MATERNAL LIPID METABOLISM, CYTOKINE PROFILE, AND FETAL SUSTAINABILITY DURING GESTATION: IMPLICATION OF DIETARY OMEGA-3 POLYUNSATURATED FATTY ACIDS

By

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ABSTRACT

The quantity and quality of dietary fats consumed during pregnancy have profound implication on both maternal and fetal health during and after pregnancy. Our laboratory has previously shown that omega (n)-3 polyunsaturated fatty acids (PUFA) regulate offspring lipid and lipoprotein metabolism, and neurotrophin signalling in offspring brain. However, the effects of n-3 PUFA on pregnancy/fetal outcomes are controversial; this is likely due to differences in the amount and/or the source of n-3 PUFA in these studies. My thesis examined the *in-utero* effects of breeding chow diets, differing in the quantity and quality of dietary fats, on maternal metabolic regulation and pregnancy outcome in C57BL/6 mice. Female mice (7 weeks old) were fed specific diets for 2 weeks before mating and throughout pregnancy; tissues and blood samples were collected before and during gestation at day 6.5, 12.5 and 18.5. My findings revealed that a breeding chow diet containing n-3 PUFA from fish oil maintained maternal metabolic profile to meet fetal lipid requirement during gestation, prevented placental inflammation and sustained more fetuses till late gestation (Chapter 2 and 3). A limitation to this study was that the diets varied in both the quantity (5% vs. 11% w/w), and the quality (providing n-3 PUFA from fish oil at 8% vs. soybean oil at 3% w/w) of fat. I designed my second study using semi-purified diets (containing purified protein, carbohydrate, vitamins and mineral premixes) where the amount of fat was kept consistent (20% w/w), while the amount of n-3 PUFA was varied to give a diet high (9%), low (3%) and very low (1%) in n-3 PUFA from fish oil, respectively. My findings revealed that a maternal diet high in n-3 PUFA prevented dyslipidemia prior to pregnancy and maintained maternal lipid profile required for successful pregnancy. High n-3 PUFA diet also maintained plasma progesterone level during gestation, reduced inflammatory cytokines and sustained higher number of fetuses (Chapter 4). My findings also show that maternal diet high in n-3 PUFA increased the accretion of longer chain n-3 PUFA into fetal brain and regulates neurotrophin

signalling as gestation progressed (Chapter 5). Overall, my thesis findings demonstrate the importance of high n-3 PUFA intake during pregnancy.

CO-AUTHORSHIP STATEMENT

For the literature review presented in Chapter 1, some information used has been published in the *Journal of Nutrition & Intermediary Metabolism (2016) 5, 23 – 33,* and as a book chapter titled "Omega-3 fatty acids in the prevention of maternal and offspring metabolic disorders" in *Personalized Nutrition as Medical Therapy for High-Risk Diseases, CRC press, Taylor & Francis (2020) 283 – 302.* I prepared these manuscripts, and I am the first author on these publications. Dr. Sukhinder Kaur Cheema reviewed and edited the manuscripts.

For the work presented in Chapter 2, published in *Reproduction (2017) 154, 153 – 165,* I, Olatunji Anthony Akerele, conceived the idea, and was involved in the design of the study, conducting the experiment, analysing the data and preparation of the manuscript; I am the first author. Dr. Sukhinder Kaur Cheema received the funding, supervised the project, aided in study design and interpreting the results, reviewed and edited the manuscript.

For the work presented in Chapter 3, published in *Prostaglandins, Leukotrienes and Essential Fatty Acids (2018) 137, 43 – 51,* I, Olatunji Anthony Akerele, conceived the idea, and was involved with the design of the study, conducting the experiment, analysing the data and preparation of the manuscript; I am the first author. Dr. Sukhinder Kaur Cheema received the funding, supervised the project, aided in study design and interpreting the results, reviewed and edited the manuscript.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ACACA	Acetyl-CoA carboxylase 1
ALA	Alpha linolenic acid
ANOVA	Analysis of variance
ABCA1	ATP Binding cassette sub-family A member 1
ABCG1	ATP-binding cassette sub-family G member 1
BDNF	Brain derived neurotrophic factor
CHD	Coronary heart disease
CHMS	Canadian Health Measures Survey
CREB	cAMP response element binding protein
COMA	Committee on Medical Aspects of Food and Nutrition Policy
COX	Cyclooxygenase
CVD	Cardiovascular disease
DGAT	Diacylglycerol acyltransferase
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EL	Endothelial lipase
ELOVL	Elongation of very long chain fatty acids
EPA	Eicosapentaenoic acid
FABPpm	Plasma membrane fatty acid binding protein
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport proteins

FO	Fish oil-based diet
GDM	Gestational diabetes mellitus
HDL	High-density lipoprotein
HMGCR	3-Hydroxy-3-methyl-glutaryl Coenzyme A reductase
IFN-γ	Interferon gamma
ISSFAL	International Society for the Study of Fatty Acids and Lipids
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-10	Interleukin-10
LA	Linoleic acid
LDL	Low-density lipoprotein
LIF	Leukemia inhibitory factor
LOX	Lipooxygenase
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCP-1	Monocyte chemoattractant protein-1
MFSD2A	Major facilitator superfamily domain 2A
MMP	Matrix metalloproteinase
MUFA	Monounsaturated fatty acid
N-3	Omega-3
N-6	Omega-6
NEFA	Non-esterified fatty acids
NGF	Nerve growth factor
PC	Phosphatidylcholine

pCREB	Phosphorylated CREB
PE	Phosphatidylethanolamine
PG	Prostaglandins
PL	Phospholipid
PLA_2	Phospholipase A_2
PPAR	Peroxisome proliferator-activated receptor
PTB	Pre-term birth
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell
RXR	Retinoid X receptor
SCD1	Stearoyl-Coenzyme A desaturase 1
SFA	Saturated fatty acid
StAR	Steroidogenic acute regulatory protein
SR-B1	Scavenger receptor class B type I
SREBP-1c	Sterol regulatory element-binding protein 1c
SO	Soybean oil based diet
TC	Total cholesterol
TG	Triacylglycerol
TNF-α	Tumour necrosis factor
TrKB	Tropomyosin receptor kinase
uNK cells	Uterine natural killer cells

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CHAPTER ONE

Introduction and Overview

INTRODUCTION

Maternal nutritional status during pregnancy is a major factor in healthy prenatal development, as well as programming for adult diseases (Barker *et al*., 1989; Laker *et al.*, 2013; Lithell *et al.*, 1996). The development of several chronic diseases has been clearly associated with early life insults *in utero* (Barker *et al.*, 1993; Laker *et al*., 2013). Extreme nutritional deficiency, such as low protein and essential fatty acid intake, at critical periods of pregnancy increases the risk of cardio-metabolic diseases in the offspring, which manifest at childhood or later in life (Barker *et al.*, 1989; Voortman *et al*., 2015). Conditions characterized by severe undernutrition during pregnancy, as typified by the Biafran famine (Barker *et al.*, 1993; Laker *et al*., 2013) and Dutch Hunger Winter (Schulz, 2010), have the potential to impact fetal development and health outcomes negatively. A number of studies have shown that the quantity, as well as the quality of dietary fats, consumed during pregnancy have profound implications on maternal health during and after pregnancy, and fetal health outcome (Coletta *et al.*, 2010; Schwab *et al.*, 2014).

The quantity and quality of essential fatty acids consumed during pregnancy is crucial in growth and development of the fetus, and to maintain maternal metabolism (Coletta *et al*., 2010; Greenberg *et al.*, 2008). Linoleic acid (LA) and alpha-linolenic acid (ALA) are the essential omega (n)-6 and n-3 polyunsaturated fatty acids (PUFA), respectively. Once consumed, these fatty acids are converted to longer chain n-6 and n-3 fatty acids, such as arachidonic acid, an n-6 PUFA, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the n-3 PUFA (Abedi & Sahari, 2014). The longer chain n-3 and n-6 PUFA fatty acids, play critical roles during fetal growth and development (Birch *et al*., 2007; Singh, 2005; Uauy *et al*., 1992). Earlier study revealed that the mean n-3 PUFA intake of about 90% of Canadian women is only 82 mg per day (Denomme *et al*., 2005), which is far below the recommendation of the international organizations such as ISSFAL (200 mg/day DHA or 300 mg/day; EPA + DHA) (Table 1.1) (GOED, 2014).

Table 1.1: Global Recommendations for Omega-3 Polyunsaturated Fatty Acids Intake

Organization	Org. Type	Target Population	Recommendation
World Health Organization (WHO)	Authoritative Body	General adult population	n-3 PUFAs: 1-2% of energy/day
Food and Agriculture Organization of the United Nations (FAO)	Authoritative Body	0-6 months	DHA: 0.1-0.18%Energy
		6-24 months	DHA: 0.1-0.18%Energy
		2-4 years	DHA: 10-12 mg/kg birth weight
		4-6 years	EPA + DHA: 100-150 mg
		6-10 years	EPA + DHA: 150-200 mg
		Pregnant/Lactating Women	EPA + DHA: 0.3 g/d of which at least should be 0.2 g/d DHA
International Society for the Study of Fatty Acids and Lipids (ISSFAL)	Expert Scientific Organization	General adult population for cardiovascular health	At least 500 mg/day of EPA+DHA
		Pregnant/Lactating Women	DHA: 200 mg/day
NATO Workshop on n-3 and n-6 Fatty Acids	Workshop	General Adult Population	300-400 mg EPA+DHA/day
World Association of Perinatal Medicine	Working Group	Pregnant and Lactating Women	200 mg DHA/day
		Infants, when breastfeeding is not possible	0.2-0.5% weight total fat
World Gastroenterology Organisation	Expert Scientific Organization	General Adult Population	3-5 servings/week of fish

A summary of global *n*-3 PUFA intake recommendation; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; n-3 PUFA: omega-3 polyunsaturated fatty acids; NATO: North Atlantic Treaty Organization.

According to the Canadian Health Measures Survey (CHMS), only 15% of Canadian adults consume fish at least twice a week; as such, only 2.6% of Canadian adults (20 – 79 years old) meet the recommended longer chain n-3 PUFA index (Langlois & Ratnayake, 2015). Of keen interest is the fact that the mean n-3 PUFA intake among Canadian women of reproductive age (20 – 39 years old) was lower, compared to older adults (Langlois & Ratnayake, 2015). Surprisingly, only 27 % and 25 % of women consume adequate amounts of longer chain n-3 PUFA during pregnancy and lactation, respectively (Jia *et al*., 2015). Apparently, most women do not get enough longer chain n-3 PUFA during and after pregnancy. The requirement for longer chain n-3 PUFA increases during pregnancy because the fetus accumulates about 50 – 70 mg of longer chain n-3 PUFA during pregnancy (Innis, 2005). Nevertheless, the amount of longer chain n-3 PUFA required as pregnancy progresses from early to late gestation is yet to be established.

1.1 Metabolism of essential fatty acids

LA and ALA are considered essential fatty acids because humans lack the enzyme required for their endogenous synthesis via the insertion of a *cis* double bond at the 3^{rd} and 6^{th} carbon of n-3 and n-6 PUFA, respectively (Bell *et al*., 1997). Thus, LA and ALA must be obtained from dietary sources. Plant seeds and vegetable oils such as soybean, corn, safflower and sunflower oils are major sources of LA, while seeds like flax, chia and perilla are rich in ALA (Saini & Keum, 2018). Upon the consumption of these fatty acids, longer chain n-6 PUFA (arachidonic acid; AA) and n-3 PUFAs (eicosapentaenoic acid; EPA and docosahexaenoic acid; DHA) are synthesized endogenously from LA and ALA, respectively through a series of desaturation and elongation processes (Leonard *et al*., 2004) (Figure 1.1).

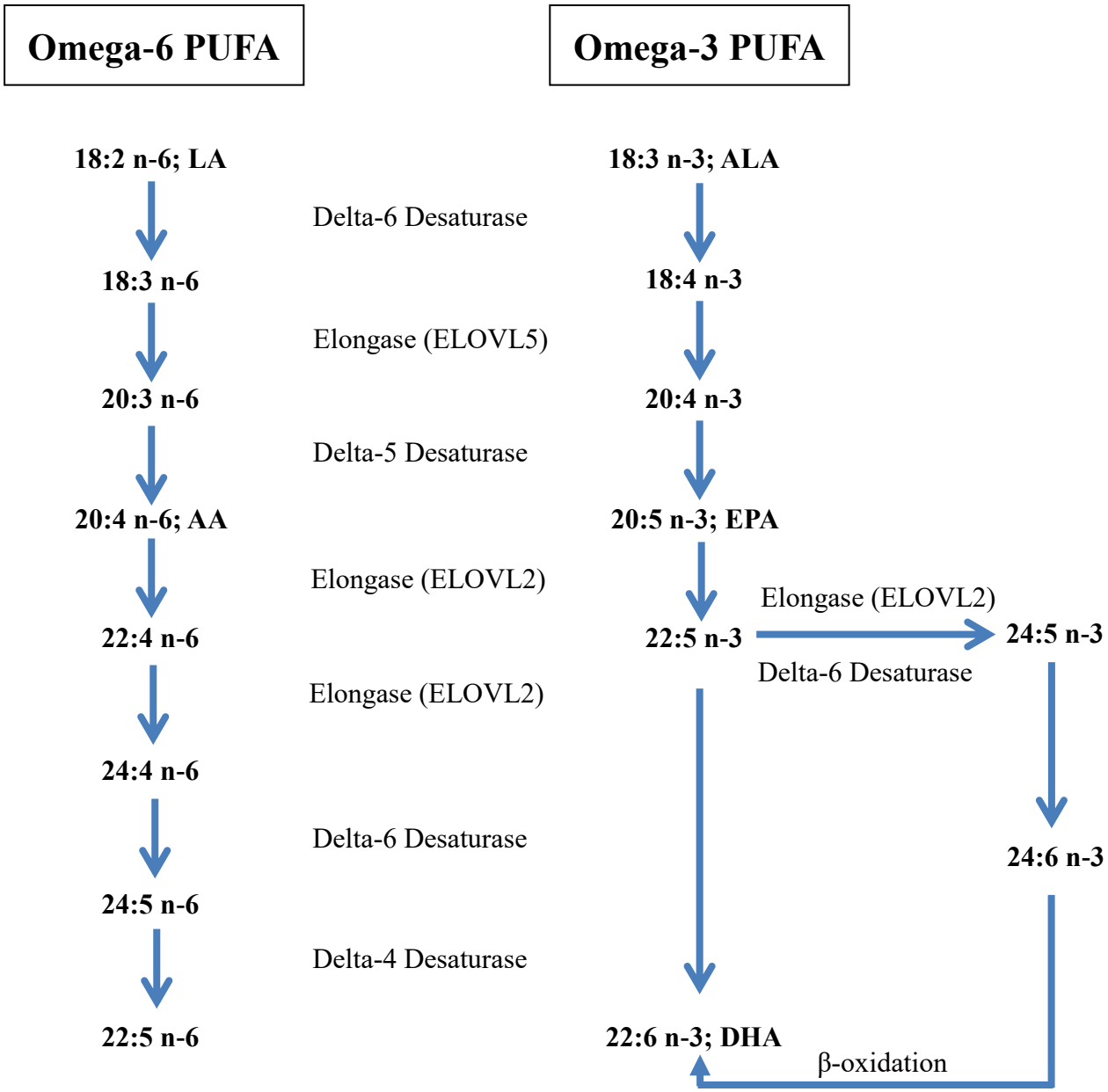

Figure 1.1: Pathway for the synthesis of longer chain essential PUFA. Modified from (Bokor *et al.*, 2010). *Journal of Lipid Research 51 (8); 2325–2333.* ALA: Alpha linolenic acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid; LA: Linoleic acid; PUFA: Polyunsaturated fatty acid.

Desaturases regulate fatty acid metabolism by removing two hydrogen atoms from a fatty acid, thus creating a carbon-carbon double bond. The double bond is inserted between the third and fourth carbon from the methyl end to create n-3 PUFA (Hastings et al., 2001). Desaturase-5 and -6 are required for the synthesis of longer chain n-3 and n-6 PUFA. In the biosynthesis of longer chain fatty acids, elongases alternate with desaturases repeatedly. Elongases catalyse carbon chain extension of fatty acids (Jump, 2009). Members of the elongation of very long chain fatty acids (ELOVL), particularly ELOVL-2 and -5 play major role in the elongation of n-6 and n-3 PUFA (Jakobsson et al., 2006).

Longer chain n-3 PUFAs such as docosapentaenoic acid (DPA), EPA and DHA can be obtained directly from fatty fish and fish oils like menhaden, salmon, sardine and herring oils (Saini & Keum, 2018). Interestingly, a number of studies using radiolabelled fatty acids have established that the rate of metabolism of essential PUFA is sex-specific; the conversion of ALA to DHA has been shown to be higher and faster in women of reproductive age compared to men of similar age group (Burdge & Wootton, 2002; Burdge & Calder, 2005). This finding was supported by a kinetic study revealing that the rate of DHA synthesis was about 4-fold higher in females, compared to males (Pawlosky *et al*., 2003). In men, the conversion rate of ALA to EPA is about 8%, while ALA to DHA conversion is between 0 - 4%. On the other hand, about 21% and 9% of ALA is converted to EPA and DHA, respectively, in women (Burdge & Wootton, 2002).

The conversion of ALA to DHA has been suggested to increase during pregnancy, essentially as a physiological adaptation that ensures adequate delivery of essential fatty acids to the developing fetus during pregnancy (Burdge, 2004; Burdge & Calder, 2005). Differences in the DHA levels between non-pregnant and pregnant women have therefore been established to reflect a huge variation in the metabolic capacity for endogenous DHA synthesis (Burdge & Wootton, 2002). Metabolic regulation is carefully controlled during pregnancy; this allows mothers to support fetal growth and development as pregnancy progresses. For example, the

fetus relies on the supply of lipids, and specific fatty acids, from maternal source for proper growth and development (Herrera & Ortega-Senovilla, 2010; Zeng *et al*., 2017), thereby establishing the importance of maternal lipids and lipoprotein metabolism during pregnancy.

1.2 Lipid and lipoproteins regulation during pregnancy

Pregnancy is a dynamic state involving several physiological changes, with a concomitant alteration in maternal metabolic profile (Lain & Catalano, 2007). Metabolic changes in the liver alter the levels of circulating triacylglycerol (TG), cholesterol, and fatty acids. However, changes in maternal lipid metabolism during pregnancy could be divided into two distinct phases: anabolic and catabolic, respectively (Grimes & Wild, 2018). Maternal lipids profile changes significantly as pregnancy transitions from anabolic to catabolic phase (Hadden & McLaughlin, 2009; Vrijkotte *et al*., 2012). The first trimester of pregnancy is characterized by increased lipid synthesis and storage (anabolic phase), in order to meet fetal lipid and energy requirement at later stage of pregnancy (Herrera, 2002; Zeng *et al.*, 2017).

Interestingly, endogenous lipid synthesis (*de novo* lipogenesis) at early gestation in humans is in-part regulated by increased insulin sensitivity (Benito *et al*., 1982; Wilcox, 2005). A similar study in rats has attributed lipid accumulation during early pregnancy to enhanced insulin responsiveness (Ramos *et al*., 2003). Knockout mouse models of the rate-limiting enzymes for endogenous lipid synthesis (acetyl-CoA carboxylase; *ACACA* and fatty acid synthase; *FAS*) showed embryonic death, demonstrating the importance of lipogenesis during pregnancy (Abu-Elheiga *et al*., 2005; Chirala *et al*., 2003). Besides *ACACA* and *FAS*, diacylglycerol acyltransferase-2 (*DGAT2*) also plays a key role in hepatic lipogenesis by catalysing the final reaction for the formation of TG; TG plays a key role in fetal growth and development by carrying essential fatty acids to the placental interface (Yen *et al*., 2008; Zammit, 2013). Maternal plasma TG level increases as pregnancy progresses in humans and in mice, however, its level falls progressively to pre-conception levels, as pregnancy approaches

parturition (Grimes & Wild, 2018; Hadden & McLaughlin, 2009; Herrera, 2002; Nikolova *et al*., 2017).

Cholesterol is also an important contributor to pregnancy progression and fetal development (Bartels & O'Donoghue, 2011; Woollett, 2005). Cholesterol is a key component of the cell membrane, where it plays pivotal roles such as regulation of membrane fluidity and permeability (Woollett, 2005). Cholesterol within the membranes has been shown to affect the function of other membrane lipids (Patton, 1970), thus indicating that cholesterol is an important mediator of fundamental lipid metabolism via signal transduction (Fielding & Fielding, 2004). Tissue cholesterol originates from either exogenous sources or from *de novo* synthesis, and the rate-limiting enzyme for endogenous cholesterol synthesis is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), which catalyses the conversion of HMG-CoA to mevalonate (Friesen & Rodwell, 2004)

Fetuses depend largely on maternal cholesterol during pregnancy, and it has been shown that fetal cholesterol levels correlate directly with maternal plasma cholesterol in rodents (McConihay *et al*., 2001). Increased delivery of maternally-derived cholesterol to fetal circulation occur during gestation, so as to meet fetal high cholesterol demand during rapid growth phase (Woollett, 2005). It has been shown that mothers with impaired cholesterol synthesis have a high risk of congenital malformations (Herman, 2003; Kratz & Kelley, 2003). In mice, plasma total cholesterol (TC) decreases as pregnancy progresses from early to late gestation (Nikolova *et al.*, 2017). Maternally derived cholesterol has been shown to cross the placenta during early gestation to support fetal growth, and also serves as a precursor for the synthesis of sex steroid hormones, particularly progesterone and estradiol, which are essential for a successful pregnancy (Grimes & Wild, 2018; Herrera, 2002; Lindegaard *et al*., 2005). Steroidogenic acute regulatory protein (*StAR*) mediates the cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of estradiol and progesterone (Hu *et al*., 2010; Stocco & Clark, 1996). Of interest is the establishment of direct relationship between

changes in sex-steroid hormones during pregnancy and lipogenesis (Goldberg & Hegele, 2012). For instance, increased circulating maternal lipids during pregnancy has been found to be driven by rise in the levels of progesterone during pregnancy (Grimes & Wild, 2018).

Pregnancy has also been associated with a significant decrease in the proportion of low-density lipoprotein (LDL) particles, with a concomitant increase in the level of high-density lipoprotein cholesterol (HDL)-c (Belo *et al*., 2002; Brizzi *et al*., 1999); this phenotype is considered to be potentially protective and less-atherogenic to mothers. Maternal HDL-c peaks at mid-gestation and decreases progressively towards late-gestation (Belo *et al*., 2002). However, dysregulation in maternal lipids metabolism during pregnancy has been implicated in a number of adverse pregnancy outcomes such as pre-eclampsia, gestational hypertension, gestational diabetes and complications during delivery (Belo *et al*., 2002; Enquobahrie *et al*., 2004; Hadden & McLaughlin, 2009; Herrera, 2002; Catov *et al*., 2007; Vrijkotte *et al*., 2012). Our laboratory and others have shown that dietary n-3 PUFA regulates lipid and lipoprotein metabolism in mice and humans, respectively, to confer cardiovascular benefits (Balogun *et al*., 2014; Ooi *et al*., 2015). However, no study has investigated the effects of maternal diet varying in the quality and quantity of n-3 PUFA on the regulation of maternal lipids and lipoprotein metabolism prior to, and at different stages of pregnancy, and its impact on pregnancy outcomes in mice.

