbreviations: ND= Not detected, 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 acid

2.2 Analyses of biochemical parameters

Plasma TC and TG concentrations were determined using cholesterol assay kit #234-60 and TG assay kit #236-17 (Genezyme Diagnostics, P.E., Canada). For the plasma TC assay, CE were hydrolyzed to FC by cholesterol esterase and FC was then oxidized to cholest-4-ene-3-one by cholesterol oxidase with a simultaneous production of hydrogen peroxide. The produced hydrogen peroxide couples with 4-aminoantipyrine and p-hydroxybenzoate, in the presence of peroxidase, yielding a chromogen with absorbance at 500 nm.

For the plasma TG assay, TG were hydrolyzed to glycerol and FFAs by lipase; glycerol was phosphorylated to glycerol-1-phosphate in the presence of ATP and glycerol kinase. Glycerol-1-phosphate was then oxidized by glycerol phosphate oxidase to yield hydrogen peroxide leading to oxidative coupling of p-chlorophenol and 4-aminoantipyrine. This produces a red colored quinoneimine dye complex which has a maximum absorbance at 520nm. The intensity of the color produced was taken as directly proportional to the concentration of TC and TG in the samples.

Non-HDL cholesterol was precipitated from plasma using kit #200-26A (DCL, P.E.I, Canada) and the supernatant was used for measuring HDL-cholesterol using total cholesterol assay kit #234-60 following the same principle as stated above .

Plasma LDL-cholesterol concentration was calculated using plasma TC, HDL-cholesterol, and TG concentration according to the method of Friedewald *et al.* (1972). Plasma FFA concentration was determined using kit# 999-34691 (Wako Chemicals Inc., USA). Coenzyme A (CoA) was acylated by the presence of serum non esterified fatty

acids in the presence of added acyl-CoA synthetase. Produced acyl-coA was oxidized by added acyl-CoA oxidase with the generation of hydrogen peroxide. This produced hydrogen peroxide permitting oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine in the presence of peroxidase to form a purple colored product, which was measured colorimetrically at 550 nm.

Plasma FC concentration was determined using kit# 435-35801 (Wako Chemicals Inc., USA). Free cholesterol in the serum was oxidized to cholesterolΔ^4-cholestenone by cholesterol oxidase which produced hydrogen peroxide. Hydrogen peroxide, 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl) aniline sodium and 4-aminoantipyrine then undergo oxidative condensation in the presence of peroxidase producing a blue color that was measured colorimetrically at 600 nm. Plasma CE was calculated by subtracting plasma FC values from plasma TC values. Fasting blood glucose concentrations were measured at the time of sacrifice using a commercially available glucometer (Lifescan Inc, CA, USA) after snipping the tail.

2.3 Fatty acid analyses

2.3.1. Fatty acid analyses of diets

To analyze the fatty acid composition of the oils used for diet preparation, lipids were extracted from safflower oil, fish oil, olive oil and lard using the method of Folch *et al.* (1957). Fatty acid methyl esters were then prepared by heating the samples 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 in order to increase

the volatility of the fatty acids (Arvidson & Olivecrona, 1962). All of the organic extractions were performed using hexane and water. Subsequently, the samples were placed at -20°C overnight to freeze the trapped water and were transferred to a new tube the following morning. The samples were then dried under nitrogen (N_2) gas and dissolved in 50 µl of carbon disulfide (Keough and Davis, 1979). The GLC parameters were set as: oven, 200°C; injector, 240°C; detector 260°C. GLC was ignited and allowed to run overnight prior to running samples in order to ensure that the baseline was stable. Samples were run for 60 minutes on an Omegawax X 320 (30 m x 0.32 mm) column from Supleco (Sigma-Aldrich, Canada) using a flame ionization detector. PUFA standards -2 and -3 (Sigma-Aldrich, Canada) were used as standards for identification of fatty acids by retention time.

Once the fatty acid compositions of four individual oils were determined using GC, total SFA, MUFA, n-6 and n-3 PUFA contents of each oil was calculated and fed into a mathematical package, "Maple" as a coefficient matrix. Data were analyzed to obtain the amount of each oil to be mixed in order to accomplish three different n-6 to n-3 PUFA ratios of 5:1, 15:1 and 30:1.

2.3.2. Fatty acid analyses of breast milk

Total lipids were extracted from breast milk using the method of Folch *et al.* (1957); and fatty acid methyl esters were prepared and the fatty acid composition was determined using GLC as described in 2.3.1.

2.3.3. Fatty acid analyses of red blood cell (RBC) membrane phospholipids (PL)

Total lipids were extracted from RBC using the method of Folch *et al.* (1957). Phospholipids were separated from total lipids using thin layer chromatography; lipid samples were dissolved in 100µl of chloroform and spotted on Whatman 250 µm layer, 20 x 20 cm flexible plates coated with silica gel (Catalogue no: 4420222, Whatman Ltd. UK). Phospholipids were separated using the solvent system hexane: ethyl ether: acetic acid (70:30:2 v/v) (Keenan *et al.*, 1982). Phospholipid spots at the point of origin were scraped and extracted using 2:1 chloroform:methanol. Fatty acid composition of total phospholipids was determined using GLC as described above.

2.4. Statistical analyses

The effect of diet on various biochemical parameters was analyzed using one-way analysis of variance (ANOVA) and a Newman-Keuls *post hoc* analysis was used to test significant differences among groups (Graph Pad Prism- Version 5.0). Values were expressed as group means ± SD. Differences were considered to be statistically significant if the associated P value was <0.05. Breast milk fatty acid and RBC fatty acid composition were expressed as weight percentage of the total extracted fatty acids. This percentage data for fatty acid compositions were then arcsine transformed before subjecting to statistical analysis in order to normalize the data distribution as data was skewed. Correlation coefficients (r) of dietary fatty acids vs. breast milk fatty acids and RBC phospholipids fatty acids vs. plasma lipids and lipoproteins were determined using Graph pad Prism software (Prism version 5.0).

Chapter 3: Results

3.1 Effects of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on regulation of metabolic pathways: One month feeding study

3.1.1. Body weight, food and caloric intake, plasma glucose and non-esterified fatty acid (NEFA) concentrations

Body weight, food and caloric intake, plasma glucose and NEFA concentrations of female mice fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios for one month are shown in Table 3.1. A significantly lower body weight was observed for females fed a 15:1 diet compared to females fed 5:1 and 30:1 diets ($p=0.008$), however, there was no difference between females fed 5:1 and 30:1 diets (Table 3.1). Furthermore, a significantly higher ovarian fat content was observed in 5:1 group while 15:1 group showed the lowest ovarian fat content ($p=0.0347$); the ovarian fat content in 30:1 group did not differ either from 5:1 or 15:1 groups.

Food intake and caloric intake did not differ significantly among various dietary groups. No significant differences were observed in NEFA concentrations and blood glucose concentrations among various dietary groups.

Table 3.1: Body weight, food intake, caloric intake, non-esterified fatty acid concentrations (NEFA) and glucose concentrations of female C57BL/6 mice fed experimental diets for one month

	5:1 Diet	15:1 Diet	30:1 Diet
BW (g)	21.67 ± 1.21^{a}	19.78 ± 1.49^{b}	21.95 ± 1.02^{a}
FI (g/week)	25.94 ± 1.50	28.87 ± 9.10	26.82 ± 6.00
CI/day	16.03 ± 0.90	17.84 ± 5.60	16.58 ± 3.70
Ovarian fat (g)	0.53 ± 0.21^{a}	0.30 ± 0.13^{b}	0.39 ± 0.07^{ab}
NEFA (mmol/L)	0.59 ± 0.13	0.54 ± 0.09	0.44 ± 0.08
Blood Glucose (mmol/L)	9.03 ± 2.55	7.40 ± 1.56	7.92 ± 1.87

Values are expressed as means ± SD, n = 8. Data were analyzed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences among various dietary groups having $p < 0.05$. BW, body weight; FI, food intake; CI, caloric intake.

3.1.2 Effects of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on red blood cell phospholipids fatty acid composition

The RBC PL fatty acid composition of female mice fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios for one month are shown in Table 3.2. No significant differences were observed in total SFA and total MUFA content among various dietary groups. The 5:1 diet group had lower stearic acid content compared to 15:1 and 30:1 diet

groups ($p=0.003$), however, there was no difference between the 15:1 and 30:1 groups. The eicosaenoic acid content was also lower in the 5:1 group compared to the 30:1 group ($p=0.04$); 15:1 group did not differ either from 5:1 or 30:1 group.

A significantly higher total n-6 PUFA ($p=0.002$) content was observed in 30:1 diet group compared to 5:1 diet group, however, 15:1 diet group did not differ from 5:1 or 30:1 diet groups. Amongst individual n-6 PUFA, AA and docosatetraenoic acid content was the lowest in 5:1 diet group, followed by 15:1 diet group compared to the 30:1 group ($p<0.0001$). However, a higher LA ($p<0.0006$) content was observed in 5:1 diet group compared to 15:1 and 30:1 diet groups.

The 5:1 dietary group had a significantly higher total n-3 PUFA content than 15:1 and 30:1 dietary groups ($p<0.0001$). Amongst individual n-3 PUFA, EPA, DPA and DHA contents were significantly higher in the 5:1 diet group compared to the 30:1 diet group. A significantly higher EPA content was observed in 5:1 diet group compared to 15:1 and 30:1 diet groups ($p=0.0007$) whereas DPA content was higher in both 5:1 and 15:1 diet groups compared to the 30:1 group ($p<0.0001$). DHA content was highest in the 5:1 diet group followed by 15:1 and 30:1 diet group ($p<0.0001$).

Table 3.2: Red blood cell phospholipid fatty acid composition of female C57BL/6 mice fed experimental diets for one month*

Diet	5:1 Diet	15:1 Diet (% w/w)	30:1 Diet
C14:0	0.21 ± 0.20	0.17 ± 0.11	0.19 ± 0.09
C16:0	19.95 ± 3.50	18.99 ± 1.82	20.30 ± 2.78
C18:0	18.84 ± 1.27^{b}	21.77 ± 3.01^{a}	22.53 ± 0.72^{a}
∑ SFA	38.99 ± 3.26	40.92 ± 4.57	43.02 ± 2.34
C18:1n9	11.19 ± 0.97	11.8 ± 1.18	11.43 ± 0.53
C18:1n7	1.49 ± 0.32	1.98 ± 0.80	1.50 ± 0.16
C20:1n9	0.4 ± 0.06^{b}	0.41 ± 0.06^{ab}	0.49 ± 0.07^{a}
∑MUFA	13.46 ± 1.11	14.38 ± 1.85	13.69 ± 0.62
C18:2n6	15.93 ± 1.07^{a}	14.31 ± 1.22^{b}	13.56 ± 0.53^{b}
C18:3n6	0.17 ± 0.02	0.15 ± 0.07	0.19 ± 0.02
C20:4n6	12.03 ± 1.31^{c}	16.10 ± 2.76^{b}	19.07 ± 1.03^{a}
C22:4n6	0.74 ± 0.13^{c}	1.37 ± 0.17^{b}	2.10 ± 0.14^{a}
∑ n-6 PUFA	28.87 ± 2.30^{b}	31.93 ± 4.02^{ab}	34.92 ± 1.60^{a}
C20:5n3	3.81 ± 1.6^{a}	1.46 ± 0.94^{b}	0.41 ± 0.10^{b}
C22:5n3	2.54 ± 0.48^{a}	2.01 ± 0.77^{a}	0.85 ± 0.30^{b}
C22:6n3	12.07 ± 1.13^{a}	8.81 ± 1.93^{b}	6.77 ± 0.78^{c}
∑ n-3 PUFA	18.66 ± 1.25^{a}	12.76 ± 2.25^{b}	8.37 ± 0.91^{c}

*Data are expressed as weight percentage of total extracted fatty acids after separation of phospholipids by thin layer chromatography. Statistical analysis was performed after

transforming the data using arcsine equation. Values are expressed as mean ± SD, n = 8. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences among various dietary groups where $p<0.05$ was considered significant.