1.2.1 Roles of n-3 PUFA in lipids and lipoproteins metabolism during pregnancy

Abnormal lipids levels during pregnancy is an independent risk factor for adverse pregnancy outcomes (Enquobahrie *et al*., 2004). Longer chain n-3 PUFA (EPA + DHA; 3-4 g/d) has been reported to reduce TG levels in dyslipidemic individuals by up to 35% (Harris, 1997; Kris-Etherton *et al*., 2002; Leslie *et al*., 2015; Skulas-Ray *et al*., 2019). The mechanism through which n-3 PUFA reduces TG has been linked to the propensity of longer chain n-3 PUFA to regulate key lipogenic genes such as peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs), liver X receptor (LXR)-alpha and retinoid X receptor (RXR)-alpha (Yoshikawa *et al*., 2002). LXR regulates the expression of SREBP1-c by

forming a heterodimer with RXR-alpha and binding to the LXR-response site in SREBP1-c, thereby inhibiting its expression (Yoshikawa *et al*., 2002). SREBP1-c has been established as the key regulator of lipid synthesis (Eberlé *et al*., 2004). As such, n-3 PUFA regulates endogenous lipid synthesis by regulating the gene expression of SREBP1-c, and consequently *ACACA* and *FAS* gene expressions, which are required to initiate *de novo* synthesis of TG (Strable & Ntambi, 2010). Longer chain n-3 PUFA has also been suggested to lower cirulating TG levels and prevent dyslipidemia by increasing β-oxidation of free fatty acids (Pégorier *et al*., 2004). N-3 PUFA has also been shown to indirectly stimulate β-oxidation of free fatty acids by regulating the expression of PPAR-alpha, which then increase fatty acid catabolism by upregulating the expression of acyl coenzyme A oxidase (Jump & Clarke, 1999).

Endogenous lipid synthesis is a normal physiological response required to maintain pregnancy, and gestation-dependent increase in lipid metabolism during pregnancy has been well documented (Hadden & McLaughlin, 2009; Herrera, 2002). As such, the risk of dyslipidemia has been shown to be high during pregnancy (Belo *et al*., 2002; Enquobahrie *et al*., 2004). Maternal dyslipidemia impairs placental function, with extended consequences on pregnancy and perinatal health outcomes (Louwagie *et al*., 2018). A number of studies have reported that n-3 PUFA regulates maternal metabolism and elicits positive pregnancy outcomes (Horvath *et al*., 2007; Makrides *et al.*, 2006). For instance, intake of n-3 PUFA has been shown to improve hyperlipidemia and restore anti-oxidant status of diabetic dams and their offspring in rats (Soulimane-Mokhtari *et al*., 2005; Yessoufou *et al.*, 2006). Nonetheless, the effects of n-3 PUFA on maternal lipids metabolism at different time points during gestation, and how this influence placental lipids profile and pregnancy outcomes is not known.

1.2.2 N-3 PUFA and placenta lipids metabolism during pregnancy

Placental development during pregnancy is a remarkable adaptation required for efficient materno-fetal interaction, and optimal fetal growth. Placental functions are precisely coordinated to ensure adequate and timely exchange of oxygen, nutrient and waste materials between the

mother and the developing fetus. As pregnancy progresses, dilated blood vessels and the resultant increase in blood flow to the placenta enhances the transfer of nutrients and oxygen to meet the demand of the growing fetus (Gude *et al*., 2004). In addition to the physiological and functional characteristics of the placenta, other changes such as preferential transfer of essential fatty acids occur during the last trimester of pregnancy in order to accommodate the metabolic requirement of the developing fetus (Duttaroy, 2009; Gude *et al*., 2004).

During pregnancy, increased transfer of DHA across the placenta, coupled with the upregulation of maternal metabolic capacity for DHA synthesis, play a key role in regulating the amount of DHA available in fetal circulation (Duttaroy, 2009). Pre-formed longer chain PUFA, especially DHA and AA are selectively and preferentially transferred from maternal circulation across the placenta to the fetus during pregnancy (Duttaroy, 2009; Montgomery *et al*., 2003). Thus, the pool of longer chain n-3 PUFA available for fetal use is predominantly regulated by maternal dietary longer chain n-3 PUFA status and the placental function (Duttaroy, 2009). The transfer of essential fatty acids across the placenta interface has been shown to occur either by passive diffusion or through membrane transporters (Duttaroy, 2009; Y. Xu *et al*., 2006). However, membrane protein-mediated translocation of fatty acids has been established to be the major means through which fatty acids are delivered to the fetus (Duttaroy, 2009; Lewis *et al*., 2018). A number of membrane transporters have been identified to be quantitatively important in the transport of n-3 PUFA across the placenta interface; these include fatty acid translocase (FAT/CD36), fatty acid transport proteins (FATP) and plasma membrane fatty acid binding protein (FABPpm) (Duttaroy, 2009; Lewis *et al*., 2018).

The activity of key lipase enzymes has been shown to be upregulated during pregnancy, which further contribute greatly to the availability of longer chain n-3 PUFA at the placental interface, and subsequent transfer to the fetus (Herrera, 2002; Waterman *et al*., 1998). During gestation, the placenta uptakes the maternal circulating non-esterified fatty acids (NEFA) released by maternal lipoprotein lipase (LPL) and endothelial lipase (EL) (Gil-Sánchez *et al*.,

2012). EL hydrolyses both phospholipids and TGs (McCoy *et al*., 2002); studies have shown that increased EL expression contributes majorly to placental fatty acid uptake (Lindegaard *et al*., 2005). Interestingly, lipolytic activity has been shown to increase exponentially at late gestation (Elliott, 1975), leading to the release of NEFA and perhaps contributes greatly to the delivery of longer chain n-3 PUFA to the fetus to promote healthy fetal growth and development. This may explain why deficiency in essential longer chain n-3 PUFA supply due to inadequate perinatal consumption or placental dysfunction has been attributed to specific adverse pregnancy outcomes (Morgan, 2014). Furthermore, n-3 PUFA has been shown to cause significant changes in the placental fatty acid composition and function during pregnancy (Jones *et al*., 2014). However, the implication of maternal diet containing different dosage of n-3 PUFA on the fatty acid composition and regulation of placental fatty acid transporters at mid- and late gestation remain unknown.

1.2.3 Roles of placenta in sex-steroid hormones synthesis and n-3 PUFA metabolism

In addition to serving as a functional interface for materno-fetal substance exchange during pregnancy, placenta also participates in the biosynthesis of sex-steroid hormones such as progesterone and estradiol, which play key roles in pregnancy maintenance (Grimes & Wild, 2018; Herrera, 2002; Lindegaard *et al*., 2005). Maternal circulating estradiol level increases during pregnancy due to increased synthesis by the placenta, and this has been suggested to contribute substantially to increased conversion of ALA to DHA during pregnancy (Giltay *et al*., 2004). The effect of estradiol on ALA to DHA conversion was subsequently proposed to be mediated by PPAR-alpha activation (Kitson *et al*., 2010). Alteration in this conversion pathway could affect the proportion of EPA and DHA in maternal circulation (Lemaitre *et al*., 2011), and perhaps impact pregnancy progression and fetal development negatively. The ALA to DHA conversion pathway is also complemented by increased mobilization of accumulated DHA reserves in the maternal tissues prior to conception (Burdge & Calder, 2005), and also by

supplementing maternal diet with DHA during pregnancy. As such, DHA intake of women prior to conception and during pregnancy may impact the amount of DHA available for fetal use.

A number of studies have reported higher levels of circulating EPA and DHA in women, particularly in lipid fractions such as free fatty acids and phospholipids, as well as total lipids, compared to men (Bakewell *et al*., 2006; Crowe *et al*., 2008; Giltay *et al*., 2004). Sex-dependent difference in DHA levels has also been reported in tissue samples, indicating that females have higher DHA in adipose tissues (Walker *et al*., 2014), red blood cells (RBC) (Metherel *et al*., 2009), and platelets (Geppert *et al*., 2010). This observation has been consistent across a number of ethnic groups (Abdelmagid *et al*., 2015). One plausible explanation for greater DHA in females is that estradiol may influence the enzymatic synthesis of longer chain fatty acids. Synthesis of DHA was observed to be 3-fold higher in women using contraceptive containing 17 α-ethylnyloestradiol, compared to those who did not use synthetic estrogen (Burdge & Wootton, 2002). Administration of oral ethinyl estradiol also increased the concentration of DHA in plasma cholesteryl esters by 42%, compared to control (Giltay *et al*., 2004). An animal study using rats revealed a higher gene expression of desaturases and elongases in females (Extier *et al*., 2010), which further increased when subcutaneously injected with estradiol, therefore enhancing the conversion of ALA to DHA (Kim *et al*., 2019). Apparently, estradiol plays a key role in upregulating the elongation and desaturation pathway in females, which may contribute to the physiological increase in plasma DHA concentration during pregnancy.

Progesterone has also been established to mediate a sex-dependent increase in the synthesis of longer chain n-3 PUFA in a dose-dependent fashion by increasing the mRNA expression of desaturase enzyme in an *in vitro* study (Sibbons *et al*., 2014). However, greater fractional conversion of ALA to DHA in women could also in part be due to a significantly lower rate of dietary ALA utilization for beta-oxidation (Williams and Burdge 2006; Abedi and Sahari 2014). Another possible biological significance of greater DHA synthesis capacity in pregnant women is to meet the demands of the fetus and neonate for this essential fatty acid.

During pregnancy, the pathway for the synthesis of longer chain n-3 PUFA has been shown to be highly efficient, so as to meet both maternal and fetal requirements (Chambaz *et al*., 1985). Since desaturase activity in developing liver of human neonates appears to be lower than that in adults (Poisson *et al*., 1993), the fetus depends on pre-formed DHA from maternal circulation in order to satisfy their DHA requirement. As such, maternal plasma phosphatidylcholine DHA increases by approximately 33% between mid- and late gestation (Postle *et al*., 1995). Increase in maternal blood volume (Gregersen & Rawson, 1959) could also be a significant adaptation to an increase in maternal DHA levels during pregnancy.

1.2.4 Dietary n-3 and n-6 PUFA intake

Drastic changes in the dietary pattern over the years to a Western diet has been implicated in a significant shift in the ratio of n-6 to n-3 PUFA from about 1 - 2:1 to about 20-30:1 (Gómez Candela *et al*., 2011). This transition has been found to promote the pathogenesis of chronic diseases such as cardiovascular disease (CVD), diabetes and obesity (Simopoulos, 2016). Metabolism of longer chain n-3 PUFAs, especially DHA and EPA, generally produce less inflammatory lipid mediators, which have been shown to reduce the risks of specific clinical conditions such CVD (Mori, 2014; Mozaffarian & Wu, 2011), while n-6 PUFA are generally considered inflammatory in nature (Calder, 2013). As such, a diet with a balanced intake of n-6 and n-3 PUFAs produce less inflammatory and less immunosuppressive eicosanoids (Abedi & Sahari, 2014), thereby protecting maternal health, and improving fetal growth and development. Most international organizations and countries have made different recommendations regarding n-3 PUFA intake; however, the recommended ratio of total n-6 to n-3 PUFA intake is approximately 5:1 (Table 1.2).

Table 1.2: International n-6:n-3 PUFA intake recommendation

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

A summary of the n-6:n-3 PUFA intake recommendation worldwide composed by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2010.http://www.issfal.org/statements/pufa-recommendations/recommendations-by-others. CHD: Coronary heart disease; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; N-3: Omega-3; LCPUFA: Long chain polyunsaturated fatty acids; NATO: North Atlantic Treaty Organization.

1.3. Roles of inflammatory cytokines in pregnancy establishment and outcomes

Pregnancy was initially thought to be characterized by either pro-inflammatory or anti-inflammatory molecules (Wegmann *et al*., 1993). However, other studies have disproved the pro- or anti-inflammatory dichotomy during pregnancy. Pregnancy in human is divided into three (3) distinct stages (trimesters), which are characterized by different proportions of anti- and pro-inflammatory molecules (Paulesu *et al*., 2010). The first trimester of pregnancy has been shown to be primarily characterised by increased production of pro-inflammatory cytokines, which play key roles in embryo reception / implantation, as well as the co-ordination of materno-fetal cross-talk (Dimitriadis *et al*., 2005; Jones *et al*., 2014; Paulesu *et al*., 2010).

Complex sequences of signalling cascades are required during implantation (a critical stage in pregnancy establishment), involving a harmonized dialogue between the active blastocyst and the endometrium; this is largely mediated by pro-inflammatory cytokines (Paulesu *et al*., 2010; Simón *et al*., 2000). Likewise, activities involving uterine contraction regulation and cervical ripening at late gestation are mediated by pro-inflammatory signals in the uterus (Kelly, 2002; Paulesu *et al*., 2010). On the other hand, mid-gestation (second trimester of pregnancy) requires anti-inflammatory molecules to establish uterine quiescence (absence of myometrial contractions and reduced risk of pregnancy loss), which is important for optimal fetal development.

Embryonic implantation occurs about 9 days after fertilization in humans and this process involves several cytokines such as interleukins (IL), interferon (IFN)-γ, and tumour necrosis factor (TNF)-α. TNF-α regulates the synthesis and activity of matrix metalloproteinase (MMP-2 and MMP-9) which is associated with the invasive phase of blastocyst implantation (Cohen *et al*., 2006; Meisser *et al*., 1999). IFN-γ is involved in the initiation of endometrial vasculature remodelling, maintenance of implantation sites, and the decidua (maternal component of the placenta) (Murphy *et al.*, 2009; Orsi, 2008). Prior to embryo implantation, activities involving

endometrial function and embryo reception regulation has been shown to be mediated by cytokines such as TNF-α (Cohen *et al*., 2006; Meisser *et al*., 1999), IL-1 (Minas *et al*., 2005; Paulesu *et al.*, 2010), IL-6 (Cork *et al.*, 2002; Dimitriadis *et al*., 2005), and IFN-γ (Murphy *et al.*, 2009; Suzuki *et al*., 1996) (Table 1.3).

IL-1 also plays an important role in pregnancy establishment; injection of IL-1 receptor antagonist into mice, prior to implantation, resulted in implantation failure (Simón *et al*., 1998). IL-1 is also involved in the stimulation of several other cytokines such as TNF-α, and IL-6 (Minas, Loutradis and Makrigiannakis, 2005). The presence of IL-1 ligands (IL-1α and IL-1β) in human embryo culture medium has been associated with high implantation rates in patients undergoing *in vitro* fertilization-embryo transfer (Karagouni *et al*., 1998).

Progression of pregnancy towards mid-gestation elicits a shift in cytokines profile toward less inflammatory/anti-inflammatory molecules (Paulesu *et al*., 2010). A handful of studies have shown that anti-inflammatory cytokines such as IL-10 play an important role in the inflammation resolution during pregnancy, especially at mid-gestation (Chatterjee *et al*., 2014; Paulesu *et al*., 2010). Inflammation resolution system is essentially required during pregnancy to regulate complex processes that could degenerate into inflammation-mediated complications. Pre-term birth (PTB) has been associated with the induction of prostaglandin synthesis before term *via* excessive production of pro-inflammatory cytokines like TNF-α, IL-6, and IL-1β which trigger pre-term labour (Keelan *et al*., 2003). Also, infusion of IL-6 and TNF-α has been shown to produce symptoms of pre-eclampsia in rats (Lamarca *et al*., 2011; LaMarca *et al*., 2005). However, other studies have associated IL-10 deficiency with the onset of hypoxia-induced pre-eclampsia features such as proteinuria, hypertension and renal pathology (Lai *et al*., 2011). As such, administration of recombinant IL-10 was observed to reverse features of pre-eclampsia in IL-10 knock out pregnant mice (Lai *et al*., 2011).

Table 1.3: Roles of pro-inflammatory cytokines in pregnancy establishment

Cytokines	Production site(s)	Roles in implantation	Reference(s)
TNF-α	Peri-implantation endometrium	Regulates the synthesis and activity of matrix metalloproteinase (MMP-2 and MMP-9)	(Cohen *et al*., 2005; Meisser *et al*., 1999)
IFN-γ	Uterus NK cells and trophoblasts	Initiates endometrial vasculature remodelling, angiogenesis at implantation sites, maintenance of the decidua	(Murphy *et al*., 2009; Orsi, 2008)
IL-1	Endometrium and blastocyst	Stimulate the secretion of other cytokines (IL-6, LIF, and TNF-α), regulates uterine receptivity, play important role in embryo implantation and decidualization	(Dimitriadis *et al*., 2005; Minas *et al*., 2005; Paulesu *et al*., 2010)
IL-6	Embryo and uterus (stroma cells and endometrial epithelium)	Regulates endometrial function and synthesis of MMP-2 and MMP-6, involved in viability of implantation sites and decidua formation	(Cork *et al*., 2002; Dimitriadis *et al*., 2005; Paulesu *et al*., 2010)

IFN-γ: Interferon gamma; IL: Interleukine; LIF: Leukemia inhibitory factor; MMP: Matrix metalloproteinase; TNF-α: Tumour necrosis factor alpha; uNK cells: Uterine natural killer cells.

IL-10 has been shown to peak on gestation day 12 in mice, which represents second trimester (Lin *et al*., 1993). Inhibition of IL-10 during pregnancy has been shown to result in neonatal growth retardation (Rijhsinghani *et al*., 1997), while administration of exogenous IL-10 has been shown to prevent fetal resorption in pregnant CBA/J x DBA/2 mice (Chaouat *et al*., 1995). Although the role of IL-10 in neonatal survival has not been clearly elucidated in mice (Svensson *et al*., 2001); however, IL-10 plays a pivotal role in inflammation regulation and the prevention of detrimental pregnancy outcomes. *GATA-3* has been implicated in the maintenance of IL-10 levels (Lee *et al*., 2000; Zheng & Flavell, 1997), thus regulating the levels of corresponding pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ, and monocyte chemotactic protein-1 (MCP-1) in maternal placental interface (Akerele & Cheema, 2016; Thaxton & Sharma, 2010).

At near term, cytokine profiles have been characterized to align towards increased production of pro-inflammatory cytokines, as they play vital roles in the coordinating processes leading to cervical ripening and uterine contraction during labour (Paulesu *et al*., 2010). More so, an increased production of major pro-inflammatory cytokines such as TNFα, IL-1β, and IL-6 in the uterus has been shown to play key role in cervical dilation and myometrial contraction during labour (Molnár *et al*., 1993).

Clearly, a balance of pro- and anti-inflammatory cytokine is very important for successful pregnancy establishment and maintenance. As such, an imbalance in cytokines profile could result in detrimental pregnancy outcomes. A plethora of evidence has shown that longer chain n-3 PUFA could alter the production, as well as the activities of pro- and anti-inflammatory cytokines (Simopoulos, 2002), which may have a profound effect on pregnancy establishment and outcomes. However, the effects of maternal diet varying in the amounts of n-3 PUFA (and/or n-6:n-3) on maternal cytokines profile during different stages of gestation and its impact on pregnancy outcome are not known.

1.3.1 N-3 PUFA and cytokine regulation during pregnancy

Metabolism of n-3 PUFA gives rise to anti-inflammatory molecules (Calder, 2013). Of keen interest is the fact that the same group of enzymes are required for the metabolism of n-6 and n-3 PUFA. The anti-inflammatory properties of n-3 PUFA is partly mediated by inhibiting the downstream production of pro-inflammatory molecules from n-6 PUFA metabolism such as prostaglandins and leukotrienes, which are regulated by cyclooxygenase and lipoxygenase enzymes, respectively (Schmitz & Ecker, 2008) (Figure 1.2).

N-3 PUFA has also been shown to elicit anti-inflammatory effects by producing pro-resolving molecules (Resolvins and protectins) and by inhibiting the production of nuclear factor kappa-B (NF-κB), which is a known transcription factor for a number of pro-inflammatory cytokines, including TNF-α and IL-6 (Calder, 2013). Also, n-3 PUFA has been shown to directly inhibit the gene expression of IL-6 and IL-1β (Yamashita *et al*., 2013). Studies have shown that supplementing maternal diet with 2 g n-3 PUFA (EPA + DHA per day) significantly decreased the production of IL-1, IL-6, and TNF-α by mononuclear cells (Trebble *et al*., 2003). Also, fish oil feeding reduces *ex vivo* production of IL-1β, IL-6, and TNF-α by macrophages in rodents (Renier *et al*., 1993). Cell culture studies also observe similar results, such that EPA and DHA inhibited the production of pro-inflammatory cytokines in macrophages and endothelial cells (Khalfoun *et al*., 1997; Lo *et al*., 1999). However, DHA has been shown to be more effective in reducing plasma TNF-α concentrations, compared to EPA with similar concentration, by 35% and 20% for DHA and EPA respectively (Mori *et al*., 2003); this effect can be attributed to the chain length of the fatty acids.

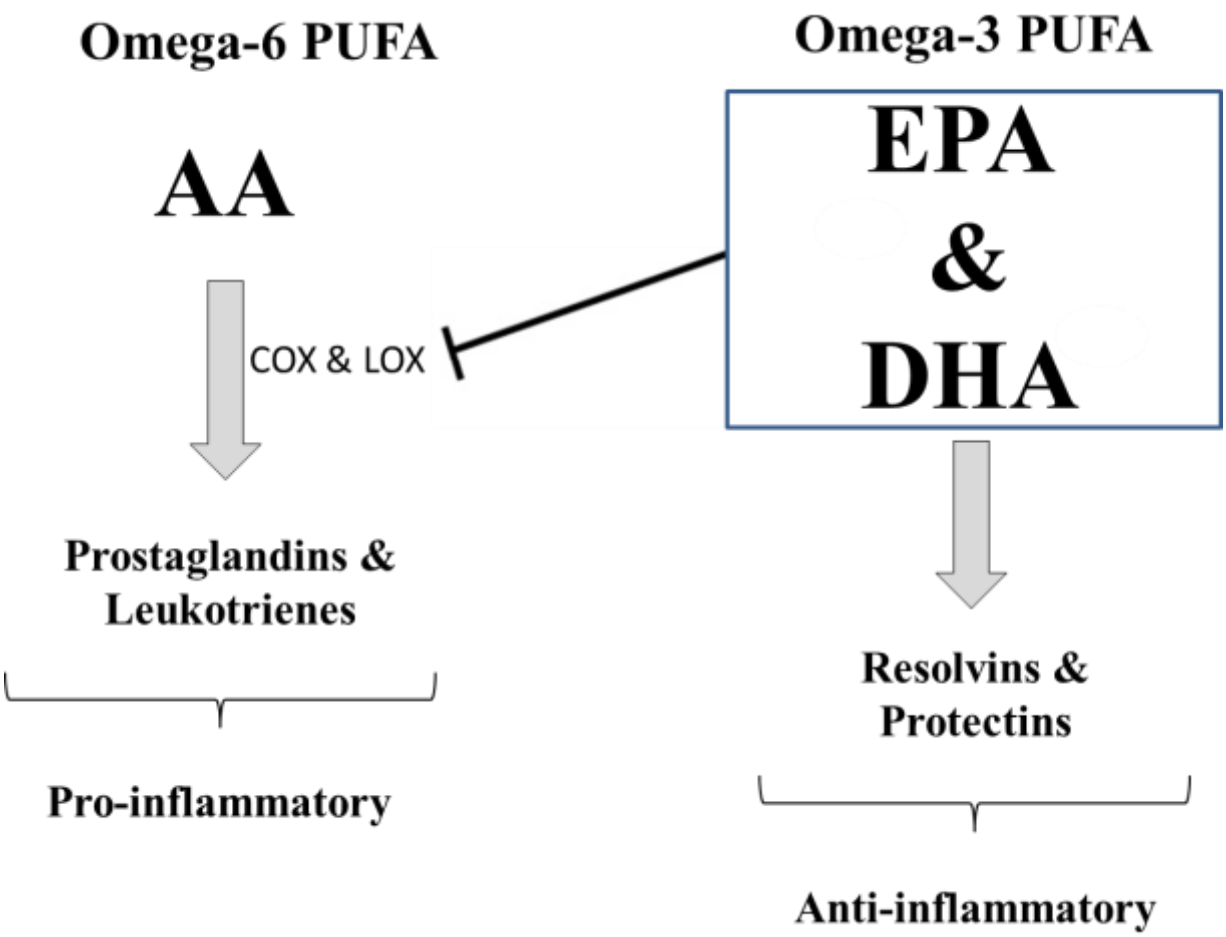

Figure 1.2: Anti-inflammatory property of omega-3 polyunsaturated fatty acids. N-3 PUFAs inhibit the downstream production of pro-inflammatory molecules such as leukotrienes and prostaglandins, and promote the secretion of protectins and resolvins. AA, Arachidonic acid; COX, Cyclooxygenase; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; LOX, Lipoxygenase.

The complexity of pregnancy establishment and progression is largely regulated by a number of cytokines and other factors playing unique roles at different stages of gestation (Jones *et al*., 2014; Paulesu *et al.*, 2010). As such, dietary intake of high amounts of n-3 PUFA prior to embryo implantation may downregulate the activities of key pro-inflammatory cytokines involved in the regulation of endometrial receptivity and labour induction; this may result in the prolongation of gestation length. From a paediatric point of view, extended gestational period and higher birth weight could be positive outcomes, compared to premature delivery. However, prolonged gestation is a predisposing factor for adverse fetal development, as well as complications during parturition (Olesen *et al*., 2003). Risk of peri-partum complications and perinatal death increases as pregnancy progresses beyond 40^{th} week of gestation (Caughey *et al*., 2007; Hilder *et al*., 1998). A majority of perinatal deaths in post-term pregnancy has been attributed to reduced placental function (Vorherr, 1975). Hence, establishing the required balance of n-6 to n-3 PUFA during gestation to prevent detrimental pregnancy outcomes is highly pertinent.

1.4 Roles of n-3 PUFA in the prevention of adverse pregnancy outcomes

The spectrum of evidence from the literature has shown that supplementing the maternal diet with n-3 PUFA during pregnancy reduces the risk of PTB, especially in high risk pregnancies (De Giuseppe *et al*., 2014; Horvath *et al*., 2007; Makrides *et al.*, 2006; Sjúrdur Fródi Olsen & Secher, 2002). Evidence from other studies has also shown that intake of marine-derived n-3 PUFA during pregnancy reduced the risk of adverse pregnancy conditions such as gestational diabetes mellitus (GDM), pre-eclampsia, maternal obesity and PTB (Haghiac *et al*., 2015; Makrides *et al*., 2006; Redman & Sargent, 2009; Rylander *et al*., 2014). A retrospective study on 84 women with GDM revealed that diabetes during pregnancy induces dyslipidemia, which was characterized by elevated TG and TC in maternal circulation (McGrowder *et al*., 2009). Intake of n-3 PUFA, especially DHA, was observed to be significantly lower in women

with gestational diabetes (Chen *et al*., 2010). More so, women with GDM have different plasma fatty acids profile compared to non-diabetic women (Chen *et al*., 2010), suggesting changes in maternal fatty acid metabolism in diabetic mothers.

GDM has also been shown to alter lipid metabolism in the offspring (Kilby *et al*., 1998), indicating that GDM is an underlying cause of dyslipidemia in the offspring. Several studies have established beneficial health effects of n-3 PUFA supplementation during pregnancy on maternal and fetal health. A prospective population based cohort study showed that fish consumption (75-100 g/d) among Norwegian women caused 30% reduction in the risk of developing type 2 diabetes, compared to those who did not consume fish (Rylander *et al*., 2014).

GDM is associated with systemic increase in the production of pro-inflammatory cytokines during pregnancy (Xu *et al*., 2014), thus exposing the fetus to an inflammatory environment during development. Supplementation of maternal diet with DHA and EPA (1200 mg/day) from week 16 of gestation to delivery exerts potent anti-inflammatory properties by lowering the expression of inflammatory cytokines in both adipose and placental tissues (Haghiac *et al*., 2015).

N-3 PUFA has also been shown to provide beneficial effects on the risk of PE. A prospective cohort study revealed that intake of EPA and DHA (100 mg/day), or fish consumption during the first trimester of pregnancy reduced the risk of PE (Oken *et al*., 2007). Observational studies found that women with higher levels of n-6 PUFA in the erythrocytes and platelets were 7.6 times more likely to have their pregnancies complicated by PE (Williams *et al*. 1995; Velzing-Aarts *et al*. 1999). As such, a 15% increase in the ratio of n-3 to n-6 PUFA has been associated with a 46% reduction in the risk of PE (Williams *et al*. 1995). An inverse association between n-3 PUFA intake during pregnancy is associated with PTB. Fish oil supplementation (2.7 g/d of EPA and DHA) at 20 weeks gestation reduced the recurrence of PTB from 33% to 21% (Olsen *et al*. 2000). Cohort studies have shown a dose dependent effect of marine foods consumption during pregnancy and the prevention of PTB (Olsen and Secher

2002; Olsen *et al.* 1993). An increased n-3 PUFA intake during pregnancy reduced the risk of PTB in high risk pregnancies (Horvath, Koletzko, and Szajewska 2007; Olsen and Secher 2002; Makrides, Duley, and Olsen 2006). As such, improving maternal n-3 PUFA status during pregnancy could be harnessed as a prophylactic strategy to prevent adverse pregnancy outcomes and improve fetal outcomes.

1.5 Omega-3 PUFA and fetal brain development

DHA is important for healthy brain development, as well as overall fetal growth during pregnancy (Singh, 2005; Uauy *et al*., 1992). The brain has the largest amount of lipids (60% dry weight), compared to other organs in the body (Chang *et al*., 2009). DHA constitutes about 10-15% of total fatty acids in the brain, which represents more than 97% of total n-3 PUFA (Makrides *et al*., 1994; O'Brien *et al*., 1964). It has been shown that there is acceleration of fetal brain growth during the second trimester (Coletta *et al*., 2010); perhaps, this is the most critical stage for DHA supplementation. However, DHA accumulation in the brain is most rapid during the third trimester of pregnancy and within the first year after birth (Clandinin *et al*., 1980; Martínez & Mougan, 1998). The fetus accrues up to 70 mg DHA per day during the last trimester, mostly in the brain (Innis, 2005), demonstrating the significance of maternal DHA status on healthy fetal brain development. Interestingly, a number of studies have shown that maternal DHA status is usually low during the third trimester, which explains a higher rate of transfer of DHA to the fetus (Montgomery *et al*., 2003). In contrast, low maternal n-3 PUFA levels (~20%) at the third trimester could be an in-built regulatory mechanism to support the synthesis of the pro-inflammatory molecules required to initiate cervical dilation and myometrial labour contractions. Nonetheless, a deficit of n-3 PUFA during pregnancy results in impaired cognitive and physiological functions in infants (Catalan *et al*., 2002), which has been suggested to be irreversible by postnatal supplementation (Nesheim & Yaktine, 2007).

Phospholipid is the primary lipid component in the brain, which is highly enriched in DHA (Sastry, 1985). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most

abundant phospholipid fractions in the brain (Rapoport, 2001). Longer chain n-3 PUFA in the form of phospholipid has been shown to be more bioavailable and efficiently metabolised by the brain, due to their higher sensitivity to phospholipases (Parmentier *et al*., 2007). However, it has been suggested that the absorption of DHA is further increased if present at the *sn*-1 position in phospholipids, as it becomes unavailable to pancreatic PLA_2 (Subbaiah *et al*., 2016). Thus, it is absorbed as lysophosphatidylcholine (LPC), and subsequently converted by acetyltransferase to PC, which readily enters the lymph and supplies DHA to the brain (Subbaiah *et al*., 2016). A member of the major facilitator superfamily (Mfsd2a) is required for the uptake of DHA into the brain (Nguyen *et al*., 2014), where DHA affects brain growth and development (Sona *et al*., 2018). However, the effect of maternal diet containing different dosages of n-3 PUFA on the regulation of Mfsd2a gene expression and fetal brain fatty acid composition at different stages of pregnancy remains unknown.

During pregnancy, the brain preferentially accumulates DHA, especially during late gestation to about two years after birth in humans and up to 21 days after birth in rodent (Green *et al*., 1999; Martinez, 1992); Martinez revealed a 30-fold increase in brain DHA. During this period, there is a rapid increase in the maturation of synapses and neuronal myelination (Dobbing & Sands, 1973), with a concomitant increase in brain weight (Carlson *et al*., 2013). It has been shown that the offspring of mothers consuming high amounts of n-3 PUFA during pregnancy have better cognitive abilities than non-consuming mothers (Daniels *et al*., 2004), suggesting that n-3 PUFA is vital for brain development and function. However, the exact mechanism through which n-3 PUFA regulates brain function during development remains unclear. The function of the brain is largely regulated by neurotrophins, and the expression of neurotrophins has been suggested to be altered by n-3 PUFA.

1.5.1 Neurotrophins and the brain

Neurotrophins are a group of trophic factors required for neuronal differentiation and survival (Huang & Reichardt, 2001); these include nerve growth factor (NGF) and brain-derived

neurotrophic factor (BDNF). Neurotrophins have been shown to be expressed in early life during the embryonic stage, as well as during middle and later stages of fetal development (Bernd, 2008; Birling & Price, 1995). Neurotrophins play a pivotal role in the development and function of the central nervous system by regulating neuronal survival, synaptic plasticity and cell differentiation (Reichardt, 2006). BDNF is one of the most studied neurotrophins in the central nervous system, and it has received tremendous attention in literature because of its importance in the development and maintenance of normal brain function (Bathina & Das, 2015). BDNF promotes neuronal development and survival, and prevents the death of peripheral sensory neurons at an early post-natal period in mice (Ernfors *et al*., 1994), while it modulates synaptic plasticity to enhance learning and long-term memory in adult mice (Egan *et al*., 2003). BDNF is synthesized as a precursor protein (pro-neurotrophin), which is then cleaved to release the mature BDNF (Chao *et al*., 2006), which binds to its high affinity receptor, tropomyosin receptor kinase B (TrKB).

Binding of BDNF to its receptor signals the downstream activation of the transcription factor cAMP-response element binding protein (CREB) to elicit brain development (Bhatia *et al*., 2011). DHA is known to differentially regulate BDNF and its target receptor at weaning and 16-weeks post-weaning in mice (Balogun & Cheema, 2014). A plethora of recent evidence from the literature have also shown that n-3 PUFA regulates BDNF in adult humans (Ferreira *et al*., 2014; Pawełczyk *et al*., 2019); however, a vast majority of neurons are formed prenatally in the brain. To date, the effects of maternal diet varying in the amount of n-3 PUFA on DHA accretion in fetal brain, and the regulation of gene expression of BDNF and its downstream signalling cascades at different stages of gestation are not known.

1.6 Controversies on n-3 PUFA and fetal sustainability during pregnancy

Literature reports are inconsistent on the effects of n-3 PUFA on fetal number/litter size in mice, and other animal models; some reported increases (Rebollar *et al*., 2014; Smits *et al*.,

2011), while others showed decreases (Fountain *et al*., 2008; Smit *et al*., 2015) or no effects (Estienne *et al*., 2006; Perez Rigau *et al*., 1995). This is likely due to differences in the amount and/or the source of n-3 PUFA in these studies. While a diet high in n-6 PUFA during gestation was not necessarily associated with an increase in litter size (Fattahi *et al*., 2018; Fountain *et al*., 2008; Ni *et al*., 2002; Shahnazi *et al*., 2018), it was found to cause intrauterine growth restriction (Reyes-Hernández & Ramiro-Cortijo., 2018). Fat-1 transgenic mice that are engineered to endogenously synthesize n-3 PUFA, and yield 1:1 tissue ratio of n-6:n-3 PUFA, show increased pregnancy rates (Hohos *et al*., 2018).

1.7 Mice as an animal models

Mice offer the advantage of short gestational periods (Croy *et al*., 2015). Their small size dramatically reduces the facilities required and allows for a large number of animals to be housed at one time, which is advantageous for doing larger and more comprehensive studies within a feasible time frame. Mice models have been extensively adopted over years by a multitude of research groups; thus, many functionally relevant antibodies, immunoassay and biochemical assay kits have been developed and are commercially available. Moreover, invasive cell types and implantation in mice are very similar to that in humans (Malassiné *et al*., 2003). Mice have also been extensively used to model innate and adaptive maternal immunity, as well as trafficking across the materno-fetal interface during pregnancy (Bonney & Matzinger, 1997).

The C57BL/6 mouse was used for this study because it is susceptible to high fat diet-induced dyslipidemia (Podrini *et al*., 2013). Moreover, our laboratory has established the C57BL/6 mouse as a model to study the effects of maternal dietary fats on offspring metabolism. These mice show significant diet-induced changes in lipid metabolism and are widely used for studies on reproduction, thus making it suitable for the study of lipid and lipoprotein metabolism during pregnancy. The C57BL/6 mouse model is also the strain of choice for the generation of numerous transgenic models to study pathological conditions related to lipid metabolism, as well

as brain function and behavioural studies, thus leaving the option of extending the scope of our study in the future.

1.8 Rationale: Aims and Objectives

Pregnancy is a dynamic process, with intricate metabolic adaptions to ensure proper establishment, smooth progression, as well as positive maternal and fetal outcomes. The quantity and quality of dietary fats consumed during pregnancy have profound implications on both maternal and fetal health during and after pregnancy. Our laboratory has previously reported the effects of maternal diets varying in the amount of n-3 PUFA on the regulation of lipid and lipoprotein metabolism, and alterations in the lipidomic profile of the offspring. However, the literature reports inconsistent effects of n-3 PUFA on pregnancy/fetal outcomes in animal models, reporting a decrease increase or no effect; this is likely due to differences in the amount and/or the source of n-3 PUFA in these studies. More so, dyslipidemia and disrupted balance of pro- and anti-inflammatory cytokines elicited adverse pregnancy outcomes. N-3 PUFA regulates lipid metabolism and inflammation; however, the regulation of maternal lipid metabolism and cytokines profile by n-3 PUFA during different gestation stages, and its impact on fetal sustainability is not known. This thesis explored the effects of maternal diet varying in n-3 PUFA prior to, and during gestation, on maternal metabolic profile, placental inflammatory cytokines, and fetal outcomes. Moreover, n-3 PUFA is known to regulate neurotrophin signalling in offspring brain. This thesis also sought to identify novel mechanisms through which n-3 PUFA elicits proper brain development during pregnancy via neurotrophin signalling using C56BL/6 mice.

The specific aims and the underlying hypotheses of my thesis were:

Aim 1: To investigate the effects of two different breeding chow diets varying in the quality and the quantity of dietary fat on maternal metabolic profile during different stages of pregnancy and its impact on pregnancy sustainability (Chapter 2).

Hypotheses: Several studies, including research from our laboratory, have shown that both the quality and the quantity of fat intake during pregnancy impact the health outcomes of the offspring. However, the effect of the quantity and the quality of dietary fat intake on maternal metabolic profile during different stages of gestation and its impact on fetal sustainability is not known. I therefore hypothesized that a maternal diet low in fat will cause an increase in lipogenesis during pregnancy to meet the requirements of the fetus. I further hypothesized that a maternal diet enriched with n-3 PUFA from fish oil will improve pregnancy outcome in terms of sustaining the number of fetuses during gestation.

Objectives: The specific objectives of this study were to investigate the effect of breeding chow diets differing in the quality and the quantity of dietary fats during gestation on:

1) the fatty acid composition of maternal RBC;

2) maternal plasma lipids and the mRNA expression of rate-limiting lipogenic genes in the liver,

3) cholesterol efflux capacity of maternal plasma using J774 cells;

4) the mRNA expression of *StAR* in the placenta and plasma concentration of progesterone and estradiol;

5) fetal sustainability as a measure of pregnancy outcome.

Aim 2: To investigate the effects of two different breeding chow diets varying in the quality and the quantity of dietary fat on the fatty acid composition of maternal uterus and the placenta at different stages of pregnancy, and its influence on the levels of pro- and anti-inflammatory cytokines in the maternal plasma and placenta (Chapter 3).

Hypotheses: There is a paucity of evidence on the effect of breeding chow diets differing in the quality and the quantity of dietary fats on the fatty acid composition of maternal uterus and the placenta at different stages of gestation, and its influence on the concentration of inflammatory and anti-inflammatory cytokines in the maternal plasma and placenta. I therefore hypothesized that a breeding chow diet containing n-3 PUFA from fish oil will cause a gestation-dependent increase in the incorporation of longer chain n-3 PUFA into the uterus and placenta, as well as

increase the accretion of longer chain n-3 PUFA in fetal brain of C57BL/6 mice, compared to a chow diet containing a plant-based n-3 PUFA. I further hypothesized that incorporation of longer chain n-3 PUFA from fish oil will cause a gestation-dependent reduction in the concentration of pro-inflammatory cytokines in maternal plasma and placenta to induce beneficial effects on pregnancy outcome.

Objectives: The specific objectives of this study were to investigate the effect of breeding chow diets differing in the quality and the quantity of dietary fats during gestation on:

1) the fatty acid composition of maternal uterus;

2) the mRNA expression placental fatty acid transporter and the incorporation of n-3 PUFA into the placenta and fetal brain;

3) concentration of pro-and anti-inflammatory cytokines in the maternal plasma and placenta.

Aim 3: To investigate the effects of maternal diet varying in the amount of n-3 PUFA on maternal lipids and lipoprotein regulation during gestation in C57BL/6 mice, and its impact on pregnancy outcome (Chapter 4).

Hypothesis: The effects of n-3 PUFA on the regulation of lipid metabolism and immune response are well known; however, no study to date has investigated the effects of different amounts of n-3 PUFA on maternal lipid profile during different stages of gestation, placental inflammatory response and its impact on pregnancy outcome. I hypothesized that a maternal diet high in n-3 PUFA will prevent gestational dyslipidemia, reduce inflammatory cytokines on the placental interface, and improve fetal outcomes, compared to low and very low n-3 PUFA diet.

Objectives: The specific objectives of this study were to investigate the effects of maternal diets containing different dosages of n-3 PUFA prior to and during gestation on:

1) the RBC and hepatic fatty acid composition, and the mRNA expression of lipogenic genes (*FAS, ACACA,* and *DGAT2*) in the liver;

2) maternal plasma and hepatic lipids profile, and the levels of sex-steroid hormones;

3) placental inflammatory cytokines, and the mRNA expression of *GATA-3*;

4) fetal numbers as an indicator of pregnancy outcomes.

Aim 4: To investigate the effects of maternal diets varying in the amount of n-3 PUFA on the fatty acid composition, and the regulation of mRNA expression of BDNF, TrKB and CREB in fetal brain at different gestation stages (Chapter 5).

Hypothesis: Neurotrophins and n-3 PUFA are important to proper functioning of the brain. However, the mechanism(s) through which *n*-3 PUFA regulate neurotrophin signalling at different gestation stages are not clear. I therefore hypothesized that maternal diet high in n-3 PUFA will cause an accretion of DHA in fetal brain during pregnancy, and consequently increase the mRNA expressions of BDNF, TrKB, and CREB in a gestation-dependent fashion.

Objectives: The specific objectives of this study were to investigate the effects of maternal diets containing different dosages of n-3 PUFA prior to and during gestation on:

1) placental fatty acid composition and fatty acid transporters;

2) the mRNA expression of Mfsd2a and incorporation of n-3 PUFA into fetal brain;

3) the regulation of mRNA expression of BDNF, TrKB and CREB in fetal brain.

1.8.1 Study Design and Limitation

The first set of studies outlined in this thesis were carried out to better understand the *in utero* effects of breeding chow diets, differing in the quantity and the quality of dietary fats, on maternal metabolic regulation and pregnancy outcome in C57BL/6 mice. A limitation of this study was that the breeding chow diets varied in both the quantity (5% vs. 11% w/w fat), and the quality (providing n-3 PUFA from fish oil at 8% vs. soybean oil at 3% w/w, respectively) of fat. In follow-up studies, I fed semi-purified diets (20% fat w/w) where the amount of n-3 PUFA was varied to give a diet high (9%), low (3%) and very low (1%) in n-3 PUFA (from fish oil), to C57BL/6 mice.

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CHAPTER TWO

A low fat diet enriched in fish oil increased lipogenesis and fetal outcome of C57BL/6 mice

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2.1 ABSTRACT

There is clear evidence that the nutritional strategy employed during pregnancy has profound influence on the offspring health outcomes. However, the effect of the quality and the quantity of maternal fat intake on maternal metabolic profile during different stages of pregnancy and its impact on pregnancy sustainability is not known. Female C57BL/6 mice (7 weeks old) were fed diets varying in the quantity of fat (5% *vs.* 11%) for two weeks prior to mating and throughout pregnancy. The 5% fat diet was enriched with longer-chain omega (n)-3 polyunsaturated fatty acids (PUFA) from fish oil. Maternal plasma and tissues were collected before mating and during pregnancy at day 6.5, 12.5 and 18.5. Plasma lipids, glucose, insulin, progesterone and estradiol levels were measured. Cholesterol efflux capacity of maternal plasma, as well as the mRNA expression of placental steroidogenic acute regulatory protein and hepatic lipogenic genes (acetyl-CoA carboxylase-1, fatty acid synthase, diacylglycerol acyltransferase-2 and stearoyl-CoA desaturase-1) was determined. Feto-placental weight and fetuses sustained throughout gestation were recorded. A low fat maternal diet enriched with n-3 PUFA increased maternal plasma triacylglycerol and the mRNA expression of rate limiting lipogenic enzymes, along with increasing cholesterol efflux capacity ($P<0.05$), likely to meet fetal lipid demand during pregnancy. Furthermore, a low fat diet enriched with longer-chain n-3 PUFA increased the maternal plasma concentration of progesterone and estradiol during pregnancy ($P<0.05$), which coincides with an increase in the number of fetuses sustained till day 18.5. These novel findings may be important when designing dietary strategies to optimize reproductive capability and pregnancy outcomes.

2.2 INTRODUCTION

The susceptibility of offspring to developing pathological conditions primarily originate from compromised intrauterine environment via nutritional insults (Barker *et al*., 1989; Perera & Herbstman, 2011). We have previously shown that the quantity and the quality of fat consumed during pregnancy and lactation has a profound effect on the aortic function, as well as the expression of brain derived neurotrophic factor in the offspring (Balogun & Cheema, 2014; Kanta *et al.*, 2010). Furthermore, we have established that the fatty acid composition of maternal diet has the potential to induce long lasting changes in the tissue fatty acid composition of the offspring (Chechi *et al*., 2010); this is very important because fatty acids play a key role in maintaining metabolic functions (Martínez-Fernández *et al*., 2015). The quality as well as the quantity of maternal fat intake is capable of programming set points for several physiological and metabolic factors for the mother, and also in the developing embryo during pregnancy, thereby impacting the health of the mother, as well as that of the offspring (Martin-Gronert & Ozanne, 2006).