3.1.3 Effect of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma lipid levels

Plasma TC and TG concentrations of females fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios for one month are shown in Figure 3.1. A higher TC concentration was observed in 30:1 group compared to 5:1 and 15:1 groups ($p=0.0046$), however, no significant difference was observed between 5:1 and 15:1 groups (Figure 3.1A). No significant difference was observed in plasma TG levels among various dietary groups (Figure 3.1B).

Plasma LDL-cholesterol concentration and HDL-cholesterol concentrations of females fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios for one month are shown in Figure 3.2. A higher LDL-cholesterol concentration was observed in 30:1 group compared to 5:1 and 15:1 groups ($p=0.002$) however, there was no difference between 5:1 and 15:1 groups (Figure 3.2A). No significant differences were observed in plasma HDL-cholesterol concentrations among various dietary groups (Figure 3.2B).

Plasma LDL/HDL ratio and CE concentration of females fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios for one month are shown in Figure 3.3. Similar to plasma LDL-cholesterol levels, a higher LDL/HDL ratio was observed in 30:1 dietary

group compared to 5:1 and 15:1 groups (p=0.007), however, no significant difference was observed between 5:1 and 15:1 groups (Figure 3.3A). The 30:1 diet group was associated with significantly higher CE concentrations followed by 15:1 and 5:1 groups (p=0.0008) (Figure 3.3B). However, no significant differences were observed in plasma FC levels (means ± SD; 5:1=0.23 ± 0.03 mmol/L, 15:1=0.23 ± 0.04 mmol/L, 30:1=0.24 ± 0.04 mmol/L) among the various dietary groups.

Figure 3.1: Plasma concentrations of (*A*) total cholesterol and (*B*) triglycerides of C57BL/6 female mice fed diets varying in n-6 to n-3 PUFA ratios for one month

Values are expressed as means ± SD, n = 8. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences between various dietary groups having $p < 0.05$.

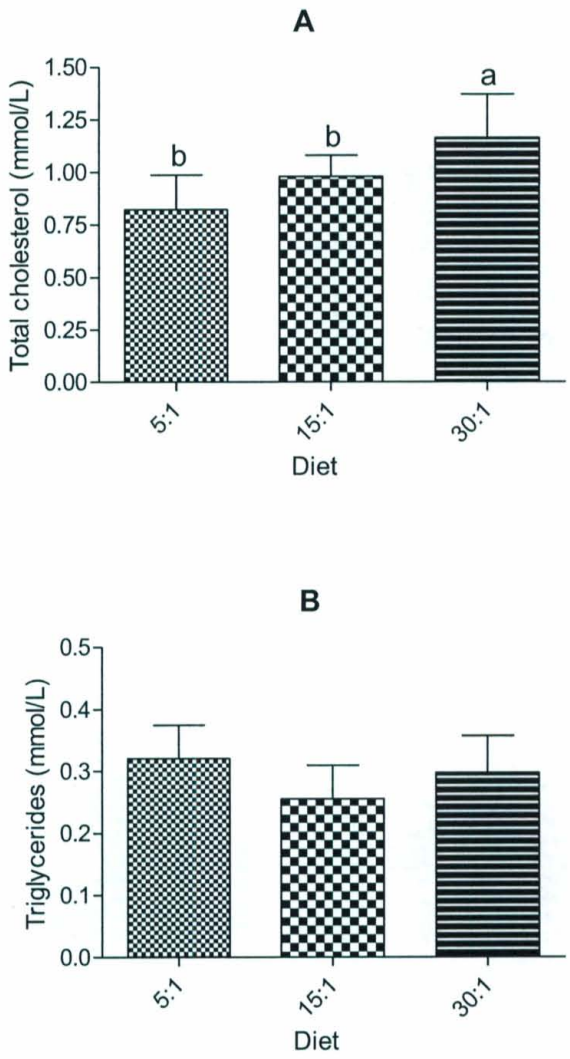


Figure 3.2: Plasma concentrations of (*A*) LDL-cholesterol and (*B*) HDL-cholesterol of C57BL/6 female mice fed diets varying in n-6 to n-3 PUFA ratios for one month

Values are expressed as means ± SD, n = 8. Data were assessed using one-way ANOVA. Significant efffects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences having $p < 0.05$.

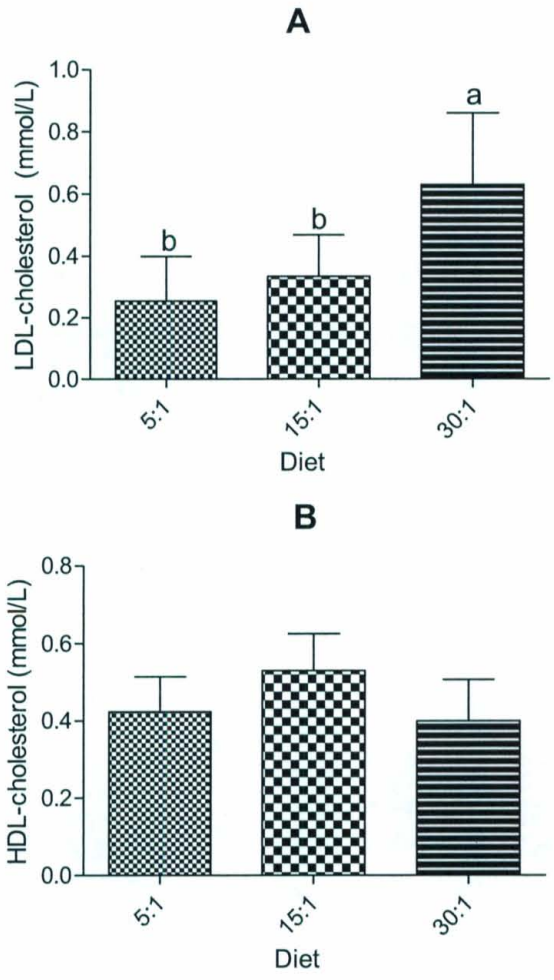
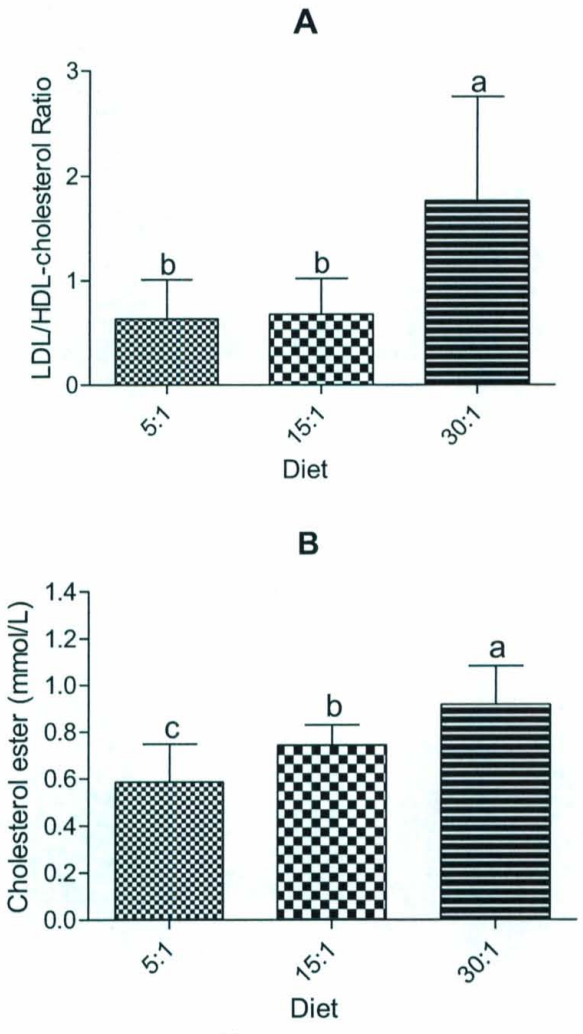


Figure 3.3: Plasma LDL/HDL cholesterol ratio (*A*) and cholesterol ester concentration (*B*) of C57BL/6 female mice fed diets varying in n-6 to n-3 PUFA ratios for one month

Values are expressed as means ± SD, n = 8. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences having $p < 0.05$.



3.2: Effects of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratio on regulation of metabolic pathways of the offspring

3.2.1: Maternal body weight, food and caloric intake, plasma glucose and NEFA concentrations

Body weight, food and caloric intake, plasma glucose and NEFA concentrations of females fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during pregnancy and lactation are shown in Table 3.3. No significant differences were observed in the body weight, food intake and caloric intake among various dietary groups (Table 3.3). NEFA concentration and fasting blood glucose concentration did not show significant differences among various dietary groups, however, the 30:1 group showed a trend ($p=0.22$) towards higher NEFA and blood glucose levels (Table 3.3).

3.2.1.1: Pregnancy rate, pup survival rate and sex ratio of the offspring at the time of weaning

Pregnancy rate was defined as the number of pregnant mice divided by the number of female mice used for mating (Table 3.4). Survival rate was defined as the number of live pups at weaning divided by the number of live pups at birth (Table 3.4). Sex ratio was defined as the number of male pups divided by the number of female pups obtained from a dietary group of mothers counted at the time of weaning (Table 3.4).

The pregnancy rates of the mice fed high n-6 to n-3 PUFA ratio diet (30:1) and low n-6 to n-3 PUFA ratio diet (5:1) were similar. However, pregnancy rate of mice fed n-6 to n-3 PUFA ratio of 15:1 diet was slightly lower. The survival rates of pups were

similar in the three dietary groups. Male to female ratio was almost 50% in pups obtained from mothers fed a 5:1 diet, whereas pups from mothers fed 15:1 and 30:1 diet demonstrated a male to female ratio of 2:1 and 1.4:1, respectively.

Table 3.3: Body weight, food intake, caloric intake, non esterified fatty acid concentrations (NEFA) and glucose concentrations of female C57BL/6 mice fed experimental diets during gestation and lactation

	5:1 Diet	15:1 Diet	30:1 Diet
BW (g)	31.52 ± 0.33	29.76 ± 3.38	27.56 ± 2.5
FI (g/week)	27.95 ± 5.40	26.04 ± 4.80	28.07 ± 7.80
CI (kcal/day)	22.56 ± 3.90	21.02± 4.10	22.66 ± 6.30
NEFA (mmol/L)	0.50 ± 0.22	0.44 ± 0.11	0.59 ± 0.19
Blood Glucose (mmol/L)	5.32 ± 1.72	5.61 ± 1.27	6.12 ± 0.95

Values are expressed as means ± SD, n = 4. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. BW, body weight; FI, food intake; CI, caloric intake.

Table 3.4: Pregnancy rate, pup survival rate and sex ratio of the offspring at the time of weaning

	5:1 Diet	15:1 Diet	30:1 Diet
Pregnancy Rate (%)	87.50	76.92	87.50
Pup Survival Rate (%)	80 ± 1	83 ± 1	81 ± 2
Male/Female Ratio	13/11	22/13	18/13
Litter size	7 ± 1	9 ± 1	9 ± 1

3.2.2: Effects of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratio on breast milk total fatty acid composition

Breast milk total fatty acid composition of females fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation is shown in table 3.5. No significant differences were observed in total SFA and total MUFA content among the various dietary groups. Similarly, no significant differences were observed among groups for the individual SFA and MUFA.

No significant differences were observed in total n-6 PUFA content among various dietary groups. Amongst individual n-6 PUFA, AA content was higher in 15:1 and 30:1 group compared to 5:1 group ($p=0.004$), however, there was no difference between 30:1 and 15:1 group.