As pregnancy progresses, complex metabolic adaptations occur which allow the mother to support the growth and development of the fetus. For example, during pregnancy, there is an increase in insulin secretion at early gestation as insulin stimulates hepatic *de-novo* synthesis and storage of TG (Benito *et al.*, 1982; Wilcox, 2005). Studies using knock-out mouse models of *ACACA* and *FAS*, the rate limiting enzymes for endogenous lipid synthesis, showed increased embryonic death demonstrating the importance of lipogenesis during pregnancy (Abu-Elheiga *et al*. 2005; Chirala *et al*. 2003). *DGAT2* and stearoyl-CoA desaturase-1 (*SCD1*) enzymes also play key role in hepatic lipid synthesis (Miyazaki *et al*., 2001; Zammit, 2013). *DGAT2* enzyme catalyses the final reaction for hepatic formation of TG (Yen *et al*., 2008; Zammit, 2013). Interestingly, lipogenesis has been shown to be inhibited in *SCD1* knock out mice model despite increased expression of *FAS* (Miyazaki *et al*., 2001).

An increase in TG synthesis during pregnancy contributes to fetal development by serving as a depot for fatty acids, which are released into fetal circulation via active feto-placental nutrient transport (Duttaroy, 2009). Additionally, maternally derived cholesterol also crosses the placenta at early gestation to support fetal growth and development (Herrera 2002). Cholesterol is also the precursor for the synthesis of estradiol and progesterone (sex steroid hormones), which are essential for a successful pregnancy (Hu *et al*., 2010). S*tAR* protein expression mediate cholesterol transfer within the mitochondrial, especially in the steroid-producing tissues such the ovary, testis, adrenal cortex, and the placenta (Lin *et al*., 1995; Stocco & Clark, 1996). Thus, maternal changes in lipid metabolism during pregnancy play an important role towards maintaining pregnancy and proper growth and development of the fetus.

The fetus also relies on the mother for the supply of essential fatty acids, especially DHA, a n-3 PUFA, that is important for brain and eyes (retina) development (Innis, 2007; Neuringer *et al*., 1988). The intake of maternal DHA has also been shown to reduce the risk of preterm delivery (Horvath *et al*., 2007; Olsen & Secher, 2002), and low birth weight (Imhoff-Kunsch *et al*., 2012), especially in high risk pregnancies. The essential fatty acid, alpha-linolenic acid (ALA), once obtained in the diet, is converted to DHA via elongation and desaturation. Since DHA is essential for brain and eyes development, the conversion of ALA to DHA is upregulated during pregnancy; however, this process is limited to about 9% conversion rate in women (Burdge & Wootton, 2002b; Childs *et al*., 2011). Thus, optimal growth and development of the fetus is dependent upon the nutritional, metabolic and hormonal environment provided by the mother.

Several studies, including research from our laboratory, have shown that both the quantity as well the quality of maternal fat intake impacts the health of the offspring (Balogun *et al*., 2013; Chechi *et al*., 2010; Coletta *et al*., 2010). However, the effect of the quality and the quantity of maternal fat intake on maternal metabolic profile during different stages of pregnancy and its impact on pregnancy sustainability is not known. We hypothesized that a low fat maternal

diet with longer chain n-3 PUFA will increase lipogenesis during pregnancy to meet the requirements of the fetus and will increase pregnancy outcome in terms of sustaining the number of fetuses during gestation. Our findings have revealed for the first time that a low fat maternal diet increased lipogenesis, and that a diet enriched in longer chain n-3 PUFA sustained a higher number of fetuses at late gestation.

2.3 MATERIALS AND METHODS

2.3.1 Animals and experimental design

All experimental procedures involving animals were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee (approval no: 15-11-SC). Male and female C57BL/6 mice (seven weeks old) were purchased from Charles Rivers Laboratories (MA, USA), and were housed in separate cages under controlled temperature (21 ± 1°C) and humidity (35 ± 5%) conditions with a 12-hour light/12-hour dark period cycle. Mice were kept on standard rodent chow pellets (Prolab RMH 3000) (PMI nutrition, MO, USA) for one-week acclimatization period. After this period, female mice were randomly divided into two groups, and each group was fed with breeding chow diet varying in the quantity and quality of fat (Table 2.1); 5% (w/w) fat (Pico-Vac Lab Rodent Diet, 5061; LabDiet, MO, USA) and 11% (w/w) fat (Mouse Diet, 5015; LabDiet, MO, USA) for two weeks. The 5 % fat diet contained n-3 PUFA from fish oil, while the 11 % fat diet contained n-3 PUFA from soybean oil.

Mating was carried out and female mice were checked by 6:00 am the following morning for vaginal plug formation to confirm pregnancy. Pregnant mice were continued on the assigned diets throughout gestation. Fresh food and water were provided *ad-libitum* every day. Body weight and food intake was recorded every day; no significant difference in food intake was observed (Appendix I).

Table 2.1: Macronutrient and caloric composition of the experimental diets (provided by LabDiet)

Macronutrients (% w/w)	5% Fat	11% Fat
Protein	20.0	18.9
Carbohydrate	52.9	51.8
Fat	5.0	11.1
Calories provided (%)		
Protein	24.6	19.8
Carbohydrate	62.1	54.2
Fat	13.2	26.1

Mice were sacrificed before pregnancy (non-pregnant) at early gestation (day 6.5), mid gestation (day 12.5), and late gestation (day 18.5) using isoflurane. Figure 2.1 depicts each stage of pregnancy. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4), and was separated immediately into plasma and red blood cells (RBC). Tissues were removed and weighed at the time of sacrifice, snap frozen in liquid nitrogen and stored at -80ºC until further analyses. Implantation sites and fetuses sustained throughout the gestation period were recorded. Uterine and fetal pictures were taken using Canon camera (SX500 IS). A statistical power analysis was performed to determine the sample size required to obtain significant effects of diet and gestation stage at $p<0.05$. All effort was made to minimize animal suffering.

2.3.2 Analyses of biochemical parameters and fatty acid composition

Lipids were extracted from the diets, RBC and liver samples according to the method of Folch *et al*. (1957) as per our previous publication (Chechi *et al*., 2010). Plasma biochemical parameters were quantified using commercially available kits according to the manufacturers' instructions: plasma and liver TG kit #236-17 (Genzyme Diagnostics, PEI, Canada); total cholesterol (TC) kit #234-60 (Genzyme Diagnostics, PEI, Canada); plasma glucose kit #10009582 (Cayman Chemical, US) and insulin (Mouse) ELISA Kit #KA3812 (Abnova corporation, Taiwan). Plasma progesterone and estradiol concentrations were determined using Architect Systems (B7K770 and B7K720 respectively). The fatty acid composition of the extracted lipids was determined using gas chromatography-flame ionization detection according to our previously published method (Chechi *et al*., 2010). The fatty acid composition of the diets is given in Table 2.2.

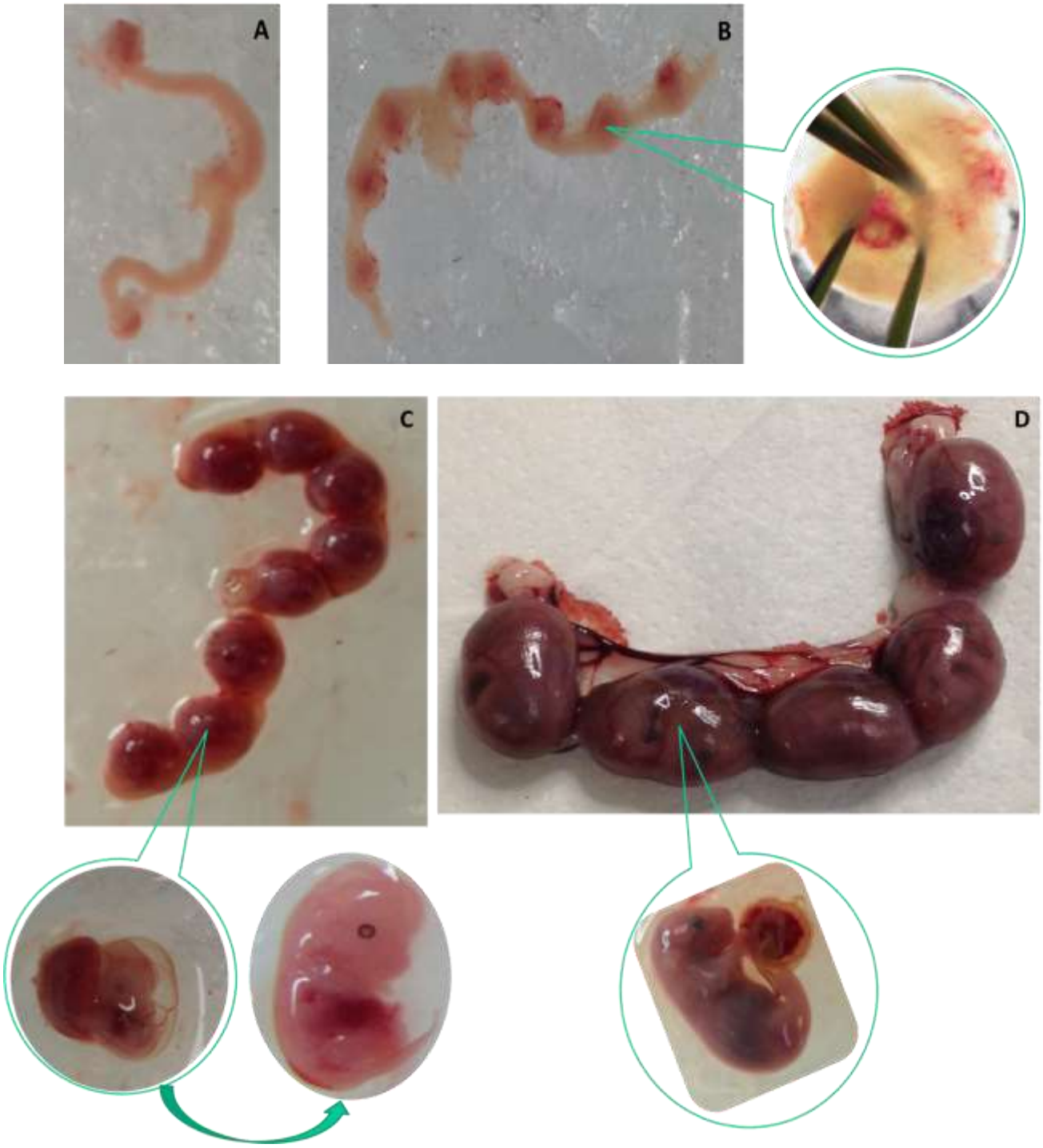

Figure 2.1: Developmental stages of mouse fetus. The uterus of a non-pregnant mouse (A); the uterus of a pregnant mouse at day 6.5 with forceps revealing the implantation site during surgery (B); the uterus of a pregnant mouse at day 12.5 showing fetus before (to the left side of the arrow) and after separation from the embryo sac and the placenta (to the right side of the arrow) (C); and the uterus of a pregnant mouse at day 18.5 showing a fully-developed fetus and placenta (D).

Table 2.2: Fatty acid composition of the diets

Fatty Acids (%)	5% Fat	11% Fat
C14:0	0.94	1.24
C16:0	14.77	20.98
C18:0	4.46	8.61
ΣSFA	**20.17**	**30.82**
C16:1	1.40	2.05
C18:1	21.40	31.73
C20:1	0.42	0.43
ΣMUFA	**23.23**	**34.21**
C18:2n6	48.85	31.87
C20:4n6	ND	0.18
Σ N-6 PUFA	**48.85**	**32.05**
C18:3n3	5.78	2.72
C20:5n3	0.88	ND
C22:5n3	0.31	ND
C22:6n3	0.86	ND
Σ N-3 PUFA	**7.76**	**2.72**
n-6:n-3	**6:1**	**12:1**

Data are expressed as weight percentage of the total extracted fatty acids. ΣSFA: sum of saturated fatty acids; ΣMUFA: sum of monounsaturated fatty acids; Σn-6 PUFA: sum of omega-6 polyunsaturated fatty acids; Σn-3 PUFA: sum of omega-3 polyunsaturated fatty acids; n-6:n-3: Omega-6 to omega-3 ratio; ND: Not detected.

2.3.3 Cholesterol efflux assay

Macrophage cholesterol efflux capacity assay of the plasma samples was determined using J774 cells according to our previously published method (Balogun et al., 2014). Briefly, the cells were seeded in 12-well plates at a density of $2x10^5$ cells/well in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic / anti-mycotic. The following day, the cells were labelled with RPMI supplemented with 1% FBS, 1μCi/ml ^{3}(H)-cholesterol (Perkin Elmer, MA, USA), 2 μg/ml acyl-CoA:cholesterol acyltransferase inhibitor (Sandoz, QC, CA), and 1x antibiotic / anti-mycotic for 24 hrs. Cells were equilibrated for 18 hrs in RPMI medium in the presence of liver X receptor agonist (1 μM) (Sigma, MO, USA), retinoic X receptor agonist (1 μM) (Sigma, MO, USA), retinoic acid (1 μM) (Sigma, MO, USA), and ATP-binding cassette A1 agonist (1 μM) (Sigma, MO, USA).

Cholesterol efflux was initiated by treating cells with 2% plasma samples from both 5% (n=8) and 11% fat group (n=8) collected at different stages of pregnancy as the efflux acceptor or 0.2% bovine serum albumin (BSA) as the negative control for 5 hrs. At the end of the efflux interval, the medium was collected from each well and centrifuged at 2000 rpm for 5 minutes. Supernatants were removed for liquid scintillation counting. Wells were washed twice with 1X PBS, and residual radioactivity in the cells was determined after scraping the cells in 1X PBS. Cholesterol efflux was calculated as ([^{3}H]-cholesterol in medium / [^{3}H]-cholesterol in medium + [^{3}H]-cholesterol in cells) x 100. All the efflux values were corrected by subtracting the percentage efflux at time zero (before active/passive efflux).

2.3.4 RNA extraction and real-time qPCR

Total RNA was extracted from liver and placenta samples using Trizol method (Chomczynski & Sacchi, 1987). Genomic DNA contamination was removed by treating with DNase enzyme (Promega, USA; #M6101). The concentration of the extracted RNA was determined using Nano Drop 2000 (Thermo Scientific, USA). RNA integrity was assessed using 1.2% agarose gel. Synthesis of cDNA from the extracted RNA was carried out using reverse-

transcription as per our previous publication (Balogun & Cheema, 2014). All primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) (Accessed on 09/06/2016) and obtained from IDT technologies (IA, USA); primer sequences and efficiencies are given in Table 2.3.

Amplification was performed using iQ SYBR Green Supermix (Bio-rad, USA). The reactions were run at a reaction volume of 10 µl and 50 ng cDNA per reaction. Samples were run using the CFX96TM Real-Time System while data output was managed using the CFX Manager™ Software Version 3.0. The delta Ct values were recorded for each of the gene of interest, corrected for amplification efficiency, and normalized with Beta-Actin (*Actb*) as the reference gene; there were no changes in the *Actb* gene expression between groups. The expression levels between the two groups were compared using the Livak method (Livak & Schmittgen, 2001), as shown in appendix II.

2.3.5 Statistical Analysis

Data were analysed using GraphPad Prism Software (version 5.0). Sample means were compared using two-way analysis of variance (ANOVA) to determine main effects of diet and time, and the interactions between them. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Results are expressed as mean ± standard deviation (SD) for n=8 in each experimental group. Real-time qPCR data were $\log_{10}$ transformed and fatty acid composition data were arcsine transformed (to normalize the data) prior to statistical analyses. Differences were statistically significant if $p<0.05$. Pearson's correlation was used to compare the relationship between plasma cholesterol and sex steroid hormones (progesterone and estradiol).

Table 2.3: Primers sequences and efficiencies

Gene(s)	Primers sequence (5' – 3')	Efficiency (%)
ACACA (S) *ACACA* (AS)	ggccagtgctatgctgagat agggtcaagtgctgctcca	89.2
FAS (S) *FAS* (AS)	ctgcggaaacttcaggaaatg ggttcggaatgctatccagg	104.7
DGAT2 (S) *DGAT2* (AS)	ctagctagttaggctaggtttcac caggaggatatagcgccagag	101.1
SCD1 (S) *SCD1* (AS)	agagtagctgagctttgggc acaccccgacagcaatatccag	92.8
STAR (S) *STAR* (AS)	tgcccatcatttcattcatcctt aaaagcggtttctcactctcc	94.8
Actb (S) *Actb* (AS)	cacgcagctcattgtagaagg atggtgggaatgggtcagaag	107.5

ACACA: Acetyl CoA carboxylase 1; *Actb:* Beta Actin; AS, anti-sense primer; *DGAT2*: Diacylglycerol acyltransferase-2; *FAS*: Fatty acid synthase; S: sense primer; *SCD1*: stearoyl-CoA desaturase-1; *StAR:* Steroidogenic acute regulatory protein.

2.4 RESULTS

2.4.1 Effects of diets on maternal RBC fatty acid composition

The RBC fatty acid composition is given in Table 2.4. The 5% diet group showed lower levels of C18:0 and total saturated fatty acids (SFA) ($p<0.005$ and $p<0.0001$, respectively), compared to the 11% group. The 5% fat group also showed lower levels of C18:1 and total monounsaturated fatty acids (MUFA), compared to the 11% group ($p<0.0001$), while C16:1n7 decreased with gestation in both groups ($p<0.05$). Moreover, the 5% diet group revealed a significant interaction between diet and gestation for C20:1; there was a significant decrease from day 6.5 to 18.5 ($p<0.05$) in the 5% group.

There was an independent effect of gestation on linoleic acid (C18:2n6; LA) and arachidonic acid (C20:4n6; AA); LA decreased as gestation progressed from day 6.5 to 18.5 for both dietary groups ($p<0.05$), while AA increased with gestation in the 5% group only ($p<0.01$). The 5% group revealed a higher amount of alpha-linolenic acid (C18:3n3; ALA), eicosapentaenoic acid (C20:5n3; EPA), docosapentaenoic acid (C22:5n3; DPA), and DHA, compared to the 11% group ($p<0.0001$). Both diet and gestation showed a significant interaction for EPA and total n-3 PUFA; EPA increased as the gestation progressed from day 6.5 to 18.5 in 11% group ($p<0.0001$). Interestingly, ALA was not detected at day 12.5 and 18.5 in the 11% diet group. Diet and gestation also had an independent effect on DHA; there was a significant increase from day 6.5 to 18.5 in both dietary groups ($p<0.001$); 5% group had higher DHA, compared to the 11% group ($p<0.0001$).

Table 2.4: Fatty acid composition of maternal red blood cells

Fatty	5% Diet			11% Diet			Main Effect		
Acids	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation Stage	Diet * Gestation
C14:0	1.70±0.15	1.44±0.43	1.54±0.12	1.23±0.15	1.46±0.07	1.25±0.23	p<0.0001	NS	NS
C16:0	29.28±0.99	29.17±1.43	30.30±1.97	29.42±0.99	29.62±0.41	30.58±0.24	NS	NS	NS
C18:0	13.84±0.57	14.31±0.51	14.60±1.00	17.38±0.57	15.81±0.52	15.91±1.02	p<0.005	NS	NS
ΣSFA	44.82±0.84	44.92±1.91	46.44±2.72	48.04±0.84	46.88±1.02	47.73±1.10	p<0.0001	NS	NS
C16:1n7	$0.91±0.20^{a}$	$0.67±0.15^{b}$	$0.66±0.25^{b}$	$0.82±0.20^{a}$	$0.71±0.24^{b}$	$0.52±0.22^{c}$	NS	p<0.05	NS
C18:1	13.03±0.94	12.98±1.14	12.09±1.44	14.60±0.50	15.19±1.49	14.46±0.78	p<0.0001	p<0.05	NS
C20:1n9	$0.41±0.31^{a}$	$0.24±0.08^{b}$	$0.27±0.20^{b}$	0.35±0.05	0.25±0.07	0.34±0.05	NS	p<0.05	p<0.05
ΣMUFA	$14.35±0.50^{a}$	$13.89±1.01^{ab}$	$13.02±0.95^{b}$	15.77±0.41	16.15±1.48	15.32±1.88	p<0.0001	p<0.05	NS
C18:2n6	$14.77±0.39^{a}$	$12.04±1.10^{b}$	$10.54±3.68^{c}$	$12.61±0.39^{b}$	$10.69±0.73^{c}$	$10.72±0.24^{c}$	NS	p<0.05	NS
C20:4n6	$13.97±0.34^{b}$	$14.52±0.58^{ab}$	$15.75±0.90^{a}$	$14.55±0.51^{b}$	$16.54±1.48^{a}$	$15.18±1.06^{b}$	NS	p<0.01	p<0.05
C22:4n6	$1.24±0.09^{b}$	$1.17±0.31^{b}$	$1.48±0.69^{a}$	1.95±0.09	2.20±0.29	2.04±0.03	p<0.0001	p<0.05	NS
ΣN-6 PUFA	$29.98±0.61^{a}$	$27.73±1.47^{b}$	$27.77±3.02^{b}$	$29.11±0.62^{a}$	$29.44±1.04^{a}$	$27.94±1.67^{b}$	p<0.0001	NS	p<0.05
C18:3n3	0.37±0.34	0.29±0.07	0.25±0.01	0.20±0.04	ND	ND	p<0.05	NS	NS
C20:5n3	1.15±0.08	0.99±0.03	0.94±0.03	$0.49±0.05^{c}$	$0.85±0.06^{b}$	$0.98±0.02^{a}$	p<0.05	p<0.05	p<0.001
C22:5n3	1.21±0.15	1.37±0.28	1.19±0.08	0.61±0.08	0.88±0.37	0.56±0.03	p<0.0001	NS	NS
C22:6n3	$7.59±0.39^{c}$	$8.74±1.38^{b}$	$9.68±0.51^{a}$	$4.79±0.39^{e}$	$5.03±0.21^{e}$	$6.45±1.80^{d}$	p<0.0001	0.001	NS
ΣN-3 PUFA	$10.32±0.30^{b}$	$11.96±1.48^{a}$	$12.53±0.57^{a}$	$6.09±0.51^{d}$	$6.76±0.21^{d}$	$7.99±1.71^{c}$	p<0.0001	p<0.001	p<0.0001
N6/N3	2.9:1	2.4:1	2.3:1	4.8:1	4.4:1	3.5:1			

Data are expressed as weight percentage of the total extractedfatty acids. Values are expressed as mean ± SD, n = 8. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c, d, and e) were significantly different for each group ($p<0.05$). ΣSFA: sum of saturated fatty acids; ΣMUFA: sum of monounsaturated fatty acids; Σn-6 PUFA: sum of omega-6 polyunsaturated fatty acids; Σn-3 PUFA: sum of omega-3 polyunsaturated fatty acids. ND: Not detected; NS: Not significant.

2.4.2 Effects of diets on maternal plasma lipids profile

The plasma TG was significantly higher in the 5% group, compared to the 11% group ($p<0.0001$) (Figure 2.2A). There was also an interaction between diet and gestation on the plasma TG ($p<0.005$) to reveal an increase from day 6.5 to 18.5 in the 5% group. However, there was no significant difference in plasma TG in the 11% group as gestation progressed from day 6.5 to 18.5. There was a significant time dependent decrease in plasma TC (Figure 2.2B) from day 6.5 to 18.5 ($p<0.0001$) in both dietary groups; this effect was more pronounced in the 5% group, compared to the 11% group (42.7% *vs.* 29.4% decrease). Furthermore, the cholesterol efflux capacity was significantly higher in the 5% group, compared to the 11% group ($p=0.0002$; Figure 2.2C). Time of gestation also had an effect on cholesterol efflux capacity ($p<0.0001$), where day 12.5 showed a significantly lower cholesterol efflux capacity in both dietary groups, compared to day 6.5 and 18.5.

2.4.3 Effects of diets on maternal glucose and insulin levels

Diet had an independent effect on plasma insulin and glucose where 5% group showed higher levels, compared to the 11% group ($p<0.0001$; Figure 2.3A and B, respectively). There was also an interaction between gestation and diet ($p<0.05$) for plasma insulin and glucose in the 5% group to show higher levels at day 12.5; however, there was no change in the 11% group.