A significantly higher total n-3 PUFA content was observed in 5:1 group followed by 15:1 and 30:1 groups ($p<0.0001$). Amongst individual n-3 PUFA, EPA ($p<0.0001$), DPA ($p<0.0001$) and DHA ($p=0.003$) contents were significantly higher in the 5:1 group followed by 15:1 group however, the levels were undetectable in the 30:1 group.

Although total PUFA content did not show a significant difference among various dietary groups, the n-6 to n-3 fatty acid ratio of 7:1 and 15:1 was observed in breast milk of 5:1 and 15:1 group. Conversely, a 42:1 n-6 to n-3 PUFA ratio was observed in breast milk of 30:1 group. Pearson's correlation coefficients (r) between dietary fatty acid composition and breast milk fatty acid compositions were 1.00, 0.87 and 0.87 for 5:1, 15:1 and 30:1 groups, respectively.

Table 3.5: Total breast milk fatty acid composition of female C57BL/6 mice fed experimental diets during pregnancy and lactation*

FA	5:1 Diet	15:1 Diet (% w/w)	30:1 Diet
C14:0	2.85 ± 1.64	3.41 ± 2.06	2.52 ± 1.28
C16:0	14.13 ± 2.50	14.95 ± 2.75	15.53 ± 1.99
C18:0	6.92 ± 4.53	5.84 ± 0.89	5.28 ± 0.48
∑ SFA	23.91 ± 3.84	24.19 ± 5.05	23.32 ± 2.83
C18:1n9	21.84 ± 6.65	29.51 ± 1.57	26.47 ± 2.05
C20:1n9	0.59 ± 0.05	0.82 ± 0.09	0.87 ± 0.24
∑ MUFA	27.34 ± 9.14	32.78 ± 4.96	34.13 ± 3.02
C18:2n6	39.60 ± 5.67	36.92 ± 1.51	39.63 ± 4.37
C18:3n6	0.50 ± 0.18	0.40 ± 0.11	0.45 ± 0.19
C20:4n6	1.44 ± 0.13[b]	1.89 ± 0.12[a]	2.02 ± 0.05[a]
∑ Omega-6	41.76 ± 5.79	39.97 ± 1.90	42.18 ± 4.46
C18:3n3	0.36 ± 0.16	0.32 ± 0.01	0.23 ± 0.20
C20:5n3	1.68 ± 0.56[a]	0.42 ± 0.10[b]	ND[c]
C22:5n3	1.30 ± 0.26[a]	0.66 ± 0.11[b]	ND[c]
C22:6n3	2.69 ± 0.61[a]	1.22 ± 0.37[b]	0.12 ± 0.21[c]
∑ Omega-3	6.46 ± 1.73[a]	2.80 ± 0.56[b]	0.36 ± 0.37[c]
∑PUFA	48.74 ± 5.52	43.02 ± 1.76	42.54 ± 4.83
Omega-6/Omega-3	6.98 ± 2.89	14.72 ± 3.32	42.18 ± 0.00

*Data are expressed as weight percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are

expressed as mean ± SD, n = 3. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences where $p< 0.05$ was considered significant.

3.2.3: Body weight, plasma glucose and non-esterified (NEFA) fatty acid concentrations of male and female offspring at weaning

Body weight, plasma glucose and NEFA concentrations of male and female offspring obtained from mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Table 3.6 and in Table 3.7. No significant differences were observed in plasma glucose and NEFA concentrations among the various dietary groups in both male and female offspring. A higher body weight was observed in 30:1 group compared to 5:1 and 15:1 groups ($p=0.0006$) in male offspring; however, there was no difference between 5:1 and 15:1 groups. In female offspring, a higher body weight was observed in 15:1 and 30:1 group compared to 5:1 group ($p=0.05$); however, there was no difference between the 15:1 and 30:1 dietary groups. No significant differences were observed in body length of both male and female offspring among the various dietary groups.

Table 3.6: Non-esterified fatty acid (NEFA) concentrations, blood glucose concentrations, body weight and body length of male offspring of C57BL/6 mice fed experimental diets during gestation and lactation

Diet	NEFA (mmo/L)	Blood Glucose (mmol/L)	Body Weight (g)	Body Length (cm)
5:1	0.46 ± 0.21	8.73 ± 2.13	6.46 ± 0.52[b]	6.40 ± 0.35
15:1	0.40 ± 0.12	7.95 ± 2.33	6.50 ± 0.96[b]	6.43 ± 0.39
30:1	0.39 ± 0.15	6.63 ± 2.73	7.64 ± 0.71[a]	6.38 ± 0.36

Values are expressed as means ± SD, n = 10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences having $p < 0.05$.

Table 3.7: Non-esterified fatty acid (NEFA) concentrations, blood glucose concentrations, body weight and body length of female offspring of C57BL/6 mice fed experimental diets during gestation and lactation

Diet	NEFA (mmo/L)	Blood Glucose (mmol/L)	Body Weight (g)	Body Length (cm)
5:1	0.47 ± 0.13	8.54 ± 1.05	5.95 ± 0.95[b]	6.15 ± 0.42
15:1	0.45 ± 0.17	6.83 ± 1.21	7.22 ± 1.28[a]	6.38 ± 0.29
30:1	0.56 ± 0.15	7.84 ± 2.04	7.01 ± 0.76[a]	6.38 ± 0.36

Values are expressed as means ± SD, n = 10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences having $p < 0.05$.

3.2.4: Effects of maternal omega-6 to omega-3 polyunsaturated fatty acid ratio on red blood cell phospholipids fatty acid composition of male offspring

The RBC PL fatty acid composition of male offspring obtained from mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Table 3.8. Total SFA content did not show a significant difference among various dietary groups. A significantly higher stearic acid content was observed in 15:1 group compared to 5:1 and 30:1 groups ($p=0.039$); however, 5:1 and 30:1 groups were not different.

A significantly higher total MUFA content was observed in 30:1 group compared to 5:1 and 15:1 groups ($p<0.0001$); however, no significant difference was observed between 5:1 and 15:1 group. Similarly, a higher oleic acid content was observed in 30:1 group compared to 5:1 and 15:1 groups ($p<0.0001$); however, 5:1 and 15:1 groups were not different.

As expected, a higher total n-6 PUFA content was observed in 30:1 group followed by 15:1 and 5:1 groups ($p<0.0001$). Despite no significant difference in LA content in the breast milk among various dietary groups, a significantly higher LA content was observed in 5:1 diet group compared to the 15:1 and 30:1 diet groups ($p=0.041$). However, no significant difference was observed between 15:1 and 30:1 groups. In contrast, a higher AA and docosatetraenoic acid content were observed in 30:1 group followed by 15:1 and 5:1 groups ($p<0.0001$).

A higher total n-3 PUFA content was observed in 5:1 group followed by 15:1 and 30:1 groups ($p<0.0001$). Amongst individual n-3 PUFA, EPA, DPA and DHA contents were significantly higher in the 5:1 diet group followed by 15:1 and 30:1 groups ($p<0.0001$). Interestingly, n-6 to n-3 PUFA ratio was sustained between 1:1 to 1:5 for all the dietary groups, however, it was closer to 1:1 in 5:1 group; 2:1 in 15:1 group and 4:1 in 30:1 group.

Table 3.8: Red blood cell phospholipids fatty acid composition of male offspring of C57BL/6 mice fed experimental diets during gestation and lactation*

FA	5:1 Diet	15:1 Diet (% w/w)	30:1 Diet
C14:0	0.53 ± 0.12	0.41 ± 0.27	0.40 ± 0.22
C16:0	23.53 ± 1.60	21.31 ± 1.41	23.51 ± 2.20
C18:0	14.44 ± 2.74^{b}	17.76 ± 3.18^{a}	14.69 ± 0.79^{b}
∑ SFA	38.50 ± 3.28	39.50 ± 4.01	38.61 ± 2.37
C18:1n9	10.50 ± 0.25^{b}	10.76 ± 0.76^{b}	12.27 ± 0.54^{a}
C18:1n7	1.72 ± 0.05	1.20 ± 0.60	1.50 ± 0.12
C20:1n9	0.21 ± 0.14	0.30 ± 0.16	0.37 ± 0.15
∑ MUFA	12.66 ± 0.48^{b}	12.40 ± 0.90^{b}	14.25 ± 0.57^{a}
C18:2n6	11.95 ± 0.97^{a}	10.13 ± 1.69^{b}	10.51 ± 1.11^{b}
C20:4n6	14.32 ± 1.37^{c}	19.74 ± 0.69^{b}	23.21 ± 1.58^{a}
C22:4n6	1.15 ± 0.20^{c}	3.10 ± 0.17^{b}	4.99 ± 0.32^{a}
∑ Omega-6	27.50 ± 1.10^{c}	33.13 ± 2.23^{b}	38.83 ± 2.15^{a}
C20:5n3	2.65 ± 0.46^{a}	0.55 ± 0.34^{b}	0.10 ± 0.11^{c}
C22:5n3	3.18 ± 0.62^{a}	1.95 ± 0.20^{b}	0.77 ± 0.32^{c}
C22:6n3	15.04 ± 1.38^{a}	12.11 ± 1.27^{b}	7.23 ± 0.41^{c}
∑ Omega-3	21.31 ± 2.15^{a}	14.94 ± 1.29^{b}	8.28 ± 0.34^{c}
∑ PUFA	48.82 ± 2.98	48.08 ± 3.37	47.12 ± 2.12
Omega-6/Omega-3	1.29 ± 0.10	2.22 ± 0.11	4.69 ± 0.35

*Data are expressed as weight percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SD, n = 10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences where $p < 0.05$ was considered significant.

3.2.5: Effects of maternal omega-6 to omega-3 polyunsaturated fatty acid ratio on red blood cell phospholipid fatty acid composition of female offspring

The RBC PL fatty acid composition of female offspring obtained from mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Table 3.9. No significant difference was observed in total SFA content among various dietary groups. However, a significantly higher myristic acid content was observed in 5:1 and 15:1 diet groups compared to 30:1 diet group ($p=0.002$); there was no difference between 5:1 and 15:1 groups.

No difference was observed in total MUFA content among various dietary groups. Similar to male offspring RBC PL fatty acid composition, a significantly higher oleic acid content was observed in 30:1 group compared to 5:1 group ($p=0.047$); however, 15:1 group did not differ from either 5:1 or 30:1 groups.

A higher total n-6 PUFA content was observed in 30:1 group followed by 15:1 and 5:1 groups ($p<0.0001$). A significantly higher LA content was observed in 5:1 diet group compared to 15:1 diet group ($p=0.01$), however, 30:1 diet group did not differ from

either 5:1 or 15:1 groups. Conversely, a significantly higher AA ($p<0.0001$) content was observed in 30:1 diet group compared to 15:1 diet group followed by 5:1 diet group.

A significantly higher total n-3 PUFA content was observed in 5:1 group followed by 15:1 and 30:1 groups ($p<0.0001$). Amongst individual n-3 PUFA, EPA, DPA and DHA contents were significantly higher in 5:1 group followed by 15:1 and 30:1 groups ($p<0.0001$). Although no significant difference was observed in total PUFA content among the various dietary groups, the n-6 to n-3 PUFA ratio was sustained between 1:1 to 1:5 for all the dietary groups and was closer to 1:1 in 5:1 group; 2:1 in 15:1 group and 4:1 in 30:1 group.

Table 3.9: Red blood cell phospholipids fatty acid composition of female offspring of C57BL/6 mice fed experimental diets during gestation and lactation*

FA	5:1 Diet	15:1 Diet	30:1 Diet
		(% w/w)	
C14:0	0.86 ± 0.12^{a}	0.78 ± 0.10^{a}	0.59 ± 0.12^{b}
C16:0	26.52 ± 1.53	27.60 ± 2.22	25.56 ± 2.03
C18:0	16.32 ± 3.16	18.47 ± 4.92	18.16 ± 2.33
∑ SFA	43.71 ± 2.26	46.86 ± 3.04	44.32 ± 3.25
C18:1n9	10.41 ± 0.32^{b}	10.89 ± 1.33^{ab}	11.65 ± 0.74^{a}
C18:1n7	1.66 ± 0.10	1.09 ± 0.55	1.42 ± 0.07
C20:1n9	0.22 ± 0.09	0.26 ± 0.13	0.40 ± 0.05
∑ MUFA	12.62 ± 0.66	12.33 ± 0.82	13.56 ± 0.76
C18:2n6	10.63 ± 1.13^{a}	8.72 ± 0.44^{b}	9.61 ± 1.10^{ab}
C20:4n6	13.41 ± 0.57^{c}	17.74 ± 1.20^{b}	20.78 ± 2.07^{a}
C22:4n6	1.05 ± 0.15	2.26 ± 1.12	3.76 ± 1.59
∑ Omega-6	25.17 ± 1.24^{c}	28.74 ± 1.85^{b}	34.30 ± 2.45^{a}
C20:5n3	1.99 ± 0.34^{a}	0.32 ± 0.25^{b}	0.11 ± 0.11^{b}
C22:5n3	2.87 ± 0.25^{a}	1.84 ± 0.18^{b}	0.91 ± 0.08^{c}
C22:6n3	13.00 ± 0.55^{a}	9.72 ± 1.09^{b}	6.43 ± 0.81^{c}
∑ Omega-3	18.49 ± 0.93^{a}	12.04 ± 1.30^{b}	7.80 ± 0.88^{c}
∑ PUFA	43.66 ± 1.92	40.79 ± 2.83	42.11 ± 2.97
Omega-6/Omega-3	1.36 ± 0.06	2.40 ± 0.18	4.43 ± 0.47

*Data are expressed as weight percentage of the total extracted fatty acids. Statistical analysis was performed after transforming the data using arcsine equation. Values are expressed as mean ± SD, n = 10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Superscripts represent significant differences where $p < 0.05$ was considered significant.

3.2.6: Effect of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma lipid levels of male and female offspring at weaning

Plasma TC concentration and TG concentrations of male and female offspring of mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in figure 3.4 A and B respectively. A significantly higher TC concentration was observed in 30:1 group compared to 5:1 and 15:1 groups in both male ($p<0.0001$) and female ($p=0.01$) offspring however, no significant difference was observed between 5:1 and 15:1 groups in both genders (Figure 3.4A). Female offspring showed a significantly higher TG concentration in 30:1 group compared to 5:1 and 15:1 groups ($p=0.004$) with no significant difference between 5:1 and 15:1 groups. Male offspring did not show a significant difference in plasma TG concentrations among various dietary groups; however, 30:1 group showed a trend towards higher plasma TG concentrations (Figure 3.4B).

Plasma LDL-cholesterol and HDL-cholesterol concentrations of male and female offspring of mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Figure 3.5 A and B, respectively. A significantly higher LDL-cholesterol concentration was observed in 30:1 group compared

to 5:1 and 15:1 groups ($p<0.0001$) in male offspring; however, there were no significant differences between 5:1 and 15:1 groups. On the other hand, female offspring showed lower LDL-cholesterol concentrations in 15:1 group compared to 5:1 and 30:1 groups ($p=0.009$); however, no significant difference was observed between 5:1 and 30:1 groups (figure 3.5 A). No significant differences were observed in HDL-cholesterol concentration in both male and female offspring amongst various dietary groups (figure 3.5 B).

Plasma LDL/HDL-cholesterol ratio of male and female offspring of mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Figure 3.6. A higher LDL/HDL-cholesterol ratio was observed in 30:1 group compared to 5:1 group ($p=0.02$) in male offspring; however, 15:1 group did not differ from either 5:1 or 30:1 groups. A lower LDL/HDL-cholesterol ratio was observed in 15:1 group compared to 5:1 and 30:1 groups ($p=0.02$) in female offspring however, no significant difference was observed between 5:1 and 30:1 groups (Figure 3.6).

Plasma FC and CE concentrations of male and female offspring of mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during gestation and lactation are shown in Figure 3.7 A and B, respectively. A significantly higher FC concentration was observed in 15:1 and 30:1 groups compared to 5:1 group in female offspring ($p=0.02$); however, no significant difference was observed between 15:1 and 30:1 groups. In contrast, no significant differences were observed in FC concentrations in male offspring among various dietary groups (Figure 3.7 A). Cholesterol ester concentrations of the male offspring were, on the other hand, significantly higher in the 30:1 group

compared to 5:1 and 15:1 group ($p<0.0001$); no significant difference was observed between 5:1 and 15:1 groups. Female offspring showed a lower CE concentration in the 15:1 group compared to 30:1 groups ($p=0.022$); however, 5:1 group did not differ from either 15:1 or 30:1 groups (Figure 3.7 B).

3.2.7. Correlation analysis of biochemical parameters with red blood cell phospholipid fatty acid composition

A correlation analysis was performed between plasma lipid levels and RBC PL fatty acid composition for both male (Figure 3.8) and female (Figure 3.9) offspring of mothers fed diets containing 5:1, 15:1 and 30:1 n-6 to n-3 PUFA ratios during pregnancy and lactation.

In male offspring, an increase in DHA content was associated with lower plasma TC concentrations ($r = -0.65$, $p=0.001$) and lower LDL-cholesterol concentrations ($r = -0.71$, $p=0.0003$). Similarly, increased EPA content was correlated with lower plasma LDL-cholesterol concentration ($r = -0.48$, $p=0.03$) while an increase in AA content was associated with higher plasma LDL-cholesterol concentrations ($r = 0.48$, $p=0.03$). Other fatty acids were not significantly correlated with plasma lipid levels in the male offspring.

Female offspring, on the other hand, showed no significant correlation between EPA, DHA or AA with plasma TC concentrations (Correlation coefficient (r); -0.26, -0.43 and 0.40 respectively). However, an increase in AA was associated with increased plasma TG concentrations ($r = 0.61$, $p= 0.005$) while an increase in DHA content was

associated with lower plasma TG concentrations (r = -0.66, p=0.002). Other fatty acids were not significantly correlated with plasma lipid levels in the female offspring.

Figure 3.4: Plasma concentrations of (*A*) triglyceride and (*B*) total cholesterol of male and female offspring

Values are expressed as means ± SD, n =10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences between various dietary groups having $p < 0.05$.

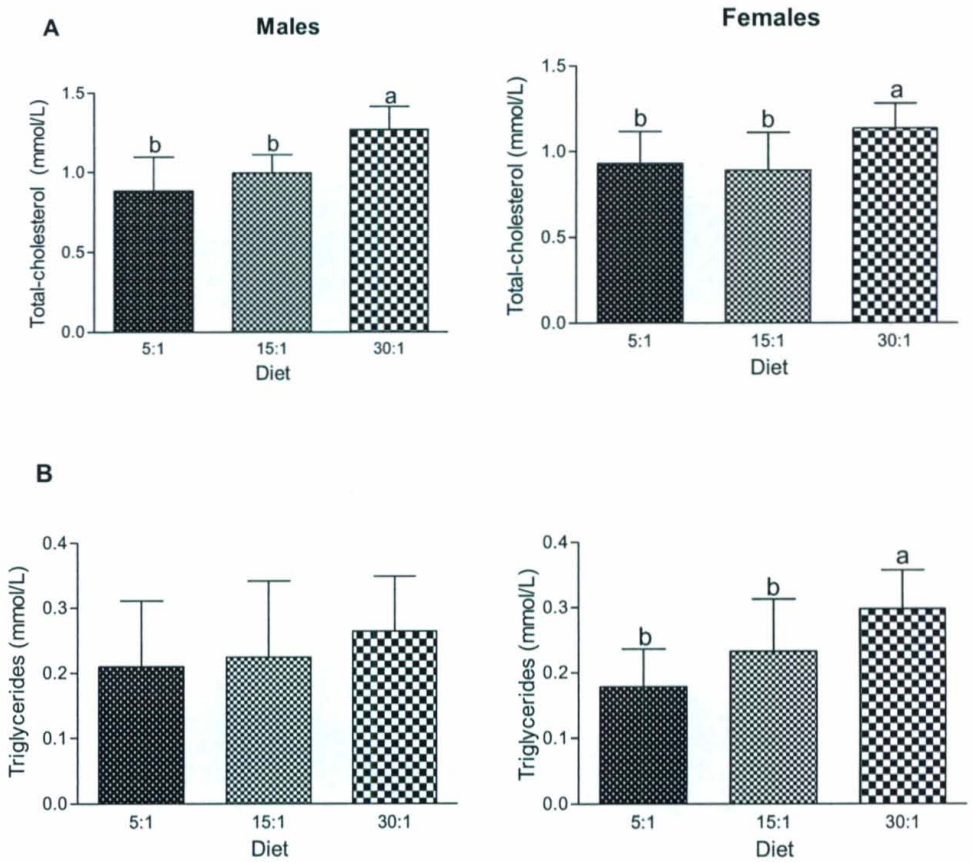


Figure 3.5: Plasma concentrations of (*A*) LDL-cholesterol and (*B*) HDL-cholesterol of male and female offspring

Values are expressed as means ± SD, n =10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences between various dietary groups having $p < 0.05$.

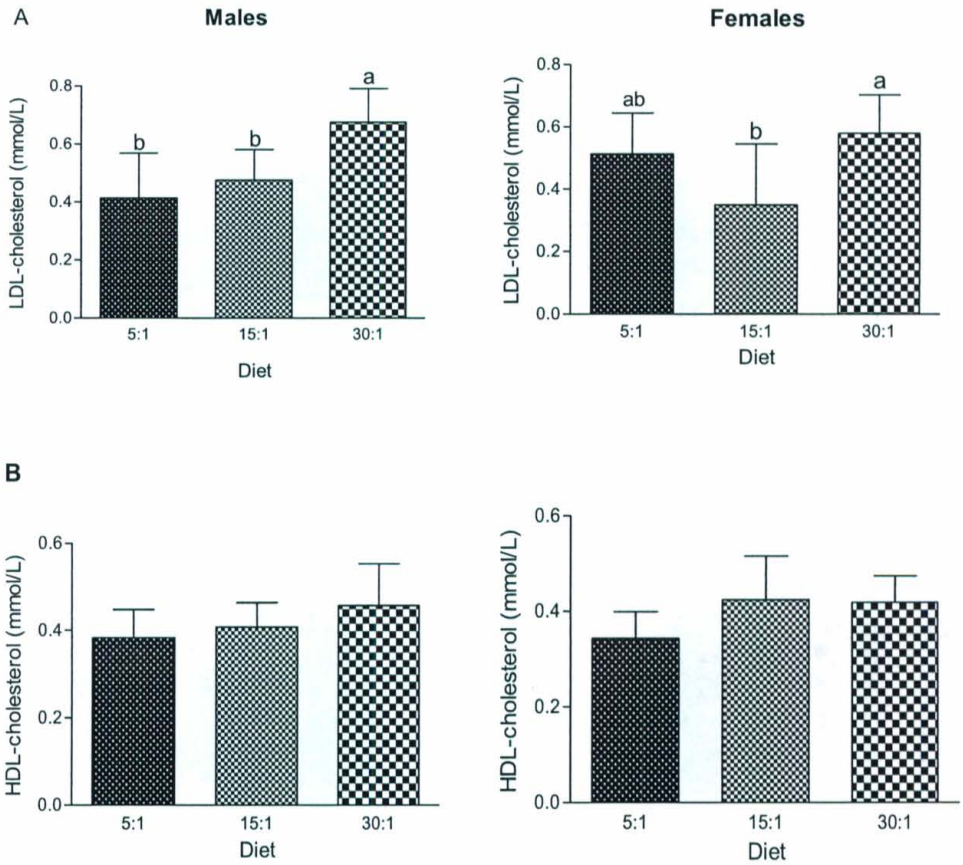


Figure 3.6: Plasma LDL to HDL- cholesterol ratio of male and female offspring

Values are expressed as means ± SD, n =10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences between various dietary groups having $p < 0.05$.