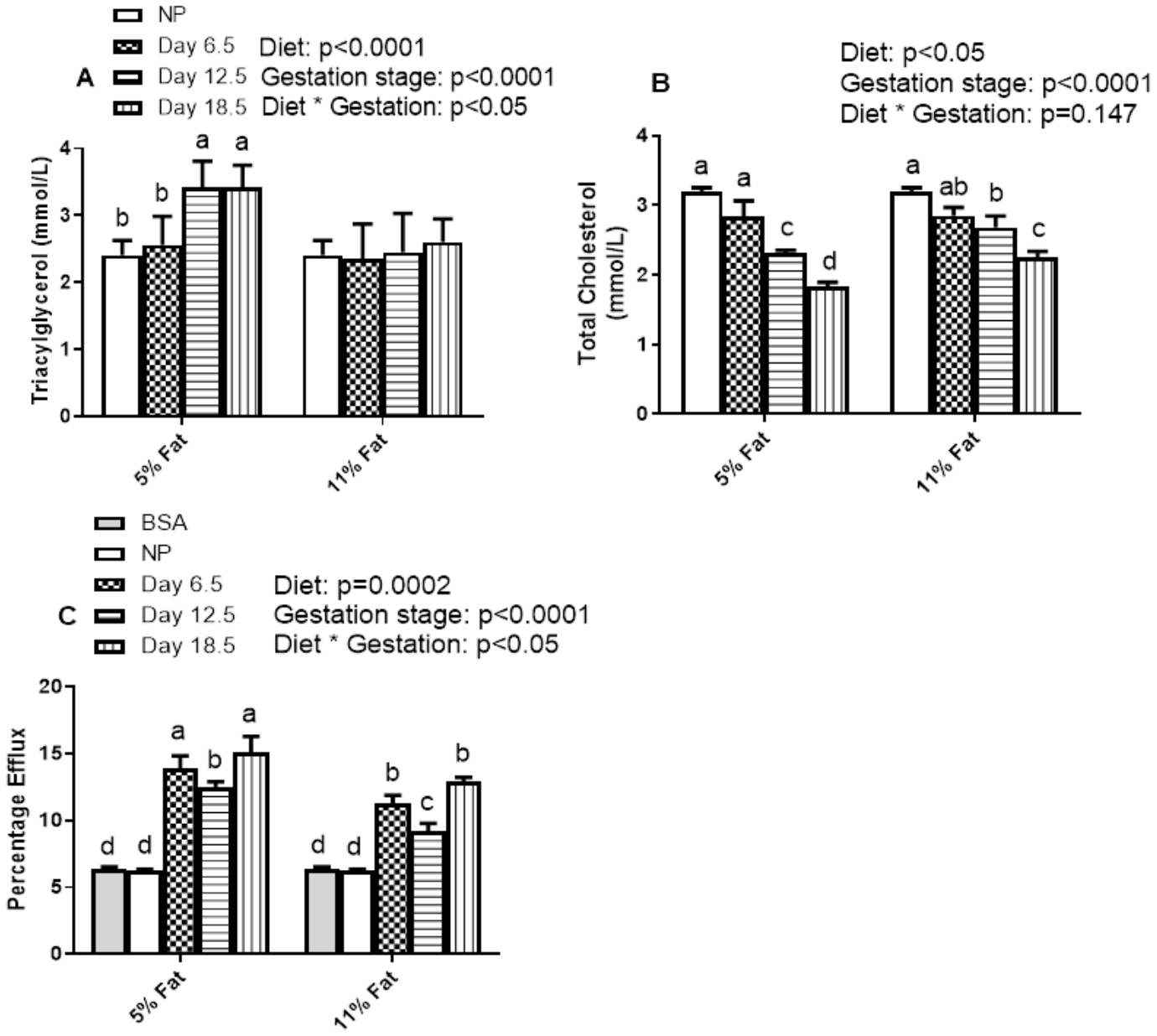

Figure 2.2: Effects of diets varying in the quantity and the quality of fat on maternal plasma lipid profile at different stages of pregnancy: plasma triacylglycerol (A), total cholesterol (B) and cholesterol efflux capacity (C) were measured for non-pregnant (NP) mice and during gestation at day 6.5, 12.5, and 18.5 as explained in the method section. Values are presented as means ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant; BSA: Bovine serum albumin.

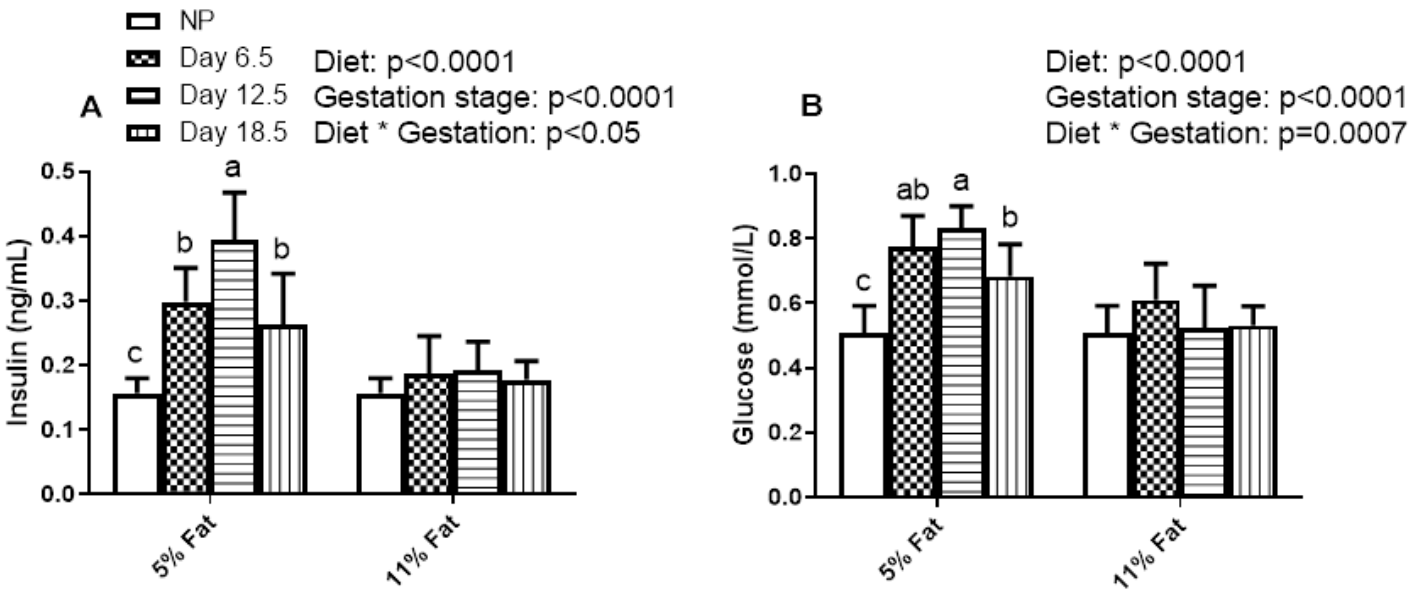

Figure 2.3: Effects of diets varying in the quantity and the quality of fat on maternal plasma insulin (A) and glucose levels (B) was measured for non-pregnant (NP) mice and during gestation at day 6.5, 12.5, and 18.5 as explained in the method section. Values are presented as means ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant.

2.4.4 Effects of diets on maternal hepatic lipid concentrations

Diet had an independent effect on liver TG concentrations ($p<0.05$; Figure 2.4A), revealing higher TG in the 5% group, compared to the 11% group. The liver TG concentration peaked at day 12.5 for both dietary groups and decreased thereafter in both dietary groups. Contrary to liver TG, there was no change in liver cholesterol concentration in the 5% group as gestation progressed (Figure 2.4B); however, there was a significant effect of gestation on liver TC in the 11% group revealing a significant decrease at day 12.5 ($p<0.05$).

2.4.5 Effects of diets on maternal mRNA expression of hepatic lipogenic genes

There was an independent effect of diet on the mRNA expression of *ACACA*, revealing a higher expression in the 5% group, compared to the 11% group ($p<0.05$; Figure 2.5A). There was also an independent effect of time on the mRNA expression of *ACACA* ($p<0.05$); *ACACA* mRNA expression increased significantly as gestation progressed from day 6.5 to 18.5 in the 5% group. Interestingly, there was no change in the mRNA expression of hepatic *ACACA* in the 11% group. Similarly, diet had an independent effect on the mRNA expression of *FAS*; the expression was significantly higher in the 5% group, compared to the 11% group ($p<0.05$; Figure 2.5B). Gestation also had an independent effect on the mRNA expression of *FAS* ($p<0.05$) in the 5% group, revealing a significant increase as gestation progressed to day 18.5. Similar to *ACACA*, there was no change in the hepatic mRNA expression of *FAS* in the 11% group. Diet had an independent effect on both *DGAT2* and *SCD1* (Figure 2.5C and D respectively), revealing a higher expression in the 5% group ($p<0.05$), compared to the 11% group.

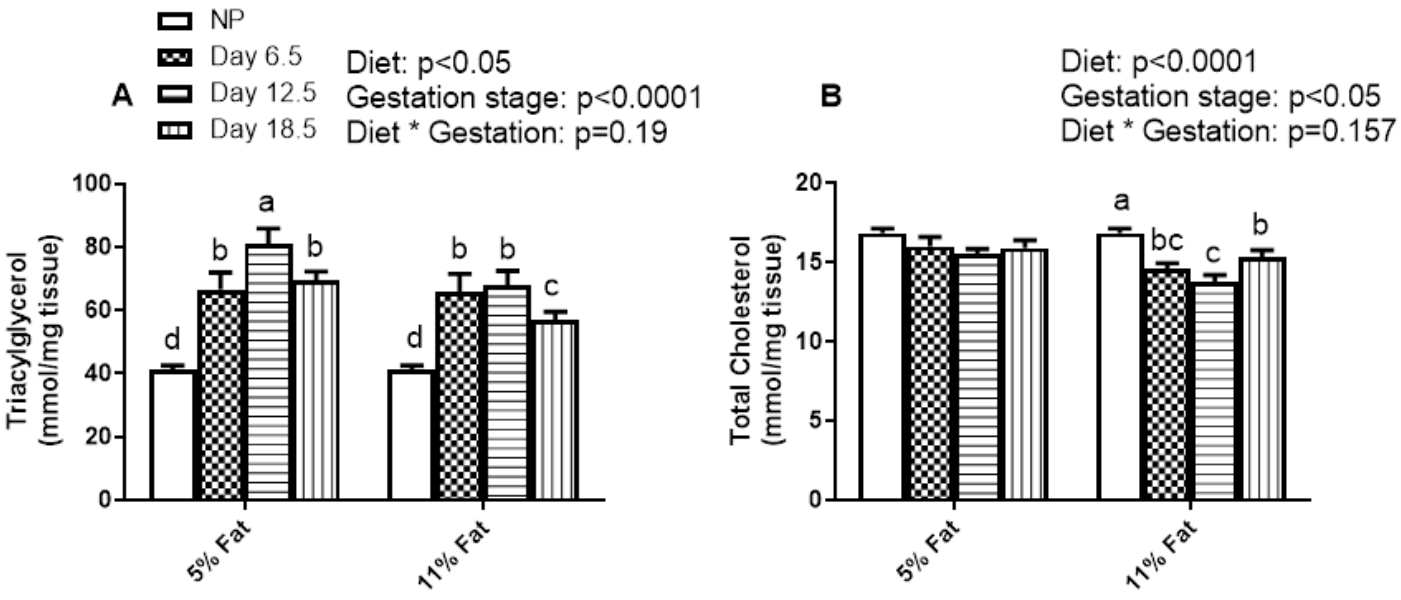

Figure 2.4: Effects of diets varying in the quantity and the quality of fat on maternal hepatic lipidsconcentration at different stages of pregnancy: liver triacylglycerol (A), and total cholesterol (B) were measured for non-pregnant (NP) mice and during gestation at day 6.5, 12.5, and 18.5 as explained in the method section. Values are presented as means ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant.

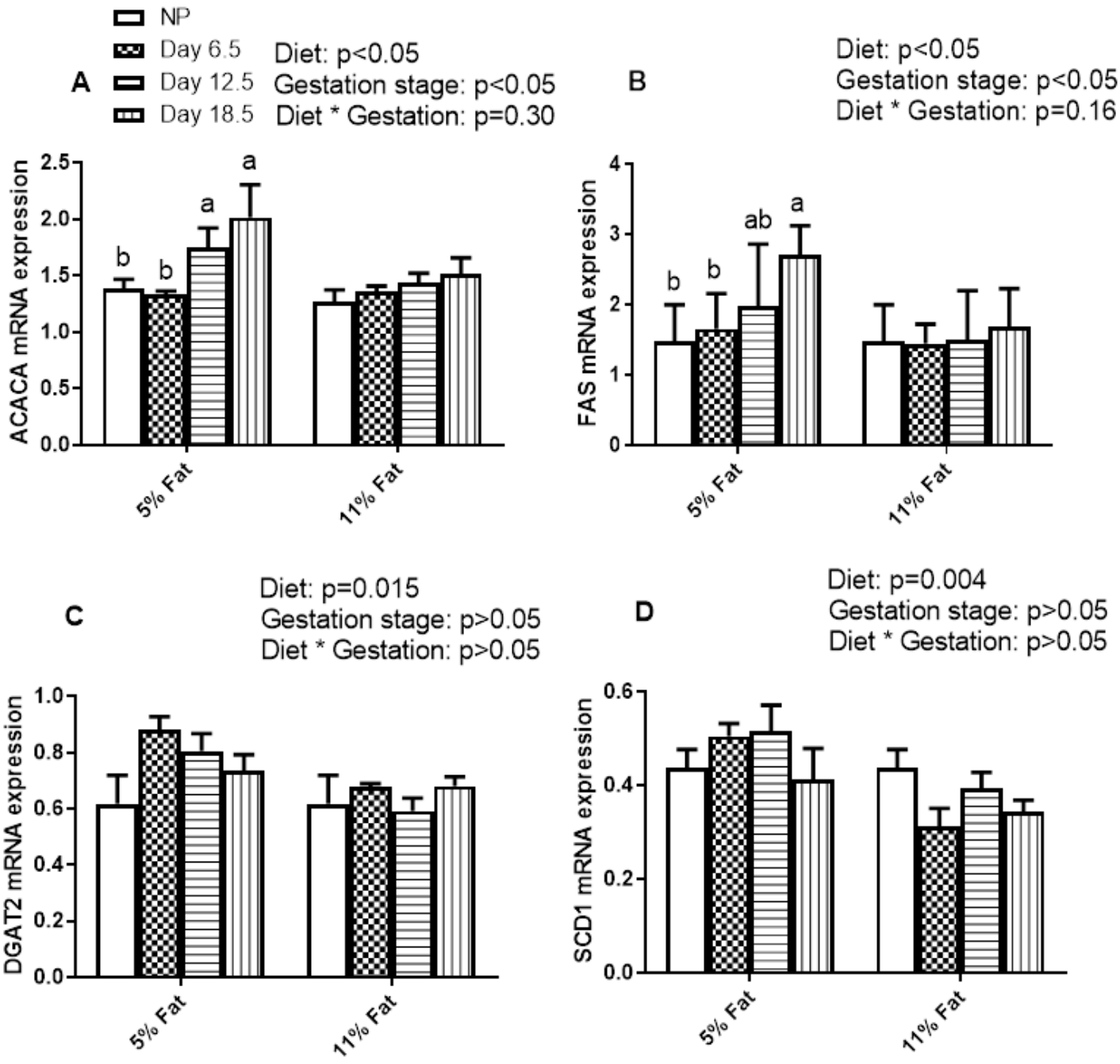

Figure 2.5: Effects of diets varying in the quantity and the quality of fat on maternal mRNA expression of hepatic lipogenic genes at different stages of pregnancy: The gene expression of acetyl CoA carboxylase; *ACACA* (A), fatty acid synthase; *FAS* (B), Diacylglycerol acyltransferase-2 (*DGAT2*) and stearoyl-CoA desaturase-1 (*SCD1*) was determined in non-pregnant (NP) and during gestation at day 6.5, 12.5, and 18.5 as explained in the method section. Values are presented as means ± SD, n=8 at each stage of pregnancy. The mRNA expression of *ACACA*, *FAS*, *DGAT2* and *SCD1* were normalized with *Actb* as the reference gene. Data were

assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant.

2.4.6 Effects of diets on maternal plasma sex-hormones level and placental *StAR* mRNA expression at different stages of pregnancy

There was an independent effect of diet on maternal plasma progesterone ($p<0.05$; Figure 2.6A) revealing a higher level in the 5% group, compared to the 11% group, especially at days 12.5 and 18.5. There was also an independent effect of gestation ($p<0.0001$), where maternal plasma progesterone levels increased significantly in both groups as gestation progressed. There was a significant inverse correlation ($p<0.05$) between maternal plasma progesterone concentration and plasma cholesterol levels in both the 5% (Figure 2.6B) and the 11% group (Figure 2.6C).

The maternal plasma estradiol concentration was also significantly higher in the 5% group ($p<0.001$) at day 12.5 and 18.5 respectively, compared to the 11% group (Figure 2.6D). There was a significant interaction between diet and gestation; estradiol concentrations increased significantly in both dietary groups as gestation progressed ($p<0.0001$). There was also an inverse correlation ($p<0.05$) between maternal plasma estradiol concentration and plasma cholesterol levels in both the 5% (Figure 2.6E) and the 11% group (Figure 2.6F).

Diet had a significant effect on the mRNA expression of *StAR* in the placenta, revealing higher expression in the 5% diet group compared to the 11% (Figure 2.7; $p<0.05$). There was also an independent effect of gestation, such that the mRNA expression of *StAR* increased in the placenta as gestation progressed from mid- to late gestation. However, the mRNA expression of *StAR* was higher in the 5% group at both mid- and late gestation ($p<0.05$), compared to the 11% group.

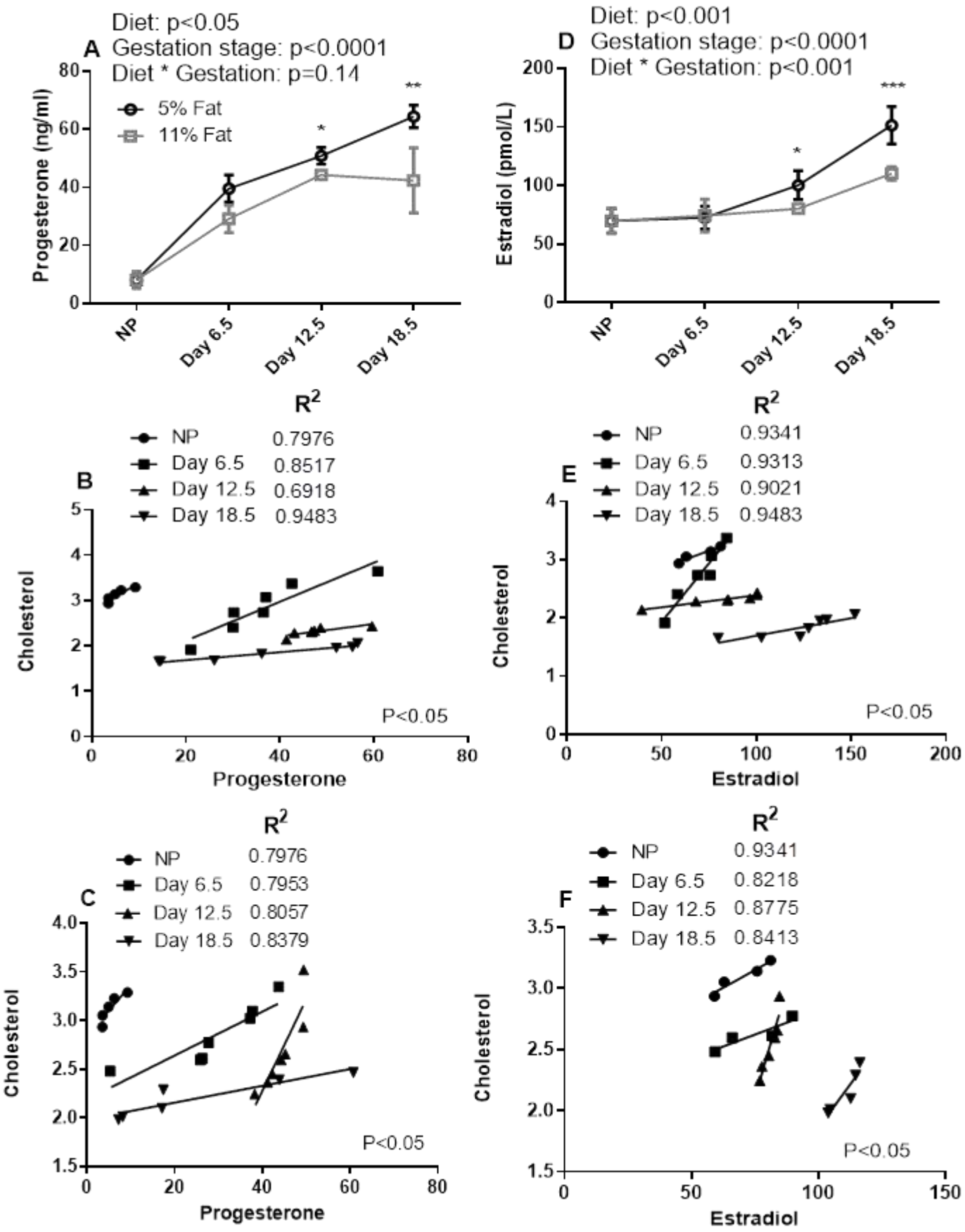

Figure 2.6: Effects of diets varying in the quantity and the quality of fat on maternal plasma sex steroid hormones at different stages of pregnancy: Plasma progesterone (A) and estradiol

(D) were measured in non-pregnant (NP) and during gestation at day 6.5, 12.5, and 18.5 as explained in the method section. Values are presented as means ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. $p<0.05$ was considered significant. Pearson's correlation analyses between plasma cholesterol and progesterone in the 5% (B) and the 11% group (C); Pearson's correlation analyses between plasma cholesterol and estradiol concentration in the 5% (E) and the 11% group (F) were carried out; $p<0.05$ represent signinficant correlation.

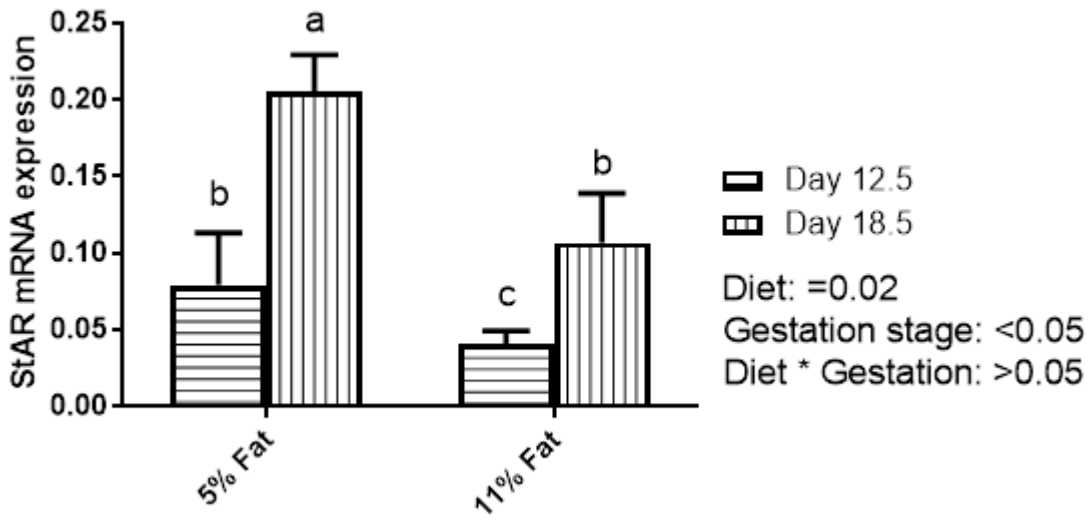

Figure 2.7: Effects of diets varying in the quantity and the quality of fat on maternal mRNA expression of placental steroidogenic acute regulatory protein (*StAR*) at different stages of pregnancy: The mRNA expression of *StAR* was measured at day 12.5 and 18.5 as explained in the methods section. Values are presented as means ± SD, n=8 at each stage of pregnancy. The mRNA expression of *StAR* was normalized with *Actb* as the reference gene. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant.