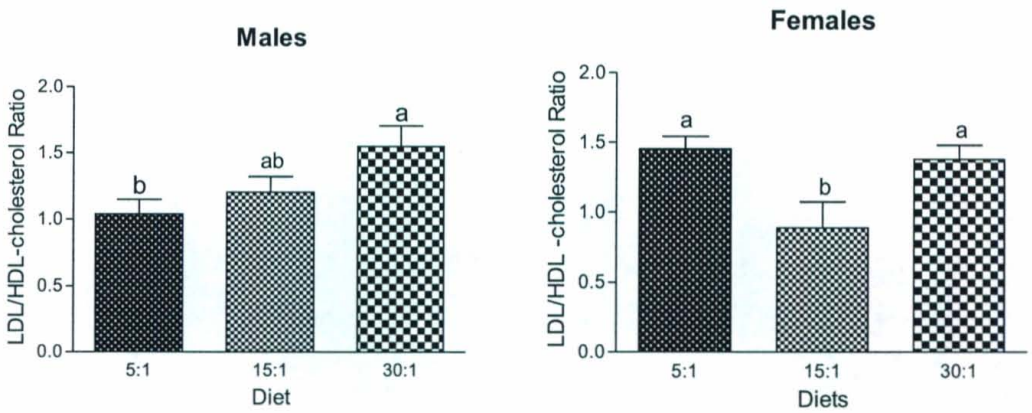


Figure 3.7: Plasma concentrations of (*A*) Free cholesterol and (*B*) Cholesterol esters of the male and female offspring

Values are expressed as means ± SD, n =10. Data were assessed using one-way ANOVA. Significant effects were further analyzed using Newman-Keuls *post hoc* tests. Letters represent significant differences between various dietary groups having $p < 0.05$.

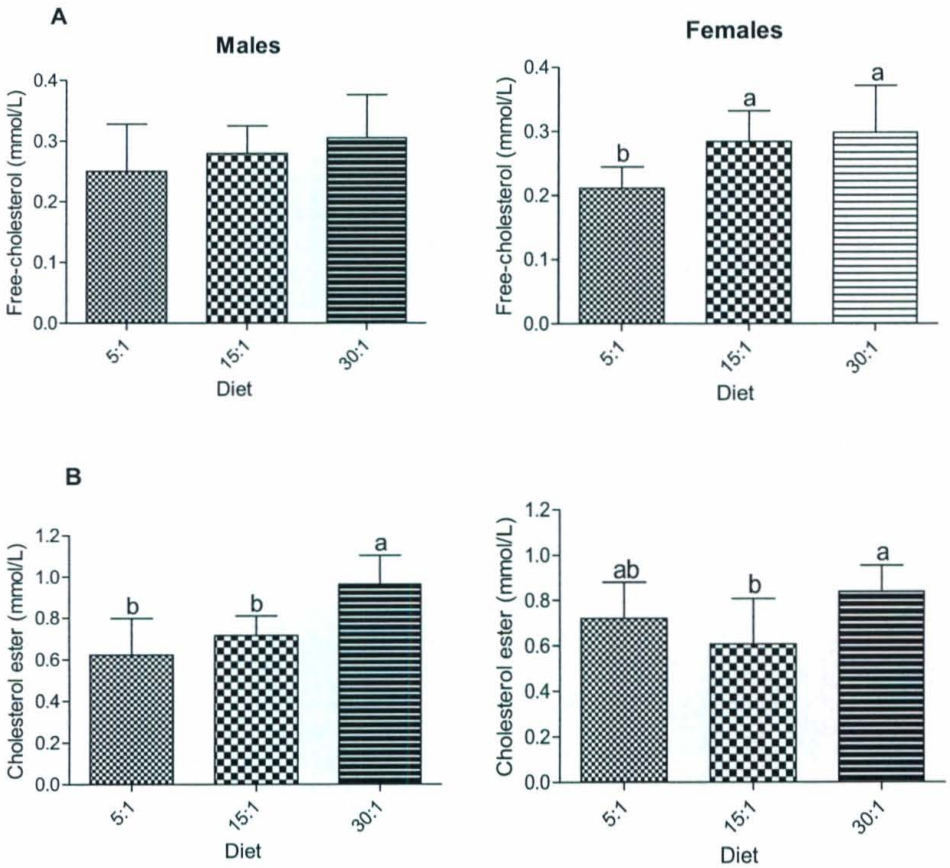


Figure 3.8: Correlation analysis between plasma lipid levels and red blood cell phospholipid fatty acid composition of male offspring: (A) total cholesterol and docosahexaenoic acid (DHA) content (B) LDL-cholesterol and arachidonic acid (AA) content (C) LDL-cholesterol and docosahexaenoic acid (DHA) content (D) LDL cholesterol and eicosapentaenoic acid (EPA) content. Correlation coefficient (r) was determined using Graph pad Prism (version 5)

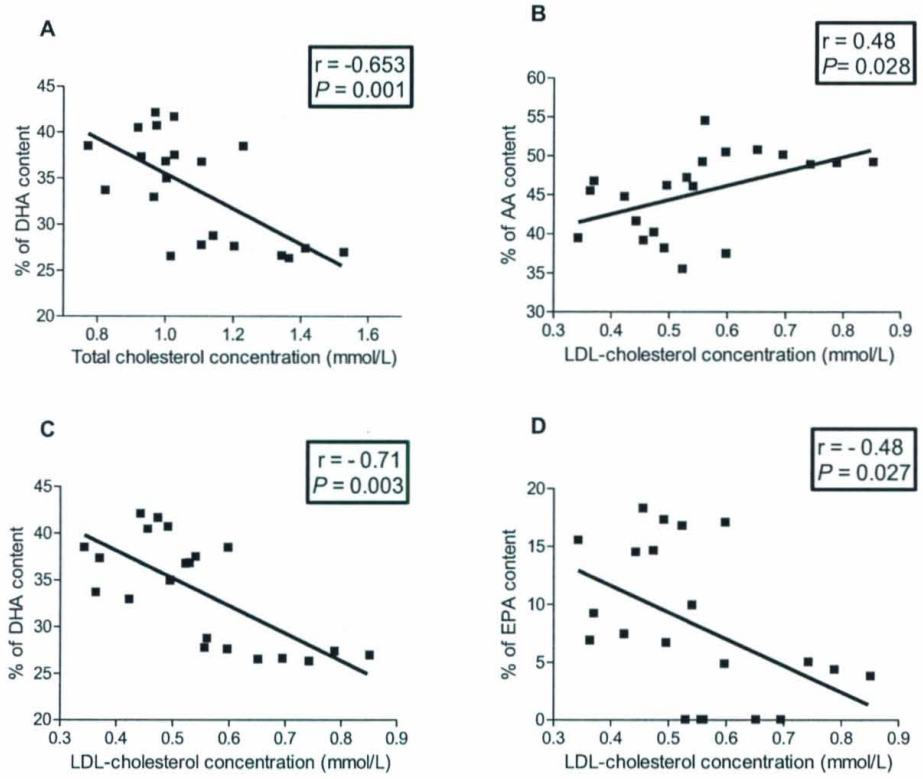
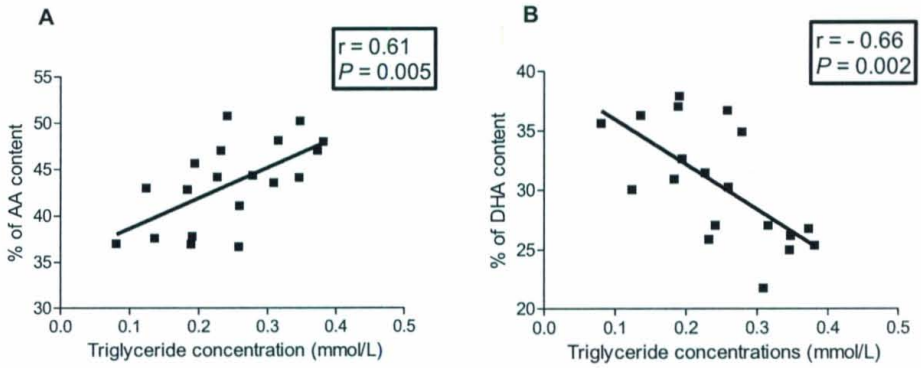


Figure 3.9: Correlation analysis between plasma lipid levels and red blood cell phospholipid fatty acid composition of female offspring. (A) triglycerides and arachidonic acid (AA) content (B) triglycerides and docosahexaenoic acid (DHA) content. Correlation coefficient (r) was determined using Graph pad Prism (version 5.0)



Chapter 4: Discussion

4.1: One month study

Increased plasma lipid levels are associated with the increased risk of many metabolic disorders including CVD, obesity, and diabetes mellitus. The objective of the current study was to determine whether feeding diets with varying n-6 to n-3 PUFA ratios will alter the regulation of metabolic pathways in C57BL/6 female mice after 4 weeks of feeding. Our findings suggest that a higher ratio of n-6 to n-3 PUFA in the diet leads to higher plasma lipid parameters compared to a lower ratio of n-6 to n-3 PUFA.

4.1.1: Effect of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on food intake and body weight

Consumption of energy dense foods, resulting in higher calorie intake and higher dietary fat intake, is one of the most important contributing factors for predisposition to metabolic syndrome (Prentice & Jebb, 2003). In the current study, no significant differences were observed in food intake and caloric intake among various dietary groups, suggesting that food intake and caloric intake were not confounding factors for the observed changes in lipid metabolism. There was a small but significant increase of body weight in 5:1 and 30:1 groups compared to 15:1 group. A higher ovarian fat content was observed in the 5:1 group compared to the 15:1 group; however, the increase in the fat content was not sufficient to account for the increase in body weight (2 g increase in body weight *vs* 0.2 g increase in fat weight). There were no significant differences in other organ weights among the dietary groups thus higher body weight in the 5:1 group could be due to higher ovarian fat and other non-measured fat depots.

The effect of dietary PUFA on body weight gain is controversial. Some studies report that n-3 PUFA reduce body weight in men (Couet *et al.*, 1997), in obese women (Kunesova *et al.*, 2006) and in mice (Ruzickova *et al.*, 2004); however, n-6 PUFA was shown to be associated with increased epididymal fat pad weight and increased body weight in mice (Massiera *et al.*, 2003). Contrarily, no difference in body weights were observed in rats fed high fat diets rich in n-3 PUFA and n-6 PUFA (Awad *et al.*, 1990; Dziedzic *et al.*, 2007). A study designed to investigate the cardiovascular effects of designer oils in C57BL/6 mice with a low ratio of n-6 to n-3 PUFA (2:1) using different sources of n-3 PUFA, together with a control diet where n-6 to n-3 PUFA ratio was 25:1, found no significant difference in body weight among the experimental groups (Riediger *et al.*, 2008).

On the other hand, a recent study by Nuernberg *et al.*, 2011 reported a higher body weight in mice fed high fat diets rich in n-3 and n-6 PUFA compared to a control diet. These authors observed a higher abdominal and perirenal fat content in both n-3 and n-6 groups, however, the liver fat content was significantly lower in the n-3 group compared to the n-6 group. It was proposed that a diet rich in n-3 PUFA may facilitate shift from fuel deposition in liver to fuel storage as fat in adipose tissue in mice. This may also explain our findings on higher ovarian fat content observed in the 5:1 group.

4.1.2: Effect of dietary omega-6 to omega-3 polyunsaturated fatty ratios on plasma triglyceride levels

No significant differences were observed in plasma triglyceride (TG) levels among various dietary groups after one month of feeding diets varying in n-6 to n-3 PUFA. Others have also reported similar findings that showed no significant differences in plasma TG levels between n-3 PUFA and n-6 PUFA fed groups after 4 weeks of dietary exposure in mice (Zampolli *et al.*, 2006; Riediger *et al.*, 2008) and in rats (Balasubramaniam *et al.*, 1985). On the other hand, several studies support the TG lowering effect of an n-3 PUFA rich diet; however, this TG lowering effect was generally detectable after 2 months of dietary exposure to n-3 PUFA rich diet. Zampolli *et al.*, (2006) reported a lower TG level in the fish oil fed group rich in n-3 PUFA compared to the corn oil fed group rich in n-6 PUFA after 20 weeks of dietary exposure in LDLr-/- mice. Studies in rats reported a hypotriglyceridemic effect of n-3 PUFA compared to n-6 PUFA after 16 weeks (Catherine Jen *et al.*, 1989), 7 weeks (Niot *et al.*, 1994) and 3 months (Froyland *et al.*, 1997). Therefore, a longer dietary exposure (more than 4 weeks) may be required to observe the TG lowering effects of n-3 PUFA.

4.1.3: Effect of dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma total -cholesterol levels

The plasma TC concentration was significantly lower in the 5:1 group and 15:1 group compared to the 30:1 group suggesting that n-3 PUFA lower plasma cholesterol levels compared to n-6 PUFA. Interestingly, even the 15:1 group had lower plasma cholesterol levels compared to the 30:1 group, suggesting a dose response effect of

changing the n-6 to n-3 PUFA ratio. No significant differences were observed in FC levels among the various dietary groups; however, low CE levels were observed in 5:1 and 15:1 groups compared to the 30:1 group thus lower TC levels appear to be due to low CE levels in 5:1 and 15:1 groups.

A recent study in C57BL/6 mice also reported a significantly lower plasma TC concentration in an n-3 PUFA fed group compared to an n-6 PUFA fed group after a one month feeding trial (Magdeldin *et al.*, 2009). Other studies in various animal models have also reported a decrease in plasma cholesterol levels after feeding diets enriched in n-3 PUFA compared to n-6 PUFA rich diet (Nieuwenhuys *et al.*, 1998; Zampolli *et al.*, 2006; Lee *et al.*, 1989).

Magdeldin *et al.*, (2009) proposed that the lowered TC levels in the plasma of n-3 PUFA fed group may be due to the ability of n-3 PUFA to inhibit fatty acid synthesis and promote mitochondrial fatty acid beta-oxidation. Pro-inflammatory mediators are released from PUFA, which bind to nuclear receptor proteins called peroxisome proliferative activators (PPARs), which play an essential role in regulating beta oxidation. The affinity of an n-6 PUFA, essentially AA, to PPARs is lower compared to EPA and DHA, thus n-3 PUFA are known to stimulate beta oxidation to a greater extent compared to n-6 PUFA, which may be responsible for a decrease in plasma lipid levels (Schmitz & Ecker, 2008).

The cholesterol lowering effect of n-3 PUFA has been suggested to be due to increased cholesterol catabolism, reduced cholesterol biosynthesis and increased biliary

excretion of cholesterol (Balasubramaniam *et al.*, 1985; Du *et al.*, 2003; Oh *et al.*, 2009). It will be interesting to investigate in future studies whether the decrease in plasma TC levels in the current study was due to a decrease in cholesterol synthesis by inhibiting HMG-CoA reductase activity, or due to an increased breakdown of cholesterol by increased activity of cholesterol 7α hydroxylase (CYP7).

4.1.4: Effect of dietary omega-6 to omega-3 polyunsaturated fatty ratios on plasma LDL- and HDL- cholesterol concentrations

A higher LDL-cholesterol concentration was observed in 30:1 group compared to the 5:1 and 15:1 groups. Low LDL-cholesterol was observed in humans who were supplemented with salmon oil, a rich source of n-3 PUFA, compared to a high n-6 PUFA group (Harris *et al.*, 1983). Animal studies have also reported a significant reduction in plasma LDL-cholesterol levels after feeding diets enriched in n-3 PUFA compared to feeding diets rich in n-6 PUFA in rats (Roach *et al.*, 1987; Ventura *et al.*, 1989; Spady, 1993) as well as in mice (Vasandani *et al.*, 2002; Zampolli *et al.*, 2006; Magdeldin *et al.*, 2009).

A likely explanation for the decreased circulating levels of plasma LDL-cholesterol in 5:1 and 15:1 groups is the increased clearance of LDL from circulation. Removal of LDL-cholesterol from the circulation is mainly regulated through liver LDLr, maintaining circulatory cholesterol homeostasis (Brown & Goldstein, 1984). It was previously shown that enhanced LDLr activity was associated with lower plasma LDL-cholesterol levels in rats fed n-3 PUFA enriched diet compared to n-6 PUFA enriched diet

(Ventura *et al.*, 1989; Spady, 1993). Therefore, low plasma LDL-cholesterol levels in the 5:1 and 15:1 groups may be due to an increased expression of LDL-r activity.

No significant changes were observed in HDL-cholesterol in the various dietary groups. Similar to our observations, recent studies in mice have also reported no change in HDL- cholesterol level between n-3 PUFA and n-6 PUFA fed groups for 4 weeks (Zampolli *et al.*, 2006), 20 weeks (Magdeldin *et al.*, 2009) and for 32 weeks (Wang *et al.*, 2009). HDL-cholesterol is of two sub-classes: HDL-2 and HDL-3; HDL-2 cholesterol involved in scavenging more cholesterol from the peripheral tissues compared to HDL-3 (Ballantyne *et al.*, 1982; Asayama *et al.*, 1990). Preliminary findings from our laboratory show that plasma from mice fed a 5:1 diet has higher cholesterol efflux capacity compared to plasma from animals fed a 30:1 diet (data not published). Although we observed no significant difference in total HDL-cholesterol levels among the various dietary groups, it is possible that 5:1 and 15:1 groups contain higher HDL-2 cholesterol levels which have better cholesterol efflux capacity compared to 30:1 group.

One of the markers for the development of CVD is the ratio of plasma LDL/HDL-cholesterol (Lemieux *et al.*, 2001; Panagiotakos *et al.*, 2003). In the current study, a lower LDL/HDL-cholesterol ratio was observed in 5:1 and 15:1 groups compared to the 30:1 group suggesting that a continuous exposure to higher n-6 to n-3 PUFA ratio may increase the risk of developing CVD. Previous dietary intervention studies have also shown that n-3 PUFA is associated with lower LDL/HDL-cholesterol ratio in humans (Dawczynski *et al.*, 2010) and in rats (Vijaimohan *et al.*, 2006).

4.1.5: Effect of dietary omega-6 to omega-3 polyunsaturated fatty acid ratio on red blood cell phospholipid fatty acid composition

The best marker of dietary intake of EPA, DHA, AA and LA is RBC PL, expressed as a percentage of total fatty acids (Matorras *et al.*, 1998).The objective of the determination of RBC PL fatty acid composition was to test the hypothesis that dietary fatty acids are reflected in the RBC PL.

As expected, a higher total n-6 PUFA content was observed in the 30:1 group compared to the 5:1 group. However, total n-6 PUFA content in the 15:1 group was not significantly different from 5:1 or 30:1 groups. These observations indicate that there is a higher incorporation of n-6 PUFA in to RBC PLs when the diet is rich in n-6 PUFA.

Amongst individual n-6 PUFA in the RBC PL fatty acids, LA content in the 5:1 group was higher compared to the 15:1 and 30:1 groups. However, AA content and docosatetraenoic acid content were low in 5:1 group compared to the 15:1 group followed by 30:1 group. These findings suggest that the conversion of LA to AA is low in the 5:1 group, which is likely due to an increased competition for elongation and desaturation enzymes between n-6 PUFA and n-3 PUFA. However, a higher content of AA with a concomitant decrease in LA indicates that LA was likely converted to AA and docosatetraenoic acid to a greater extent in the 15:1 group followed by 30:1 group, suggesting that as the amount of n-3 PUFA increase, there is a gradual decrease in the

conversion of LA to AA due to competition for desaturation and elongation enzymes between n-6 and n-3 PUFA (Emken *et al.*, 1994).

As expected, a higher total n-3 PUFA and DHA content was observed in the 5:1 group compared to the 15:1 group followed by 30:1 group, which reflected the dietary composition. A higher conversion rate of ALA to EPA, DPA and DHA was observed in the 5:1 group compared to the 15:1 group followed by 30:1 group.

Recent epidemiological studies in humans also revealed that RBC membrane fatty acids accretion is influenced by the amount and the balance of dietary n-6 and n-3 PUFA (Cartwright *et al.*, 1985; Friesen & Innis, 2010; Friesen *et al.*, 2010), confirming that there was a competitive interaction of dietary LA with AA, EPA and DHA to get incorporated into membrane lipids. It has also been shown that LA reduced the concentrations of n-3 PUFA in the RBC membrane by competing for acylation (Friesen & Innis, 2010). A study in rats also demonstrated that endogenous synthesis of n-3 PUFA from the precursor ALA is more regulated by substrate competition for existing enzymes than by their expression of desaturase and elongase genes (Tu *et al.*, 2010). Thus, it can be suggested that a higher n-6 or n-3 PUFA incorporation in to RBC PLs is favoured by increased dietary intake.

Besides the percentage of unsaturated fatty acids in RBC, the mean melting point (MMP) is also an index of membrane fluidity. DHA and EPA have been shown to have lower MMP and higher unsaturation level compared to LA and AA (Holman *et al.*, 1991; Torres & Trugo, 2009). Since the relative contribution of fatty acids with more

unsaturated chains and lower melting points, such as DHA and EPA, is high in 5:1 group, RBCs from 5:1 group will have a higher membrane fluidity compared to 15:1 and 30:1 groups. Cartwright *et al.*, (1985) reported that the supplementation of dietary EPA and DHA for 6 weeks increased the total unsaturation of the RBC membrane leading to increased lipid fluidity and reduced whole blood viscosity. Thus, it is suggested that a lower n-6 to n-3 PUFA ratio may be beneficial in reducing the risk of diseases such as CVD where the rheological properties of blood plays a considerable role.

Previously, it was suggested that low dietary intake of n-3 PUFA led to low tissue levels of n-3 PUFA which elicit systemic effects contributing to not only CVD but also to rheumatoid arthritis (Bruinsma & Taren, 2000; Simopoulos, 2006) and depression (Peet *et al.*, 1998; Lucas *et al.*, 2010). These findings further reinforce the clinical significance of the importance of a lower dietary ratio of n-6 to n-3 PUFA, and our findings have demonstrated that RBC PL fatty acid composition is reflective of the dietary n-6 to n-3 PUFA.

Overall, our findings from the one month study establish that feeding diets with varying n-6 to n-3 PUFA ratios to C57BL/6 mice for one month led to the incorporation of dietary fatty acids in RBC PL and also altered the regulation of lipid and lipoprotein metabolism. These findings formed the basis to initiate the project on investigating the effects of maternal dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism of the offspring.

4.2: Maternal nutrition study

The importance of the ratio of n-6 to n-3 PUFA has been emphasized by the potential health benefits derived from diets high in n-3 PUFA. The high n-6 to n-3 PUFA ratio found in Western diets has been shown to promote the pathogenesis of several diseases such as cancer, cardiovascular diseases, neurodegenerative diseases, and autoimmune diseases. The objective of the current study was to test the hypothesis that a maternal high fat diet with varying n-6 to n-3 PUFA ratios during gestation and lactation will alter the regulation of lipid and lipoprotein metabolism in the offspring of C57BL/6 mice at weaning. Our findings showed that, a) the breast milk fatty acid composition was reflective of the maternal fatty acid composition, b) the offspring RBC PL fatty acid composition reflected the fatty acid composition of the maternal diet, and c) high ratio of n-6 to n-3 PUFA in the maternal diet was associated with higher plasma lipid parameters compared to a lower ratio.