2.4.7 Effects of diets on pregnancy outcomes

Fetal and placental weight increased significantly from day 12.5 to 18.5 in both dietary groups ($p<0.0001$ and $p<0.05$, respectively); however, there was no effect of diet on either fetal or placental weight (Table 2.5). Both diet and gestation had a significant interaction on the whole uterine weight ($p<0.05$) to reveal an increase as gestation progressed ($p<0.0001$) in both groups, however, the 5% group showed higher uterine weight, compared to the 11% group ($p<0.05$). Interestingly, diet had an independent effect on fetal number; number of fetuses sustained from day 6.5 to 18.5 was significantly higher in the 5% group, compared to the 11% group ($p<0.05$; Figure 2.8).

Table 2.5: Pregnancy outcomes

Pregnancy	5% Fat			11% Fat			Main Effect		
Outcomes	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation Stage	Diet* Gestation
Fetal weight (g)	N/A	0.90 ± 0.02^{b}	1.11 ± 0.12^{a}	N/A	0.90 ± 0.01^{b}	1.08 ± 0.12^{a}	NS	$p<0.0001$	NS
Placental weight (g)	N/A	0.06 ± 0.14^{b}	0.09 ± 0.15^{a}	N/A	0.05 ± 0.14^{b}	0.07 ± 0.20^{a}	NS	$p<0.05$	NS
Whole Uterine Weight (g)	0.29 ± 0.07^{d}	2.85 ± 0.60^{c}	13.48 ± 3.14^{a}	0.32 ± 0.05^{c}	$2.89\pm.36^{d}$	9.45 ± 4.53^{b}	$p<0.05$	$p<0.0001$	$p<0.05$
Implantation / Fetal number	8.25 ± 1.63^{a}	7.71 ± 1.70^{a}	8.00 ± 1.83^{a}	8.43 ± 0.10^{a}	6.43 ± 1.23^{b}	5.14 ± 0.24^{b}	$p<0.05$	NS	NS

Values are presented as mean ± SD, n=8 dams at each stage of pregnancy. Data were analyzed using two-way ANOVA to determine the main effects and the interactions of diet and gestation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b c) represent significant difference between stages of pregnancy in each dietary group; $p<0.05$ was considered significant. N/A: Not available; NS: Not significant.

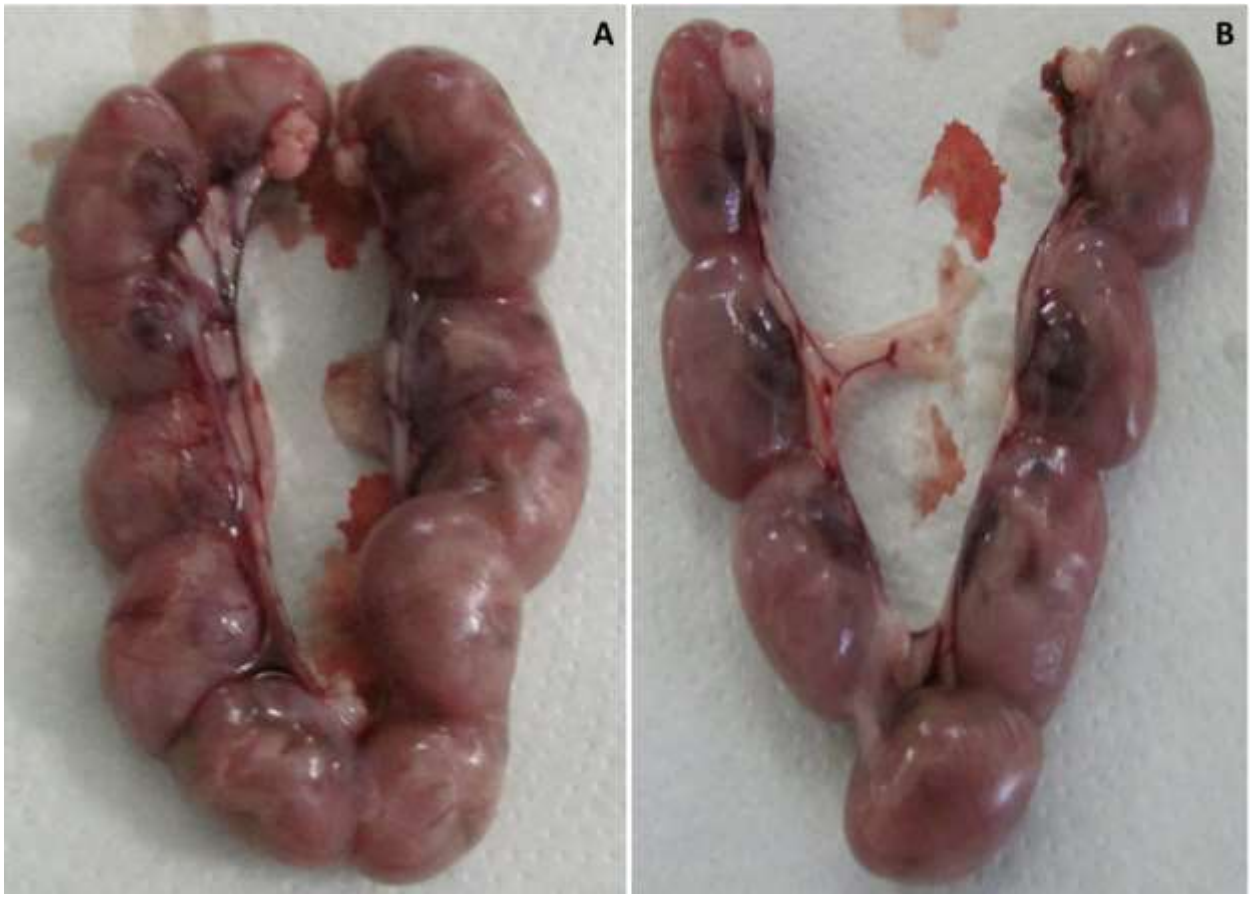

Figure 2.8: Number of fetuses sustained till day 18.5 in the 5% group (A) and 11% group (B).

2.5 DISCUSSION

The quality as well as the quantity of fat consumed during pregnancy has the potential to programme set points for several physiological and metabolic events in the mother, with a concomitant impact on the health of the offspring (Hartil *et al*., 2009; Jones *et al*., 2009; Martin-Gronert & Ozanne, 2006; Williams *et al*., 2014). We have previously shown that a maternal diet high in n-3 PUFA has protective effects on the cardiovascular health of the offspring (Balogun *et al*., 2014; Balogun *et al.*, 2013), and that the quality of maternal diet alters the tissue fatty acid composition of the offspring (Chechi *et al*., 2010). We have also shown previously that supplementing maternal diet with n-3 PUFA during pregnancy enriches offspring RBC with DHA (Balogun *et al*. 2013).

Similar to our previous findings, we found that females fed the 5% diet that contained higher levels of n-3 PUFA showed higher amounts of EPA, DPA and DHA, compared to the 11% group. It was interesting that ALA was not detected at day 12.5 and 18.5 in the 11% fat group, which coincided with an increase in EPA and DHA. The 11% diet only contained ALA as a source of n-3 PUFA, thus it is obvious that ALA is being converted to longer chain n-3 PUFA as gestation progressed to provide these fatty acids for fetal growth and development. On the other hand, the 5% group showed no change in ALA from day 6.5 to 18.5. Studies have shown that dietary EPA and DHA downregulate the conversion of ALA to longer chain n-3 PUFA by up to 70% (Arterburn *et al*. 2006; Burdge *et al*. 2003; Pawlosky *et al*. 2003); this would explain the detection of ALA throughout gestation in the 5% group as this diet contained longer chain n-3 PUFA.

Although, the conversion of ALA to DHA is generally limited in women and often not detectable in men (Burdge & Wootton, 2002a; Burdge & Calder, 2005; Hussein *et al*., 2005); the conversion becomes highly efficient during pregnancy (Burdge & Wootton,

2002a; Childs *et al*., 2011). The rate limiting step in the conversion of ALA to DHA is the conversion of intermediate DPA to DHA (Arterburn *et al*., 2006). Our data revealed lower DPA in the 11% group; this may be due to a higher conversion of DPA to DHA to increase the availability of DHA for fetal brain at late gestation. DHA is critical for brain and eyes (retina) development (Innis, 2007; Neuringer *et al*., 1988). DHA accumulation in the brain has been shown to be most rapid during the last trimester and at the first year of birth; fetus accrues approximately 70 mg DHA per day during third trimester (Clandinin *et al*., 1980). As anticipated, the highest amount of DHA was observed at day 18.5 in both dietary groups. Children from mothers with high intake of DHA during pregnancy has been shown to have higher cognitive capability and better problem solving skills compared to those with low intake (Dunstan *et al*., 2008; Judge *et al*., 2007). Our findings have established that feeding a diet containing longer chain n-3 PUFA shows higher levels of EPA and DHA in RBC, compared to the diet without longer chain n-3 PUFA, suggesting that the most effective way to supply longer chain n-3 PUFA is by providing these specific fatty acids in the diet.

As pregnancy progresses, metabolic changes occur in the mother to increase the levels of circulating lipids to supply to the fetus (Emet *et al*., 2013; Ghio *et al*., 2011; Qureshi *et al*., 1999). An increase in maternal TG during pregnancy has been shown to contribute to embryo development as it serves as a carrier for fatty acids which are later released and transferred into fetal circulation (Duttaroy, 2009). Our data revealed that both plasma and hepatic TG were significantly higher in the 5% group, compared to the 11% group likely to meet fetal fat requirement. A study by Nakashima (2009) revealed an increase in maternal plasma TG in response to a maternal low fat diet during pregnancy. We also observed a time dependent increase in plasma TG levels during pregnancy; maternal plasma TG peaked at day 18.5 in the 5% group. This is consistent with the findings of Qureshi *et al.* (1999), who reported that maternal plasma TG increased significantly during the second trimester and reached

maximum in the third trimester. An established mechanism for increased plasma TG concentration is via lipogenesis in the liver (Kersten, 2001). We observed a higher mRNA expression of lipogenic genes (*ACACA, FAS, DGAT2 and SCD1*) in the liver obtained from the 5% group, supporting our observation that the increase in plasma and hepatic TG is due to increased lipogenesis. Interestingly, there was no significant change in plasma TG concentration across gestation time in the 11% group.

We also found a significantly higher level of insulin in the 5% group, which may have played a role in increasing lipogenesis (Kersten 2001). During pregnancy, maternal lipids are used as the primary energy source to spare amino acids and glucose for fetal use (Ghio *et al*., 2011). Although the fetus has been shown to have a considerably high capacity to adapt to changes in glucose supply during pregnancy, however, lower maternal plasma glucose level has been associated with reduced fetal development (Scholl *et al*., 2001). We observed a higher plasma glucose level in the 5% group, compared to the 11% group, which could have a profound effect on the development of the fetus. Maternal glucose levels has been shown to decrease slightly during third trimester (Riskin-Mashiah *et al*., 2011); we found a similar trend, however, the reduction in maternal plasma glucose was only significant in the 5% group.

There was a time-of-gestation dependent decrease in plasma cholesterol in both groups from day 6.5 to 18.5; however, the percentage decrease was higher in the 5% group, compared to the 11% group (42.7% *vs.* 29.4%). Maternal cholesterol is an important source of fetal cholesterol at early gestation, especially for cell membranes formation (Krause & Regen, 2014). However, the significance of maternal cholesterol to fetal development decreases as gestation progresses, owing to the ability of fetal tissues to synthesize cholesterol (Herrera, 2002). We found a consistent decrease in maternal cholesterol levels as gestation progressed to day 18.5 suggesting that cholesterol is being delivered to fetal

circulation, as it plays a key role in regulating cascade of activities required for optimal fetal development (Miller, 1998). We also found an increase in cholesterol efflux capacity of plasma obtained from mothers fed the 5 % diet that contained higher levels of longer chain n-3 PUFA. This was consistent with our previous studies to show that a diet high in n-3 PUFA increases cholesterol efflux capacity (Balogun *et al*. 2014). However, further studies are required to determine the effects of these diets on cholesterol transporters, such as ABCA1 and ABCG1, and and HDL-cholesterol uptake receptor SR-B1.

Our findings revealed an inverse correlation between maternal plasma cholesterol and the concentration of progesterone and estradiol. It is well known that cholesterol is a precursor for the synthesis of steroid hormones. An increase in the level of cholesterol during pregnancy makes cholesterol available for the synthesis of progesterone and estradiol, which are indispensable in creating a suitable uterine environment for implantation and pregnancy maintenance (Miller, 1998). *StAR* is a rate-limiting regulator of steroid hormones synthesis by mediating cholesterol transfer within the mitochondrial in the steroid-producing tissues cells (Stocco & Clark, 1996). However, the placenta becomes the primary site for estradiol and progesterone synthesis during pregnancy (Yivgi-Ohana *et al*., 2009). Approximately 83% of spontaneous abortions have been directly associated with low levels of progesterone during pregnancy (Hahlin *et al*., 1990). Although progesterone treatment is controversial, a handful of studies have attempted the use of progesterone to treat recurrent miscarriages (El-Zibdeh, 2005; Palagiano *et al*., 2004; Yassaee *et al*., 2014).

Impaired progesterone synthesis and action has also been associated with preterm birth in both human and mice, partly due to its ability to suppress the expression of inflammatory cytokines at the materno-fetal interface (Blanks & Brosens, 2012; Mendelson, 2009). Progesterone also regulate uterine quiescence by preventing contractions that could disturb the growing embryo (Blanks & Brosens, 2012). In addition, oral administration of

both progesterone and estradiol has been considered to reduce miscarriage rates (Tonguc *et al*., 2011). Increased production of estradiol is very critical at mid-pregnancy as it has been shown that progesterone alone could not maintain pregnancy at this stage (Barkley *et al*., 1979). The demand for progesterone and estradiol increases as gestation progresses (Milligan & Finn 1997; Barkley *et al*. 1979); interestingly, this was also observed in our results. However, our results revealed that the level of progesterone and estradiol in maternal plasma was consistently higher in the 5% group, compared to the 11% group and this coincides with an increase in the mRNA expression of *StAR* in the placenta at mid- and late gestation.

Our finding suggests that besides the possible contribution from plasma cholesterol for progesterone and estradiol synthesis, the presence of n-3 PUFA in the 5% group may also be regulating the synthesis of these hormones. A study by Richardson *et al*. (2013) showed that feeding cows with dietary n-3 fatty acids increased serum progesterone levels. Although the mechanism through which n-3 PUFA might be regulating the synthesis of progesterone and estradiol has not been comprehensively examined, our results show for the first time that higher level of these hormones may elicit higher number of fetuses as the number of fetuses sustained till day 18.5 was significantly higher in the 5% group, compared to the 11% group.

In conclusion, our findings demonstrate for the first time that a low fat maternal diet enriched with longer chain n-3 PUFA increased the mRNA expression of rate limiting enzymes for lipogenesis and increased cholesterol efflux, likely to meet fetal lipid demand during pregnancy. In addition, our findings indicate that supplementing maternal diet with longer chain n-3 PUFA increased the maternal plasma concentration of progesterone and estradiol during pregnancy, which may be responsible for an increase in the number of fetuses sustained till day 18.5 as proposed in Figure 2.9. The effects of longer chain n-3 PUFA in eliciting positive pregnancy outcomes by regulating sex-steroid hormones has been well established in human. However, these novel findings may be important when designing

dietary strategies to optimize reproductive capability and maternal and fetal health in mice, and other animals.

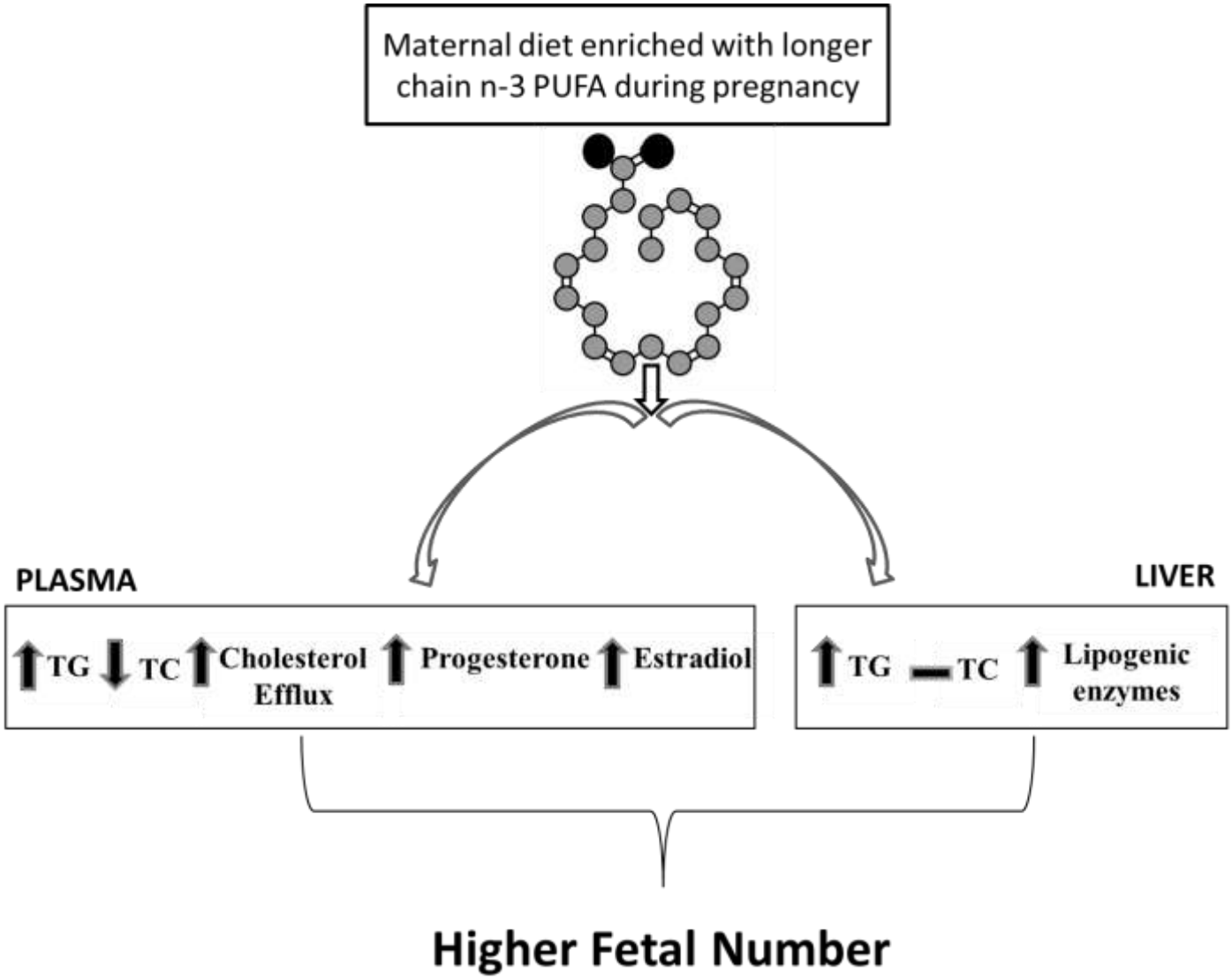

Figure 2.9: Schematic representation of the effects of a low fat maternal diet supplemented with longer chain n-3 PUFA on maternal metabolic profile and fetal outcome. N-3 PUFA: Omega-3 polyunsaturated fatty acids; TC: total cholesterol; TG: triacylglycerol.

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CHAPTER THREE

A diet enriched in longer chain omega-3 fatty acids reduced placental inflammatory cytokines and improved fetal sustainability of C57BL/6 mice

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3.1 ABSTRACT

Omega (n)-3 polyunsaturated fatty acids (PUFA) are important regulators of inflammatory response that may impact pregnancy outcome. The effects of breeding chow diets containing n-3 PUFA from either fish oil (FO) or soybean oil (SO) were investigated on tissue fatty acid composition, inflammatory cytokines, and pregnancy outcome. Female C57BL/6 mice (7 weeks old) were fed FO or SO diets for 2 weeks before mating and throughout pregnancy. Animals were sacrificed before and during pregnancy at day 6.5, 12.5 and 18.5. The FO diet increased the incorporation of n-3 PUFA in placenta, with a concomitant decrease in the concentration of pro-inflammatory cytokines. The FO diet increased the mRNA expression of placental specific PUFA transporter, which coincided with accretion of n-3 PUFA in fetal brain. Sites of fetal resorption were noticeable in the SO group but not in the FO group. N-3 PUFA may improve fetal sustainability via altering inflammatory cytokine levels in placenta.

3.2 INTRODUCTION

Pregnancy relies on a careful balance between pro- and anti-inflammatory cytokines (Challis *et al.,* 2009; Zourbas *et al*., 2001). As such, an imbalance in the levels of pro- and anti-inflammatory cytokines can result in aberrant inflammation, with a concomitant adverse pregnancy outcome such as spontaneous abortion, preterm labor, and intrauterine growth restriction (Cotechini & Graham, 2015; El-Shazly *et al*., 2004; Renaud *et al.*, 2011; Zenclussen *et al*., 2003). Studies have shown that the consumption of longer chain n-3 PUFA during pregnancy reduces the risk of pre-term delivery by modifying gestation length and increasing offspring weight at birth (De Giuseppe *et al.*, 2014; Olsen *et al*., 1992, 1994; Szajewska *et al*., 2006). However, the mechanism through which n-3 PUFA regulates pregnancy duration and fetal sustainability during different stages of pregnancy is vaguely understood.

N-3 PUFAs are considered to be less inflammatory/anti-inflammatory, while n-6 PUFAs are generally associated with increased inflammation (Calder, 2009). Interestingly, n-3 and n-6 PUFA are metabolized by the same group of enzymes; thus, n-3 PUFA exerts its anti-inflammatory properties by inhibiting the metabolism of n-6 PUFAs such as arachidonic acid (AA) into downstream inflammatory metabolites (Schmitz & Ecker, 2008). As such, low intake of n-3 PUFA has been implicated in the pathogenesis of several inflammatory diseases during pregnancy (Coletta *et al*., 2010). Pregnancy was initially categorized as a single event characterized by either pro-inflammatory or anti-inflammatory cytokines (Wegmann *et al*., 1993). However, recent studies revealed that each stage of pregnancy is regulated by different proportions of anti- and pro-inflammatory molecules which are localized in maternal reproductive tissues, such as the placenta and the uterus (Paulesu *et al*., 2010; Zourbas *et al*., 2001). Early and late gestation involves increased production of pro-inflammatory cytokines in the uterine *milieu* (Paulesu *et al*., 2010; Singh *et al*., 2011).