4.2.1: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on food intake and body weight of the offspring at weaning

There were no significant differences in food intake, caloric intake and body weight of mothers in the three dietary groups, suggesting that food intake and caloric intake of the mothers were not confounding factors for the observed changes in the lipid metabolism of the offspring. Interestingly, the 30:1 group showed a higher body weight compared to both 5:1 and 15:1 groups. No differences were observed in the body length and the organ weights of the offspring amongst the various dietary groups. However, we

were not able to collect fat from animals at weaning and we did not measure the carcass weight thus the difference in body weight could be due to a number of unexplained reasons.

The effects of maternal dietary n-3 PUFA and n-6 PUFA on body weight of the offspring is controversial. Similar to our observations, the offspring of female Sprague-Dawley rats fed a low n-6 to n-3 PUFA ratio had a significantly lower body weight compared to the offspring of mothers on high n-6 to n-3 PUFA ratio (Korotkova *et al.*, 2002; Korotkova *et al.*, 2005). These and other investigators reported reduced adipose tissue mass and reduced adipocyte size in the n-3 PUFA fed group (Massiera *et al.*, 2003; Yessoufou *et al.*, 2006). Thus it appears that there is a strong association between high dietary n-3 PUFA, low body weight, and reduced adipose tissue mass. Studies have shown that fatty acid oxidation in the body depends on the quality of dietary fat and the fatty acid chain length in the adipose tissue (Yamazaki *et al.*, 1987; Halminski *et al.*, 1991). It is therefore logical to assume that n-3 PUFA reduces body fat deposition, and a consequent reduction in body weight via increased fatty acid oxidation.

We were not able to collect any fat tissue in the offspring of various dietary groups at weaning. A post weaning study of the offspring would therefore be necessary to investigate the effects of different dietary n-6 to n-3 PUFA ratios on body fat deposition and body weight gain.

4.2.2: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma non esterified fatty acids and blood glucose of offspring at weaning

Intrauterine stress, especially maternal under-nutrition is known to be associated with onset of diabetes and CVD in the offspring at later life (Barker *et al.*, 1993; Barker, 1995; Barker, 1997). An increased level of NEFA in the serum and increased blood glucose levels are important markers of diabetes mellitus (Min *et al.*, 2005). In the current study, no significant differences were observed in blood glucose and plasma NEFA levels in both dams and in offspring at weaning.

No significant differences were reported in plasma NEFA and glucose levels in offspring at postnatal day 90, of dams fed a high fat diet (21%) rich in n-6 PUFA (n-6 to n-3 PUFA ratio of =28:1) and n-3 PUFA (n-6 to n-3 PUFA ratio of =2.5:1) during perinatal period (Ibrahim *et al.*, 2009). A recent study on rats also reported no difference in glucose levels; however, lower plasma NEFA levels were reported in both male and female offspring of dams fed a high DHA diet at weaning, possibly due to reduced mobilization of fat from adipose tissue (Muhlhausler *et al.*, 2010). In addition, it is interesting to observe a higher male to female ratio in 15:1 and 30:1 groups compared to 5:1 group however the reason behind is unknown, which need to be explored in the future.

4.2.3: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratio on breast milk total fatty acid composition

Breast milk is the ultimate energy source of an infant and provides fatty acids for the developing membrane lipids and storage in adipocytes as TGs during the lactation period (Novak & Innis, 2011). In the present study, dams were fed the experimental diets, for 2 weeks before mating, during gestation and also during the lactation. No significant differences were observed in total SFA and total MUFA content in the breast milk among the various dietary groups; this finding is similar to the dietary fatty acid composition establishing that maternal dietary fatty acid composition is reflected in the breast milk.

Similarly, total n-6 PUFA and LA content were not different among various dietary groups. However, a significantly lower AA content was observed in the 5:1 group compared to the 15:1 and 30:1 groups. Previous studies have shown that the AA content in the milk depends on maternal storage levels and the conversion of LA to AA (Arterburn *et al.*, 2006; Brenna *et al.*, 2007). Thus it is reasonable to assume that there may be a lower conversion rate of LA to AA in the 5:1 group compared to the 15:1 and 30:1 group likely due to competition between n-6 and n-3 PUFA classes for the elongation and desaturation enzymes. Nonetheless, further research is required to confirm the above hypothesis.

A higher EPA and DHA content was observed in the 5:1 group followed by the 15:1 and 30:1 groups, thus breast milk reflected the fatty acid content of the various maternal diets. Studies have shown higher levels of DHA and EPA levels in breast milk when lactating dams were fed diets high in n-3 PUFA (Jen *et al.*, 2009; Novak & Innis,

2011; Arterburn *et al.*, 2006; Brenna *et al.*, 2007). Docosahexaenoic acid plays a critical role in the perinatal period, especially in neuronal and visual development (Guesnet *et al.*, 1997).

In the current study, the n-6 to n-3 PUFA ratio in the breast milk closely reflected the maternal dietary n-6 to n-3 PUFA ratio in all the dietary groups. Interestingly, breast milk n-6 to n-3 PUFA ratio reflected nearly the same PUFA ratio as that of the maternal diet and the ratios were 7:1, 15:1 and 42:1 in 5:1, 15:1 and 30:1 groups. The n-6 to n-3 PUFA ratio in breast milk is relatively stable during the first 3 months of lactation in humans and gets higher as lactation progresses (Jang *et al.*, 2011), which could be the reason for a higher n-6 to n-3 PUFA ratio observed in the breast milk of 30:1 mice. Similar to our observation, similar n-6 to n-3 PUFA ratios have been reported in breast milk at 1 week and 2 weeks after delivery as that of the maternal diet in mice (Kagohashi *et al.*, 2007; Kagohashi *et al.*, 2010). Previous studies and the current study highlight the influence of maternal dietary fatty acids, and the n-6 to n-3 PUFA ratio, on breast milk composition, which can have a major impact on the offspring's health status in later life.

4.2.4: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma total -cholesterol levels of offspring at weaning

A growing body of evidence suggests that perinatal nutrition affects the health of the offspring later in life (Lucas, 1998), thus the effects of maternal dietary n-6 to n-3 PUFA ratios on the offspring's metabolic parameters are of considerable interest. In the current study, lower plasma total cholesterol levels were observed in 5:1 and 15:1

groups compared to 30:1 group in both male and female offspring at weaning obtained from dams fed various dietary ratios of n-6 and n-3 PUFA.

There are few studies of the effects of maternal n-3 PUFA and n-6 PUFA supplementation during the perinatal period on offspring lipid parameters. A lower plasma cholesterol level was reported in macrosomic rat offspring where diabetic dams were fed EPA+DHA (n-6 to n-3 PUFA ratio of = 0.49) compared to a control group (n-6 to n-3 PUFA ratio of =25.8) at day 60 and 90 days after birth (Yessoufou *et al.*, 2006). However, other studies in Sprague-Dawley rats reported no change in plasma cholesterol levels in male and female offspring of dams fed diets containing (n-6 to n-3 PUFA ratios of = 2.5, 8.3 and 17.5) at 3 weeks (Korotkova *et al.*, 2002; Korotkova *et al.*, 2005).

Dietary intervention studies in adult mice have reported that n-3 PUFA, mainly EPA and DHA, reduced the expression of SREBP-1 which acts as a transcription factor of the lipogenic genes (Kim *et al.*, 1999; Nakatani *et al.*, 2003). The n-3 PUFA, such as EPA and DHA, are capable of reducing the expression of many lipogenic enzymes, i.e. sterol-CoA desaturase (SCD) and fatty acid synthase (FAS), thereby inhibiting VLDL production. Feeding diets high in n-3 PUFA to adult C57BL/6 mice also inhibit HMG-Co A reductase activity, leading to reduced serum cholesterol levels compared to an n-6 PUFA diet (Du *et al.*, 2003; Oh *et al.*, 2009). It will be important to investigate in the future whether varying the maternal n-6 to n-3 PUFA ratios alter HMG Co A reductase activity or affect VLDL secretion from liver.

The lipid lowering effect of n-3 PUFA could also be due to the regulation of PPARs which are activated by n-3 PUFA and are known to up-regulate genes of fatty acid oxidation (Couet *et al.*, 1997). The n-3 PUFA are more potent in vivo activators of PPAR-α than n-6 PUFA (Engler & Engler, 2000; Frenoux *et al.*, 2001), thus n-3 PUFA may lead to decreased plasma lipid levels by up regulating PPARs to increasing fatty acid oxidation (Schmitz & Ecker, 2008).

The pattern of CE levels was similar to that of TC levels with no significant changes in the FC levels among the dietary groups in male offspring. It is likely that the lower TC levels in the 5:1 and 15:1 groups are due to low CE levels in the male offspring. On the other hand, the female offspring obtained from mothers fed a 5:1 diet showed lower FC levels compared to 15:1 and 30:1 groups; however, no difference was observed in the 15:1 and 30:1 groups. A lower CE level was observed in 15:1 group compared to 30:1 group; however, 5:1 group did not differ from either 15:1 or 30:1 groups in female offspring.

The synthesis of CE level is regulated by the enzyme acyl coenzyme A: cholesterol acyltransferase 2 (ACAT2, EC 2.3.1.26) which converts FC to CE from FC in the liver (Joyce *et al.*, 1999). Fish oil, a rich source of n-3 PUFA, has been shown to decrease the activity and mRNA expression of ACAT (Smit *et al.*, 1991; Botham *et al.*, 2003). Plausible explanations for reduced TC levels in 5:1 and 15:1 groups could be due to all or one of the mechanisms discussed above. Therefore, further investigation is needed to confirm whether the programming effects of lower n-6 to n-3 PUFA ratio are

due to inhibition of HMG Co-A reductase activity, reduced SREBP expression, reduced ACAT expression or by increasing beta oxidation due to activation of PPARs.

4.2.5: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma triglyceride levels of offspring at weaning

No significant differences were observed in TG levels in male offspring obtained from mothers fed various n-6 to n-3 PUFA ratios, while there was a trend towards lower TG levels in the 5:1 and 15:1 groups. The female offspring of mothers fed 5:1 and 15:1 diets showed significantly lower TG levels (40% and 22% decrease respectively) compared to the 30:1 group.

There are only a few studies to show the effects of maternal n-3 PUFA and n-6 PUFA supplementation during pregnancy and lactation periods on plasma TG levels; data from these studies are inconsistent. Three studies on rats reported no change in plasma TG levels in male and female offspring of mothers fed n-3 PUFA diet and n-6 PUFA diet at 105 days (Ibrahim *et al.*, 2009) and at 3 weeks (Korotkova *et al.*, 2002; Korotkova *et al.*, 2005). However, Joshi *et al.* (2003) reported a lower TG level at 6 months in male offspring of dams fed an n-3 PUFA rich diet during pregnancy, while female offspring showed no difference.

Studies using adult animal models have also reported differential effects of dietary n-3 PUFA on plasma TG levels. Some studies in mice reported no change in TG levels when fed n-3 PUFA diet (Zampolli *et al.*, 2006; Riediger *et al.*, 2008), while other studies

on rats revealed that n-3 PUFA lowers plasma TG levels mainly through the down regulation of TG synthetic enzymes (Rustan *et al.*, 1992; Ribeiro *et al.*, 1991; Geelen *et al.*, 1995) and by increasing in liver beta-oxidation (Yamazaki *et al.*, 1987; Halminski *et al.*, 1991), compared to n-6 PUFA. Nevertheless, a majority of the data support the school of thought that dietary n-3 PUFA reduce plasma TG level compared to n-6 PUFA (Harris & Bulchandani, 2006).