Implantation is a critical step in the establishment of pregnancy, involving a harmonized dialogue between maternal endometrium and the semi-allograft blastocyst, and this stage is largely regulated by increased production of inflammatory cytokines such as TNFαIL-6, IL-12, IFNγ, and MCP-1 in maternal utero-placental interface (De *et al*., 1993; Paulesu *et al*., 2010; Singh *et al*., 2011). Similarly, activities involving labour stimulation, uterine contraction and cervical ripening at late gestation are all mediated by pro-inflammatory cytokines (Kelly, 2002; Paulesu *et al*., 2010).

Cytokines profile shift towards less inflammatory cytokines as pregnancy progresses towards second trimester. As such, anti-inflammatory cytokines such as IL-10 is well characterized in maternal utero-placental interface at mid-gestation where it plays a key role in inflammation resolution (Paulesu *et al*., 2010). Uterine quiescence is highly essential during mid-gestation for optimum fetal growth; thus, a well-coordinated inflammation resolution system is required to prevent persistent inflammation, which could degenerate into complications during pregnancy (Chatterjee *et al*., 2014). Apparently, successful pregnancy establishment and progression is dependent on the establishment of a healthy balance between pro- and anti-inflammatory cytokines at different stages of pregnancy (Chatterjee *et al*., 2014; Zourbas *et al*., 2001).

Increased intake of n-6 PUFA, as typified by western diet has been shown to cause significant changes in the fatty acid composition of membrane phospholipids with a resultant effect on inflammatory response (Simopoulos, 2016), whilst increased levels of uterine n-3 PUFA has been shown to prevent pathological inflammatory response in pregnant mice (Yamashita *et al*., 2013). The average intake of n-3 PUFA among Canadian women is 82 mg/d, which is below the recommendations by the International Society for the study of Fatty Acids and Lipids (300 mg/d) (Denomme *et al*., 2005). However, there is paucity of evidence on the effect of diets differing in the types and amount of n-3 PUFA on the fatty acid

composition of maternal uterus and the placenta at different stages of pregnancy, and its influence on the levels of pro- and anti-inflammatory cytokines in the maternal plasma and placenta. We hypothesized that a breeding chow diet containing longer chain n-3 PUFA from fish oil will cause a gestation dependent increase in the incorporation of longer chain n-3 PUFA into maternal uterus and placenta, as well as increase the accretion of longer chain n-3 PUFA in fetal brain of C57BL/6 mice, compared to a breeding chow diet containing n-3 PUFA from soybean oil. We further hypothesized that incorporation of longer chain n-3 PUFA from fish oil will reduce the concentration of pro-inflammatory cytokines in maternal plasma and placenta in a gestation dependent manner to induce beneficial effects on pregnancy outcome, compared to a diet containing soybean oil.

3.3 MATERIALS AND METHODS

3.3.1 Ethics statement

All experimental procedures involving animals were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee (approval no: 15-11-SC). All effort was made to reduce the number of animals and to minimize animal suffering.

3.3.2 Animals and experimental design

Male and female C57BL/6 mice were purchased from Charles River Laboratories (MA, USA) at seven weeks of age, and housed in separate cages under controlled temperature (21 ± 1°C) and humidity (35 ± 5%) condition with a 12-hour light/12-hour dark period cycle. Mice were kept on standard rodent chow pellets (#RMH 3000; LabDiet, St. Louis, MO, USA) for one-week acclimatization period. After this period, female mice were randomly divided into two groups and were assigned to either a fish oil (FO) based (#5061, LabDiet, St. Louis,

MO, USA) or a soybean oil (SO) based (#5015, LabDiet, St. Louis, MO, USA) breeding chow diet (Table 2.1) for two weeks, as per our previous publication (Akerele & Cheema, 2017).

Mating was carried out and female mice were checked by 6:00am the following morning for vaginal plug formation to confirm pregnancy, representing day 0.5 (Croy *et al*., 2015). Pregnant mice were continued on the assigned diets throughout gestation. Fresh food and water were provided *ad-libitum* every other day. Body weight and food intake was recorded every day; no significant difference in food intake was observed, and there was no difference in maternal weight across the two dietary groups before pregnancy and at each stage of pregnancy (Appendix I). Mice were sacrificed before pregnancy (non-pregnant), at early-gestation (day 6.5), mid-gestation (day 12.5), and late-gestation (day 18.5) using 2.5% isoflurane. Blood was collected by cardiac puncture in tubes containing EDTA (4.5 mM, pH 7.4) and was separated immediately into plasma and red blood cells. Maternal and fetal tissues were removed and weighed at the time of sacrifice. All fetuses were removed from the uterus; placenta was carefully separated from the fetus and washed free of maternal blood in ice-cold phosphate buffered saline (PBS). Fetal brain was collected from each fetus. Tissues were snap frozen in liquid nitrogen and stored at -80ºC until further analyses. Pictures of fetal resorption were taken using Canon camera (SX500 IS).

3.3.3 Fatty acid composition analyses

Lipids were extracted from maternal uterus, placenta and fetal brains according to the method of Folch *et al.*, (1957), and the fatty acid composition was determined as per our previous publication (Akerele & Cheema, 2017). Brain samples for all fetuses in each litter were collected, analyzed individually and results averaged for each dam. The fatty acid

composition of the breeding chow diets has been previously published (Akerele & Cheema, 2017), and is provided as Table 2.2 of Chapter 2 .

3.3.4 Cytokine assay

Cytokines concentration in the maternal plasma and placental samples was determined using BD Cytometric Bead Array (CBA) mouse inflammation kit (#552364, BD Biosciences, ON, Canada) according to the manufacturer's instruction. The linearity and efficiency of the kit was established for placental cytokines. Briefly, mouse inflammation standard was reconstituted in assay diluent and incubated at room temperature for 15 minutes before carrying out serial dilutions (1:2 up to 1:256). The mouse inflammation capture beads were mixed and transferred to each assay tube prior to the addition of standards or samples. The placenta samples were homogenized in 1mL of PBS with 10 µl of protease inhibitor cocktail (#P8340, Sigma-Aldrich, Canada).

Clear placental supernatant was separated by centrifuging the placental homogenate at 800 x g for 10 minutes and used for cytokine assay. Detection reagent was added to each tube and incubated for 2 hours at room temperature protected from light. Each tube was then washed with wash buffer and centrifuged at 200 x g for 5 minutes. The supernatant was discarded, and the pellet was re-suspended in 300 µl of wash buffer. Cytokines concentration was determined using FACSAria flow cytometer (#650110C8, BD Bioscience, Canada). Data analysis was performed using Flow Cytometry Analysis Program (FCAP) Array software (version 3.0).

3.3.5 RNA extraction and real-time qPCR

Total RNA was extracted from placenta samples using Trizol method (Chomczynski & Sacchi, 1987). Genomic DNA contamination was removed by treating with DNase enzyme (#M610A, Promega, USA). The concentration of the extracted RNA, and the A260/A280 was

determined using Nano Drop 2000 (Thermo Scientific, USA). RNA integrity of each sample was confirmed using 1.2 % agarose gel. Synthesis of cDNA from the extracted RNA was carried out using reverse-transcription method as per our previous publication (Balogun & Cheema, 2014). All primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) (accessed on 02/06/2018) and obtained from IDT technologies (IA, USA); primer sequences and efficiencies are given in Table 3.1. Amplification was performed using iQ SYBR Green Supermix (#1708880, Bio-rad, USA) and samples were run using the CFX96TM Real-Time System while data output was managed using the CFX ManagerTM Software Version 3.0. The delta Ct values were recorded for each of the gene of interest, corrected for amplification efficiency, and normalized with Beta-Actin (*Actb*) as the reference gene; there was no change in the expression of *Actb* between groups. The expression levels between the two groups were compared using the Livak method (Livak & Schmittgen, 2001).

Table 3.1: Primers Sequences

Gene	Primers sequence (5' – 3')	Primer Efficiency (%)
FAT / CD36 (S)	atgggctgtgatcggaactg	98.11
FAT / CD36 (AS)	gtcttcccaataagcatgtctcc	
FABPpm (S)	agcggctgaccaaggagtt	97.09
FABPpm (AS)	gacccctgccacggagat	
Actb (S)	cacgcagctcattgtagaagg	107.47
Actb (AS)	atggtgggaatgggtcagaag	

Actb, beta actin; AS, antisense; *FABPpm*, placental plasma membrane fatty acid binding protein; *FAT / CD36*, fatty acid translocase; S, sense.

3.3.6 Statistical analysis

Data were analysed using GraphPad Prism Software (version 5.0). Sample means were compared using two-way analysis of variance (ANOVA) to determine main effects of diet and time, and the interactions between them. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Results are expressed as mean ± standard deviation (SD) for n=8 dams in each experimental group. Placenta and brain samples for all fetuses in each litter were individually analysed, and results were averaged for each dam. Fatty acid composition data were arcsine transformed prior to statistical analyses. Differences were statistically significant if $p<0.05$.

3.4 RESULTS

3.4.1 Effects of diets on maternal uterine fatty acid composition

There was no effect of diet on saturated fatty acids (SFA) but there was an independent effect of gestation time on SFA in both diet groups ($p<0.0001$) (Table 3.2). Myristic acid (C14:0) decreased significantly at day 12.5 and 18.5 compared to day 6.5 in both diet groups ($p<0.05$). Animals fed the FO diet showed no difference in palmitic acid (C16:0) during pregnancy, while stearic acid (C18:0) increased at day 12.5, compared to day 6.5 ($p<0.05$). On the other hand, animals fed the SO diet showed an increase in palmitic acid and stearic acid as gestation progressed from day 6.5 to day 18.5 ($p<0.05$).

Gestation time also had a significant effect on monounsaturated fatty acids (MUFA). Palmitoleic acid (C16:1n7), oleic acid (C18:1) and total MUFA decreased at day 12.5 and 18.5 in both diet groups, compared to day 6.5 ($p<0.05$). Eicosenoic acid (C20:1n9) increased at day 18.5 in both diet groups ($p<0.05$), compared to day 6.5, and there was a significant interaction between diet and gestation time ($p<0.05$).

Animals fed the FO diet had lower levels of arachidonic acid (C20:4n6; AA), adrenic acid (C22:4n6) and total n-6 PUFA ($p<0.05$), compared to the SO diet group. Linoleic acid (C18:2n6; LA) decreased with gestation time in both diet groups ($p<0.05$), while AA, adrenic acid and total n-6 PUFA increased significantly at day 18.5 in both diet groups, compared to day 6.5 ($p<0.05$). Animals fed the FO diet had higher levels of eicosapentaenoic acid (C20:5n3; EPA), docosapentaenoic acid (C22:5n3; DPA), docosahexaenoic acid (C22:6n3; DHA) and total n-3 PUFA, compared to the SO group ($p<0.05$). Alpha linolenic acid (C18:3n3; ALA**)** decreased as gestation progressed from day 6.5 to 18.5 in the FO group ($p<0.05$). Interestingly, there was no detection of ALA at day 12.5 and 18.5 in the SO group. DPA, DHA and total n-3 PUFA increased from day 6.5 to 12.5 in both diet groups ($p<0.05$), and DPA also revealed an interaction between diet and gestation time ($p<0.05$).

Table 3.2: Fatty acid composition of maternal uterus

Fatty Acids	FO Diet (High n-3)			SO Diet (Low n-3)			Main Effect		
	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation	Diet*Gestation
C14:0	0.79 ± 0.13^{a}	0.48 ± 0.04^{c}	0.57 ± 0.04^{c}	0.69 ± 0.14^{b}	0.51 ± 0.04^{c}	0.54 ± 0.07^{c}	NS	$p<0.05$	NS
C16:0	19.12 ± 1.58	18.19 ± 0.54	18.97 ± 0.36	17.56 ± 0.47^{b}	17.85 ± 0.70^{b}	19.04 ± 0.75^{a}	NS	$p<0.05$	NS
C18:0	11.73 ± 1.74^{c}	17.23 ± 2.32^{a}	14.53 ± 4.18^{b}	11.80 ± 3.89^{c}	18.67 ± 2.36^{a}	16.74 ± 2.05^{a}	NS	$p<0.05$	NS
ΣSFA	31.64 ± 5.58^{b}	35.90 ± 1.80^{a}	34.07 ± 4.08^{a}	30.05 ± 4.16^{b}	37.03 ± 2.28^{a}	36.32 ± 2.37^{a}	NS	$p<0.0001$	NS
C16:1n7	2.33 ± 0.29^{a}	1.47 ± 0.10^{b}	1.74 ± 0.52^{b}	2.18 ± 0.75^{a}	1.25 ± 0.14^{b}	1.22 ± 0.19^{b}	NS	$p<0.05$	NS
C18:1	19.01 ± 2.08^{a}	13.98 ± 0.72^{bc}	16.25 ± 0.20^{b}	20.64 ± 5.53^{a}	14.11 ± 0.73^{b}	15.68 ± 1.84^{b}	NS	$p<0.05$	NS
C20:1n9	0.46 ± 0.10^{d}	0.76 ± 0.06^{cd}	0.86 ± 0.43^{c}	0.96 ± 0.18^{c}	1.44 ± 0.39^{a}	1.18 ± 0.07^{b}	NS	$p<0.05$	$p<0.05$
ΣMUFA	21.80 ± 4.67^{a}	16.21 ± 2.49^{b}	18.85 ± 3.91^{b}	23.78 ± 4.47^{a}	16.81 ± 2.39^{b}	18.08 ± 2.19^{b}	NS	$p<0.05$	NS
C18:2n6	14.00 ± 2.80^{a}	9.79 ± 0.42^{b}	9.74 ± 2.40^{b}	9.67 ± 2.32^{b}	$8.23\pm0.4b^{c}$	6.77 ± 1.16^{d}	$p<0.05$	$p<0.05$	NS
C20:4n6	7.16 ± 1.72^{d}	12.40 ± 2.12^{b}	11.90 ± 0.17^{b}	9.33 ± 3.95^{c}	15.27 ± 2.15^{a}	13.22 ± 0.67^{b}	$p<0.05$	$p<0.05$	NS
C22:4n6	1.38 ± 0.32^{c}	2.63 ± 0.16^{b}	2.25 ± 0.67^{b}	2.60 ± 0.98^{b}	2.99 ± 0.51^{ab}	3.44 ± 0.56^{a}	$p<0.05$	$p<0.05$	$p<0.05$
ΣN-6 PUFA	22.54 ± 0.80^{cd}	24.82 ± 0.87^{b}	23.89 ± 0.44^{b}	21.60 ± 0.9^{d}	26.49 ± 0.27^{a}	23.43 ± 0.65^{bc}	$p<0.05$	$p<0.05$	NS
C18:3n3	0.72 ± 0.25^{a}	0.24 ± 0.00^{c}	0.18 ± 0.01^{d}	0.35 ± 0.11^{b}	ND	ND	$p<0.05$	$p<0.05$	NS
C20:5n3	0.22 ± 0.07^{a}	0.27 ± 0.02^{a}	0.25 ± 0.07^{a}	0.09 ± 0.01^{b}	0.27 ± 0.01^{a}	0.10 ± 0.04^{b}	$p<0.05$	$p<0.0001$	NS
C22:5n3	1.10 ± 0.29^{c}	1.67 ± 0.58^{a}	1.50 ± 0.38^{a}	0.66 ± 0.10^{d}	1.06 ± 0.14^{c}	1.25 ± 0.12^{b}	$p<0.05$	$p<0.05$	$p<0.05$
C22:6n3	3.53 ± 0.60^{b}	6.45 ± 0.21^{a}	6.74 ± 1.53^{a}	2.32 ± 0.09^{c}	4.63 ± 0.67^{b}	4.96 ± 0.81^{b}	$p<0.05$	$p<0.05$	NS
ΣN-3 PUFA	5.57 ± 1.30^{c}	8.63 ± 1.68^{a}	8.67 ± 0.55^{a}	3.42 ± 0.67^{d}	5.96 ± 0.98^{c}	7.22 ± 0.78^{b}	$p<0.05$	$p<0.05$	NS

Data are expressed as weight percentage of total fatty acids. Values are expressed as mean ± SD, n = 8. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c and d) were significantly different for each group ($p<0.05$). FO, fish oil based diet; ΣMUFA, sum of monounsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not detected; NS, not significant; ΣSFA: sum of saturated fatty acids; SO, soybean oil based diet.

3.4.2 Effects of diets on maternal placental fatty acid composition

There was no effect of diet on SFA, but gestation time had an independent effect on myristic acid, stearic acid and total SFA (Table 3.3). Myristic acid decreased significantly ($p<0.05$), while stearic acid increased in both diet groups from day 12.5 to 18.5 ($p<0.0001$). Gestation time had no effect on palmitic acid in both diet groups. There was no change in total SFA in the FO group, while total SFA increased at day 18.5 in the SO group ($p<0.05$). There was a significant effect of diet on palmitoleic acid, eicosenoic acid and total MUFA, revealing that the FO group had higher levels of palmitoleic acid ($p<0.0001$) and lower levels of eicosenoic ($p<0.05$) acid and total MUFA, compared to the SO group ($p<0.05$). Gestation time also had an independent effect on all MUFAs; palmitoleic acid, oleic acid, and total MUFA decreased from day 12.5 to 18.5 in both diet groups ($p<0.05$). Eicosenoic acid increased from day 12.5 to day 18.5 in the FO group, and decreased from day 12.5 to day 18.5 in the SO group ($p<0.05$).

Diet had a significant effect on AA, adrenic acid and total n-6 PUFA. The FO group had lower levels of AA ($p<0.0001$), adrenic acid ($p<0.05$) and total n-6 PUFA ($p<0.05$), compared to the SO group. Total n-6 PUFA decreased from day 12.5 to 18.5 in the FO group ($p<0.05$), while there was no change in the SO group. Diet also had a significant effect on n-3 PUFAs; the amount of ALA, DPA, DHA and total n-3 PUFA was significantly higher in the FO group, compared to the SO group ($p<0.0001$). ALA decreased from day 12.5 to 18.5 in the FO group ($P<0.05$), while it was not detectable at day 18.5 in the SO group. EPA increased from day 12.5 to 18.5 in the FO group, while there was no change in the SO group ($p<0.05$). There was no change in DPA as gestation progressed from day 12.5 to 18.5 in both diet groups, while DHA and total n-3 PUFA increased significantly from day 12.5 to 18.5 in both groups ($p<0.0001$). The effect of the FO diet on ALA and DHA was dependent on the gestation time ($p<0.05$).

Table 3.3: Fatty acid composition of maternal placenta

Fatty Acids	FO Diet (High n-3)		SO Diet (Low n-3)		Main Effect		
	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Diet	Gestation	Diet * Gestation
C14:0	0.42±0.03^{a}	0.31±0.04^{b}	0.42±0.06^{a}	0.34±0.07^{b}	NS	p<0.05	NS
C16:0	17.21±0.37	16.78±0.19	16.56±0.43	16.56±0.86	NS	NS	NS
C18:0	19.90±0.83^{b}	22.00±0.53^{a}	20.67±0.62^{b}	22.55±1.03^{a}	NS	p<0.0001	NS
Σ SFA	38.24±1.11^{b}	39.88±0.74^{ab}	38.37±0.83^{b}	40.27±0.40^{a}	NS	p<0.05	NS
C16:1n7	1.22±0.04^{a}	1.07±0.07^{b}	1.06±0.07^{b}	0.91±0.06^{c}	p<0.0001	p<0.0001	NS
C18:1	11.09±0.77^{a}	10.03±0.60^{b}	11.40±0.42^{a}	10.43±0.78^{b}	NS	p<0.05	NS
C20:1n9	0.27±0.03^{d}	0.51±0.03^{c}	1.74±0.31^{a}	1.08±0.28^{b}	p<0.05	p<0.05	NS
Σ MUFA	12.61±0.67^{b}	11.63±0.65^{c}	14.09±0.79^{a}	12.44±0.82^{b}	p<0.05	p<0.05	NS
C18:2n6	11.89±1.40^{a}	10.26±0.13^{ab}	10.58±0.32^{ab}	9.19±0.69^{b}	NS	p<0.05	NS
C20:4n6	13.34±0.45^{c}	14.69±0.84^{b}	15.35±0.55^{ab}	16.34±1.59^{a}	p<0.0001	p<0.05	NS
C22:4n6	3.86±0.28^{a}	2.29±0.19^{b}	4.03±0.14^{a}	3.31±1.31^{a}	p<0.05	p<0.05	NS
Σ N-6 PUFA	29.41±1.98^{a}	27.52±0.79^{b}	30.62±0.51^{a}	29.35±1.73^{a}	p<0.05	p<0.05	NS
C18:3n3	0.26±0.00^{a}	0.19±0.01^{b}	0.10±0.01^{c}	ND	p<0.05	p<0.05	<0.05
C20:5n3	0.52±0.09^{b}	0.69±0.05^{a}	0.47±0.12^{b}	0.65±0.45^{ab}	NS	p<0.05	NS
C22:5n3	1.60±0.16^{a}	1.56±0.16^{a}	0.84±0.13^{b}	0.77±0.11^{b}	p<0.0001	p<0.05	NS
C22:6n3	7.59±0.35^{b}	9.69±0.63^{a}	5.48±0.31^{d}	6.48±0.42^{c}	p<0.0001	p<0.0001	<0.05
Σ N-3 PUFA	10.05±0.39^{b}	12.40±0.72^{a}	8.06±0.46^{c}	9.06±0.44^{b}	p<0.0001	p<0.05	NS

Data are expressed as weight percentage of total fatty acids. Values are expressed as mean ± SD, n = 8. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c and d) were significantly different for each group ($p<0.05$). FO, fish oil based diet; ΣMUFA, sum of monounsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not detected; NS, not significant; ΣSFA: sum of saturated fatty acids; SO, soybean oil based diet.

3.4.3 Effects of diets on fetal brain fatty acid composition

There was no effect of either diet or gestation time on SFA (Table 3.4). There was a significant effect of diet on eicosenoic acid; the FO group had higher levels of eicosenoic acid, compared to the SO group ($p<0.05$). Gestation time had an independent effect on oleic acid and total MUFA, showing a decrease from day 12.5 to 18.5 in both diet groups ($p<0.0001$), while there was no change in palmitoleic acid. There was no effect of diet on n-6 PUFAs in both diet groups. However, LA decreased from day 12.5 to day 18.5 in both diet groups ($p<0.0001$). There was an independent effect of diet on n-3 PUFAs, revealing that the FO group had higher amount of EPA, DPA, DHA, and total n-3 PUFA in fetal brain, compared to the SO group ($p<0.0001$). Gestation time also had a significant effect on PUFAs; ALA decreased from day 12.5 to 18.5 in both diet groups ($p<0.05$), while EPA, DHA and total n-3 PUFA increased from day 12.5 to day 18.5 in both diet groups ($p<0.0001$).

3.4.4 Effects of diets on maternal placental fatty acid transporters

Diet had a significant effect on the mRNA expression of placental plasma membrane fatty acid binding protein (FABPpm), revealing higher expression in the FO group, compared to the SO group ($p<0.0001$; Figure 3.1). However, there was no effect of gestation time on the mRNA expression of FABPpm in both diet groups. There was no effect of diet on the mRNA expression of fatty acid translocase FAT/CD36 (Appendix III).