The most plausible explanation for the reduced TG levels in the offspring of dams fed a lower n-6 to n-3 PUFA ratio in the current study may be attributed to stimulation of increased peroxisomal and mitochondrial beta oxidation due to higher accretion of n-3 PUFA. A high maternal n-3 PUFA diet has been shown to increase n-3 PUFA status in human infants (Connor *et al.*, 1996; Elias & Innis, 2001; Helland *et al.*, 2001). It was found recently that DHA is preferentially transported to the foetus compared to LA (Larque *et al.*, 2003). In the current study, the 5:1 group showed the highest total n-3 PUFA and DHA levels followed by 15:1 and 30:1 group suggesting a higher incorporation of n-3 PUFA. Since, n-3 PUFA are better in-vivo activators of PPAR-α than n-6 PUFA (Engler & Engler, 2000; Frenoux *et al.*, 2001), cellular beta oxidation is likely to be enhanced in the 5:1 and 15:1 groups thereby resulting in lower TG levels compared to the 30:1 group.

Many studies using adult rodents have shown that reduced TG levels in n-3 PUFA fed groups were due to decreased activity of TG synthesizing enzymes. It will therefore be important to further investigate whether the programming effects of plasma

TG levels in the current study were due to increased beta oxidation or decreased activity of TG synthesizing enzymes.

4.2.6: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratios on plasma LDL-cholesterol and HDL-cholesterol levels of offspring at weaning

The association between CVD and high levels of LDL-cholesterol is well established (Munro & Cotran, 1988; Kinsella *et al.*, 1990). Lower LDL-cholesterol levels were observed in 5:1 and 15:1 groups compared to the 30:1 group in male offspring. However, in the female offspring, 15:1 group showed lowest LDL-cholesterol levels compared to the 30:1 group.

The effects of n-3 PUFA on plasma LDL-cholesterol levels are inconsistent. In line with the low LDL-cholesterol levels in the male offspring in the current study, Yamashita *et al.* (2005) also reported significant reductions in plasma LDL-cholesterol concentrations in apoE-/- LDLr-/- double knockout mice fed diets enriched in n-3 PUFA. On the other hand, fish oil supplementation with 6 g/day of EPA and DHA has been reported to increase LDL-cholesterol in humans (Connor *et al.*, 1993). An increase in LDL-cholesterol concentration was also reported in hypertriglyceridemic participants after treatment with DHA enriched eggs (Maki *et al.*, 2003). However, it has been

suggested that a higher amount of fish oil could lower LDL-cholesterol concentrations (Harris *et al.*, 1983).

One plausible explanation of low LDL-cholesterol levels observed in the 5:1 and 15:1 groups in male offspring may be due to enhanced expression of LDLr. Previous reports have demonstrated that an increase in LDLr activity lowers plasma LDL-cholesterol levels after supplementation with n-3 PUFA in rats (Ventura *et al.*, 1989; Spady, 1993). On the other hand, decreased ACAT activity (Smit *et al.*, 1991; Botham *et al.*, 2003) and TG levels (Rustan *et al.*, 1992; Ribeiro *et al.*, 1991; Yamazaki *et al.*, 1987; Halminski *et al*, 1991) also result from n-3 PUFA supplementation, which may lead to lower synthesis of VLDL, resulting in lower levels of LDL Whether a decrease in LDL-cholesterol levels in the male offspring in the current study is due to an increase in LDLr mRNA expression or a decrease in ACAT activity needs to be further investigated.

The reasons for increased LDL-cholesterol levels after fish oil supplementation have never been clearly determined. Nonetheless, the growing body of evidence for increased LDL-cholesterol levels by fish oil diet suggest alterations in the physical properties of LDL particle. It has been suggested that the size of VLDL secreted by the liver is small when fed fish oil and small VLDL particles readily convert to LDL, resulting in increased LDL-cholesterol levels (Packard *et al.*, 1984; Mori *et al.*, 2000). Studies have shown that fish oil feeding leads to increased LDL particle size, which is less atherogenic in nature (Contacos *et al.*, 1993; Mori *et al.*, 2000). It is likely that the above is true for higher LDL-cholesterol levels observed in the female offspring in the

current study; future studies are essential to establish whether the LDL particle size is changed.

No significant changes were observed in HDL-cholesterol levels in either male or female offspring among the various dietary groups. Studies in adults also have shown no change in HDL- cholesterol level between n-3 PUFA and n-6 PUFA fed groups for 4 weeks, 20 weeks and for 32 weeks (Zampolli *et al.*, 2006; Magdeldin *et al.*, 2009; Wang *et al.*, 2009). Since both female and male offspring demonstrated low total cholesterol levels in the 5:1 and 15:1 groups, it is possible that these groups may contain higher HDL-2 cholesterol levels which have better cholesterol efflux capacity compared to 30:1 group. Therefore, it will be interesting to further investigate cholesterol efflux capacity of HDL particles among the various dietary groups.

A lower LDL to HDL-cholesterol ratio was observed in the 5:1 group compared to the 30:1 group in male offspring. Previous studies have also shown that n-3 PUFA are associated with lower LDL/HDL-cholesterol ratio in humans (Dawczynski *et al.*, 2010), and in rats (Vijaimohan *et al.*, 2006). Low LDL-cholesterol profile in 5:1 group in male offspring may have led to low LDL/HDL-cholesterol ratio. On the other hand, female offspring in 15:1 group showed the lowest LDL/HDL-cholesterol levels compared to the 5:1 and 30:1 groups however, there was no significant difference between the 5:1 and 30:1 group. Higher LDL/HDL-cholesterol ratio in 5:1 group is a result of increased LDL-cholesterol levels.

4.2.7: Effect of maternal dietary omega-6 to omega-3 polyunsaturated fatty acid ratio on red blood cell phospholipid fatty acid composition of offspring

The RBC PL fatty acid composition of the offspring was determined as an indirect marker to evaluate the effect of various maternal dietary fatty acid ratios on the fatty acid status of the offspring. No significant difference was observed in the percentage of total SFA content in RBC PLs in either male or female offspring reflecting the maternal dietary SFA content.

A lower n-6 to n-3 PUFA ratio was associated with a lower C18:1n9 level in RBC PL, which may be due to reduced expression of stearoyl-CoA desaturase-1 enzyme (SCD-1), a rate-limiting enzyme in the synthesis of monounsaturated fatty acids (Ntambi & Miyazaki, 2003). Previously, feeding diets enriched in n-3 PUFA to rats have been shown to reduce SCD-1 expression (Levy *et al.*, 2004).

An increased LA content in RBC PLs in 5:1 group may be a result of poor conversion of LA to long chain n-6 PUFA. It has been shown that both Δ5 and Δ6 desaturase prefer n-3 PUFA to n-6 PUFA (de Gomez and Brenner, 1975; Hagve and Christophersen, 1984). As the 5:1 group contains a higher level of n-3 PUFA, the reduced conversion of LA to AA in 5:1 group appears to be due to a higher competition of n-3 PUFA for the elongation and desaturation enzymes.

The long chain n-3 PUFA, essentially EPA and DHA, are critical for normal growth and development (Clandinin *et al.*, 1980b). Higher total n-3 PUFA, DPA and DHA content were observed in the 5:1 group followed by 15:1 and 30:1 groups reflecting

the maternal dietary fatty acid composition. Previous reports have also shown a high EPA, DHA content and a significantly lower n-6 to n-3 fatty acid ratio in RBC PLs where mothers were fed fish oil during pregnancy (Dunstan *et al.*, 2004; Arbuckle & Innis, 1993; Carlson *et al.*, 1986; Hrboticky *et al.*, 1990). The RBC PL composition is a good indicator of fatty acid composition of tissue and brain of the offspring (Sanjurjo *et al.*, 1995; Maurage *et al.*, 1998). Therefore, RBC PL fatty acid composition can also be used to predict the fatty acid composition of whole body fatty acid status.

A strong negative correlation between percentages of DHA and EPA in RBC PL and plasma total- and LDL-cholesterol levels, and a positive correlation between percentages of AA and plasma LDL-cholesterol levels in male offspring further supports the effect of dietary PUFA on plasma lipid and lipoprotein profiles. Similarly, plasma TG levels in female offspring were negatively correlated with percentages of DHA in RBC PLs and positively correlated with percentages of AA in RBC PLs. These correlation analyses suggest that n-3 PUFA are associated with reduced plasma lipid and lipoproteins, whereas n-6 PUFA are associated with increased plasma lipoprotein levels.

The fluidity of RBC membranes is highly dependent upon the lipid composition (Colin *et al.*, 1992). Since increased unsaturation results in an increase in membrane fluidity, increased incorporation of DHA and EPA in to RBC PLs in 5:1 group may increase the membrane fluidity of RBCs compared to 15:1 and 30:1 groups. Thus increased flexibility of RBCs is likely to allow easier passage through narrow blood vessels in tissue thereby improving blood supply and oxygen delivery. Moreover these altered properties may favour the flow of blood and reduce thrombus formation leading to

a reduced risk of atherosclerosis. Therefore in the current study, RBC PL fatty acid compositions of offspring demonstrate the critical role of maternal dietary n-6 to n-3 fatty acid ratio, on tissue accretion of fatty acids in the offspring.

4.3. Conclusion and Future work

Current recommendations to replace SFA with PUFA have led to an increased consumption of n-6 PUFA, shifting the n-6 to n-3 PUFA ratio from 2-3:1 in the diets of early hunter gatherers to 25:1 in the Western diet, which may increase the risk of CVD. In the current study, feeding diets with varying n-6 to n-3 PUFA ratios to C57BL/6 mice for one month altered the regulation of lipid and lipoprotein metabolism. A decrease in n-6 to n-3 PUFA ratio decreased plasma TC levels, which could be due to a decrease in HMG-CoA reductase activity, or an increased activity of CYP7. Future investigations are needed to understand the mechanisms behind TC lowering effects of n-6 to n-3 PUFA ratios. We also observed lower LDL-cholesterol levels as the n-6 to n-3 PUFA ratio was lowered, which could be due to increased LDLr gene expression that is a topic for future investigation.

The findings from the one month feeding study formed the basis to investigate the effects of maternal dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism of the offspring at weaning. The lower plasma TC in offspring obtained from dams fed 5:1 and 15:1 diets is possibly due to inhibition of HMG Co-A reductase activity, reduced SREBP expression, reduced ACAT expression or by increasing beta oxidation due to activation of PPARs. Future investigations are needed to understand the mechanisms responsible for the cholesterol and TG lowering effects of diets varying in n-6 to n-3 PUFA ratios.

The offspring were studied at weaning to understand the effects of pre-weaning diets. However, longer term studies should be planned where offspring can be fed, the same diet as that of the mothers or by switching the diets to investigate whether the effects of the pre-weaning diets are maintained when post-weaning diets are altered. Previously it was suggested that PUFA metabolism is affected by sex hormones (Extier *et al.*, 2010). Therefore differential effects observed in male and female offspring may be due to differences in sex hormones, which need to be explored in the future.

A limitation of the current study is that the observations cannot be directly related to humans due to differences in the lipid and lipoprotein metabolism.. In addition, menhaden oil was used in the current study as a source of n-3 PUFA that provided high amounts of EPA and DHA; whether alpha-linolenic acid, the essential n-3 PUFA, has similar effects on the regulation of lipid and lipoprotein metabolism is not known. Future studies can thus be designed using different animal models to relate changes in lipid and lipoprotein metabolism to humans, and also by using different sources of n-3 PUFA to establish whether the type of n-3 PUFA is important. .

Overall, findings from the current thesis support the role of maternal dietary n-6 to n-3 PUFA ratios on the regulation of lipid and lipoprotein metabolism of the offspring of C57BL/6 mice. Our findings also show that the programming effects are different in male and female offspring.

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