Table 3.4: Fatty acid composition of fetal brain

Fatty Acids	FO Diet (High n-3)		SO Diet (Low n-3)		Main Effect		
	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Diet	Gestation	Diet*Gestation
C14:0	1.92±0.26	2.14±0.42	2.26±0.08	2.29±0.11	NS	NS	NS
C16:0	28.47±1.16	28.14±0.89	30.33±0.96	29.43±0.71	NS	NS	NS
C18:0	14.33±1.30	15.64±0.65	14.03±0.64	16.12±0.56	NS	NS	NS
ΣSFA	44.71±1.44	45.31±2.76	46.62±1.54	47.84±1.19	NS	NS	NS
C16:1n7	5.90±0.82	5.79±0.87	6.71±0.18	6.29±0.16	NS	NS	NS
C18:1	22.00 ± 1.24^{a}	16.89 ± 1.08^{b}	22.88 ± 0.43^{a}	17.68 ± 0.52^{b}	NS	p<0.0001	NS
C20:1n9	2.44 ± 0.84^{a}	2.77 ± 1.06^{a}	1.80 ± 0.51^{b}	0.91 ± 0.34^{c}	p<0.05	NS	p<0.05
ΣMUFA	30.34 ± 2.00^{a}	25.45 ± 1.05^{b}	30.75 ± 1.77^{a}	24.88 ± 0.58^{b}	NS	p<0.0001	NS
C18:2n6	2.28 ± 0.60^{a}	0.67 ± 0.08^{b}	2.38 ± 0.38^{a}	0.77 ± 0.05^{b}	NS	p<0.0001	NS
C20:4n6	10.39±0.55	10.31±0.54	11.53±0.84	11.41±0.24	NS	NS	NS
C22:4n6	3.07±0.88	2.88±0.69	2.93±0.29	3.78±0.71	NS	NS	NS
ΣN-6 PUFA	12.67±2.80	10.98±0.56	13.91±1.22	12.18±0.22	NS	NS	NS
C18:3n3	0.63 ± 0.07^{a}	0.41 ± 0.03^{c}	0.53 ± 0.07^{b}	0.30 ± 0.06^{d}	NS	p<0.05	NS
C20:5n3	1.77 ± 0.23^{b}	2.82 ± 0.63^{a}	0.75 ± 0.27^{d}	1.22 ± 0.53^{c}	p<0.0001	p<0.05	NS
C22:5n3	0.80 ± 0.21^{a}	0.70 ± 0.18^{a}	0.14 ± 0.00^{c}	0.25 ± 0.07^{b}	p<0.0001	NS	NS
C22:6n3	6.51 ± 0.58^{c}	11.47 ± 0.75^{a}	4.38 ± 0.45^{d}	9.54 ± 0.52^{b}	p<0.0001	p<0.0001	NS
ΣN-3 PUFA	9.21 ± 1.58^{c}	15.39 ± 1.15^{a}	5.80 ± 0.62^{d}	11.32 ± 1.02^{b}	p<0.0001	p<0.0001	NS

Data are expressed as weight percentage of total fatty acids. Values are expressed as mean ± SD, n = 8. Main effects and interactions were determined by two-way ANOVA after arcsine transformation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c, and d) were significantly different for each group ($p<0.05$). FO, fish oil based diet; ΣMUFA, sum of monounsaturated fatty acids; Σn-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σn-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not detected; NS, not significant; ΣSFA: sum of saturated fatty acids; SO, soybean oil based diet.

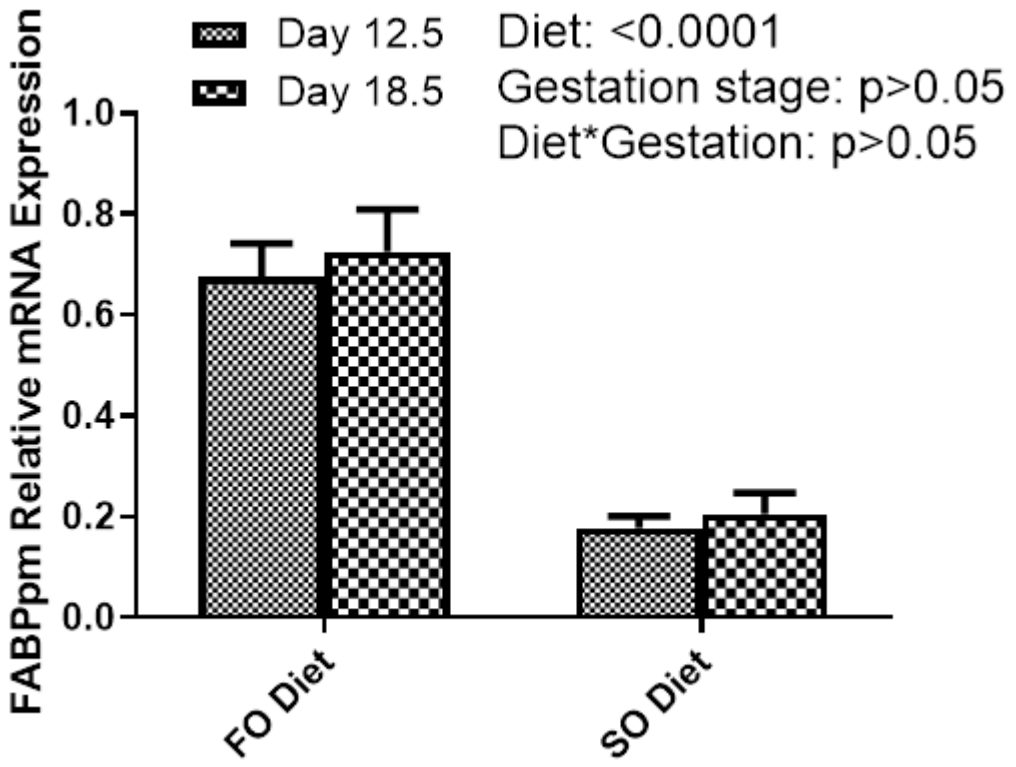

Figure 3.1: Effects of dietary n-3 polyunsaturated fatty acids on maternal mRNA expression of placental membrane fatty acid binding protein (FABPpm) was determined during gestation at day 12.5 and 18.5 as explained in the Material and Method section. Values are presented as mean ± SD, n=8 at each stage of pregnancy. The mRNA expressions were normalized with *Actb* as the reference gene. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and gestation; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. $p<0.05$ was considered significant. *Actb*; beta actin; FO, fish oil based diet; NS, not significant; SO, soybean oil based diet.

3.4.5 Effects of diets on maternal plasma cytokines

Diet had no independent effect on plasma cytokines. However, there was a significant effect of gestation time on the concentrations of IFN-γ, TNFα, MCP-1, IL-12, IL-6 and IL-10 in both diet groups (Figure 3.2). The concentrations of IFN-γ decreased from day 12.5 to 18.5 in the FO group, while it increased from day 12.5 to 18.5 in the SO group ($p<0.0001$). There was no change in TNFα as gestation progressed from day 6.5 to 18.5 in the FO group, while it increased from day 6.5 to 18.5 in the SO group ($p<0.05$). MCP-1 decreased significantly at day 18.5 in the FO group, compared to the SO group ($p<0.05$). IL-12 decreased as pregnancy progressed from day 6.5 to 18.5 in the FO group, while it increased in the SO group ($p<0.0001$). IL-6 decreased significantly from day 12.5 to 18.5 in the FO group, compared to the SO group ($p<0.0001$). IL-10 increased significantly from day 6.5 to 18.5 in the FO group, while there was no change in IL-10 levels in the SO group as pregnancy progressed from day 6.5 to 18.5 ($p<0.0001$). There were significant interactions between diet and gestation time on the concentration of IFN-γ, IL-12 and IL-10 in both diet groups ($p<0.05$).

3.4.6 Effects of diets on placental cytokines

Diet had an independent effect on IL-6, TNFα and MCP-1 ($p<0.05$), revealing lower levels in the FO group, compared to the SO group (Figure 3.3). There was also an independent effect of gestation time on TNFα and MCP-1, revealing that TNFα increased as gestation progressed from day 12.5 to 18.5 in both dietary groups ($p<0.0001$). In contrast, MCP-1 decreased as gestation progressed from day 12.5 to 18.5 in both diet groups ($p<0.0001$). There was no effect of gestation time on IL-6; neither diet nor gestation time had an effect on IL-10 ($p>0.05$).

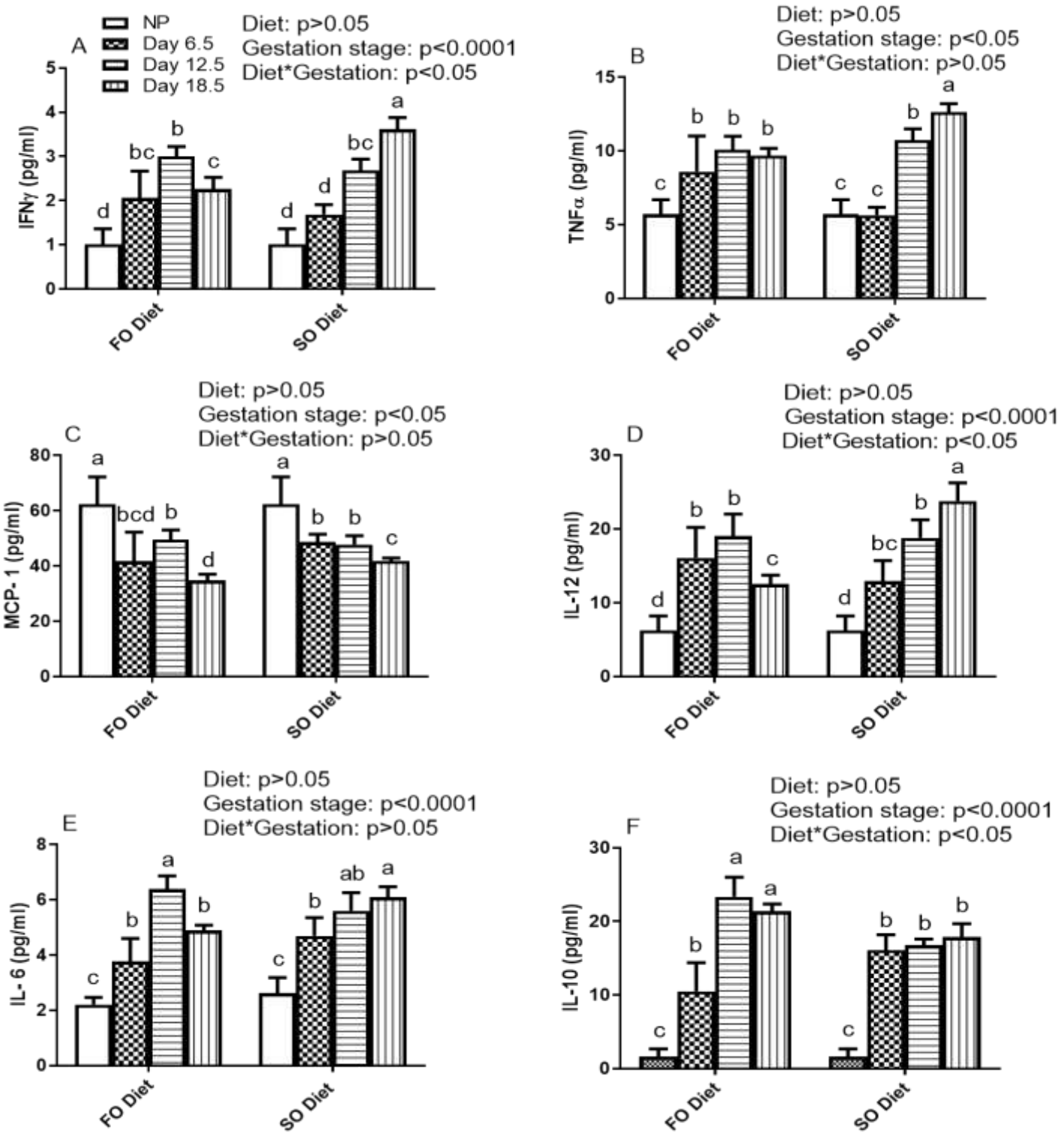

Figure 3.2: Effects of dietary n-3 polyunsaturated fatty acids on maternal plasma cytokines concentration at different stages of pregnancy Interferon gamma (IFNγ; A), tumor necrosis factor alpha (TNFα; B), monocyte chemotactic protein-1 (MCP-1; C), interleukin-12 (IL-12; D), IL-6 (E), and IL-10 (F) were measured for non-pregnant (NP) mice and during gestation at day 6.5, 12.5, and 18.5 as explained in the Materials and Methods section. Values are

presented as mean ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c, and d) represent significant difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant. FO, fish oil based diet; NS, not significant; SO, soybean oil based diet.

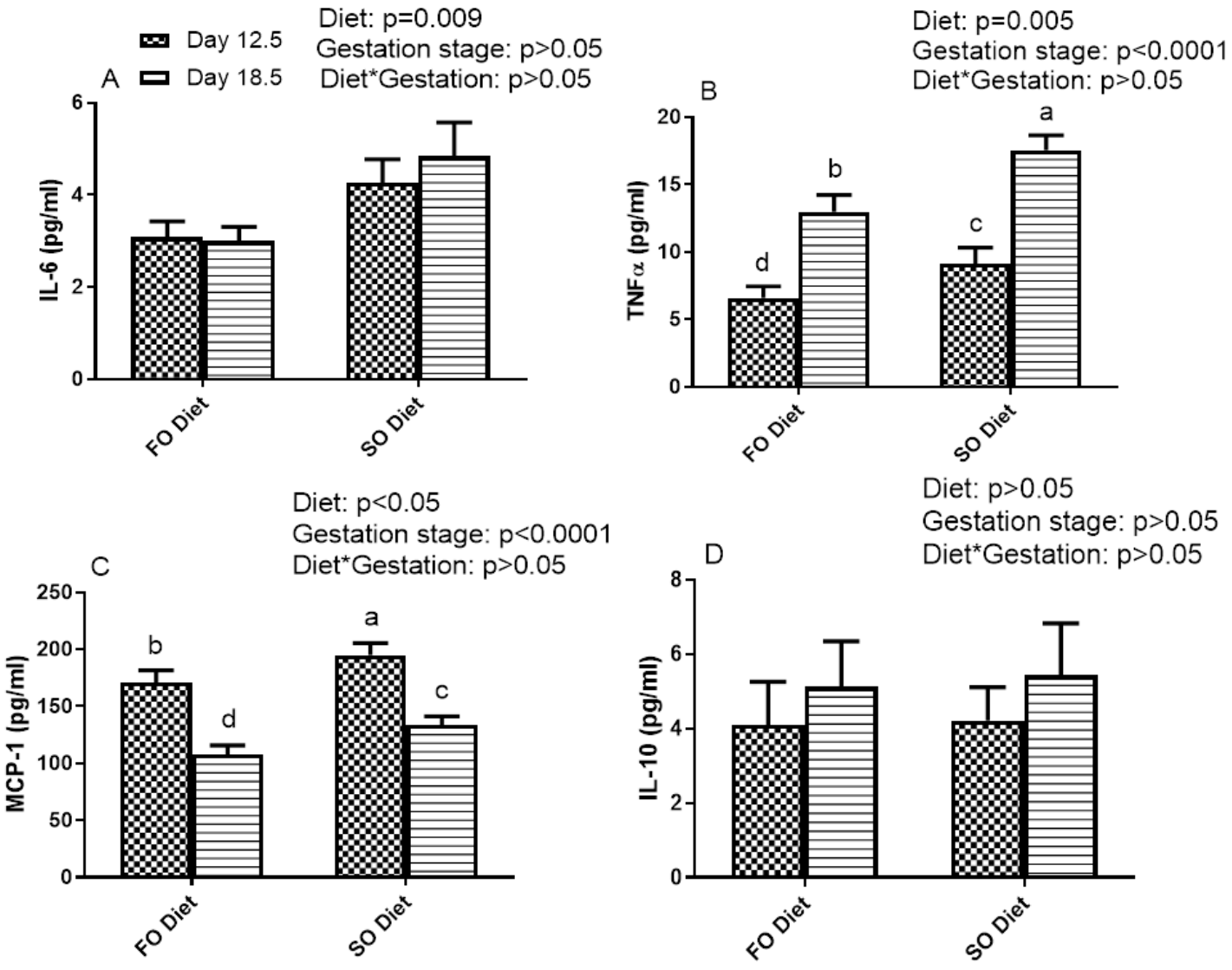

Figure 3.3: Effects of dietary n-3 polyunsaturated fatty acids on maternal placental cytokines concentration at different stages of pregnancy; Interleukin-6 (IL-6; A), tumor necrosis factor alpha (TNFα; B), monocyte chemotactic protein-1 (MCP-1; C), and IL-10 (D) were measured for non-pregnant (NP) mice and during gestation at day12.5, and 18.5 as explained in the Materials and Methods section. Values are presented as mean ± SD, n=8 at each stage of pregnancy. Data were assessed using two-way ANOVA to determine the main effects and the interactions of diet and time; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Letters (a, b, c and d) represent significant

difference between stages of pregnancy in each diet groups. $p<0.05$ was considered significant. FO, fish oil based diet; NS, not significant; SO, soybean oil based diet.

3.4.7 Effects of diets on fetal resorption

The number of resorption sites observed in the FO and the SO diet groups, at all stages of gestation, are given in Table 3.5. No resorption sites were observed in the FO group; however, the SO group clearly revealed resorption sites as evident from the pictorial images, particularly at day 12.5 (Figure 3.4).

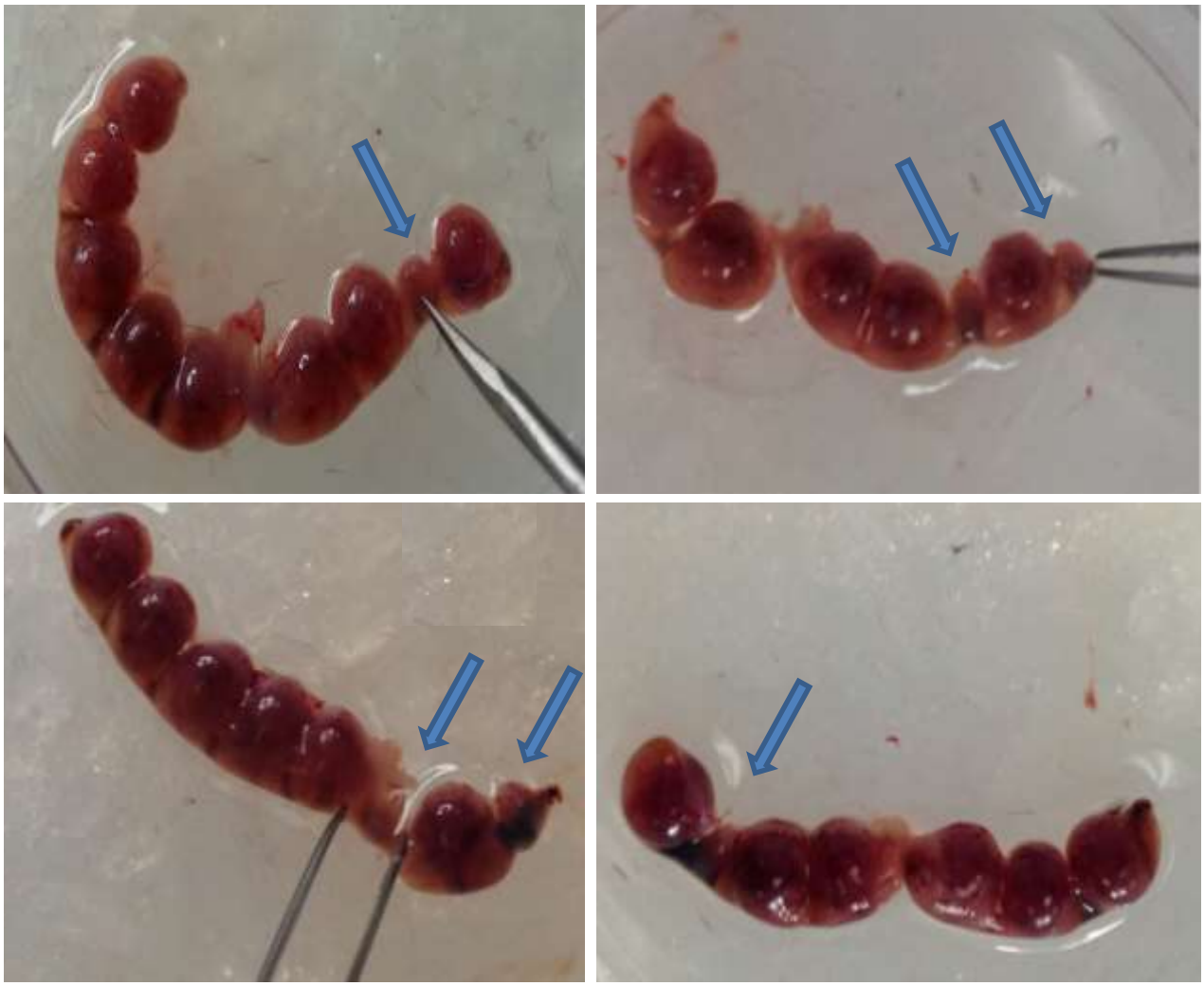

Figure 3.4: Evidence of fetal resorption in the low n-3 PUFA (SO) diet group; arrows show the resorption sites at pregnancy day 12.5. PUFA: Polyunsaturated fatty acids, SO: Soybean oil based diet.

Table 3.5: Number of fetal resorption sites during pregnancy

	FO Diet (High n-3)	SO Diet (Low n-3)
Day 6.5	NA	NA
Day 12.5	Nil	1.75±0.71
Day 18.5	Nil	2.00±0.76

Values represent number of fetal resorption sites, expressed as mean ± SD, n = 8. FO, fish oil-based diet; n-3, omega-3; NA, not applicable; SO, soybean oil-based diet.

3.5 DISCUSSION

We have previously shown that breeding chow diet containing fish oil as a source of n-3 PUFA increased the incorporation of longer chain n-3 PUFA into maternal red blood cells and improved fetal outcomes of C57BL/6 mice, compared to a diet containing soybean oil based n-3 PUFA (Akerele & Cheema, 2017). In the current study, we have shown that maternal diet containing longer chain n-3 PUFA alters the fatty acid composition of maternal uterus, placenta and fetal brain, along with causing changes in cytokine levels of maternal plasma and placenta, at each stage of pregnancy corresponding to different trimesters of pregnancy. Rodent models have proven useful to further understand the cascades of events during pregnancy in human. Reduction in uterine n-3 PUFA during pregnancy contributes to dysfunctional myometrial activity in rats, resulting in adverse pregnancy outcomes (Muir *et al*., 2018). Furthermore, the ratio of n-6 to n-3 PUFA is important in regulating uterine function (Robinson *et al*., 2002; Verma *et al*., 2018).

The transfer of longer chain n-3 PUFA into fetal tissues during pregnancy is considerably regulated by the availability of n-3 PUFA in maternal uterine and placental interface (Larque *et al*., 2011). Our findings have shown that females fed a diet containing longer chain n-3 PUFA from FO had higher levels of EPA, DPA, and DHA in maternal uterus and placental samples, compared to the SO group. Although the availability of longer chain n-3 PUFA such as DHA in the placenta is predominantly regulated by dietary intake, ALA (the essential n-3 PUFA) is also metabolised through a series of elongation and desaturation processes to DHA (Leonard *et al*., 2004). The conversion of ALA to DHA is generally high in females, and becomes more efficient during pregnancy, largely due to an increase in the demand for DHA during pregnancy (Innis, 2005; Mulder *et al*., 2014). We found that ALA was not detectable in the uterine and placental tissues in the SO group at late gestation (day 18.5). The disappearance of ALA coincided with an increase in the levels of

longer chain n-3 PUFAs (EPA, DPA and DHA) in the placental and uterine tissues at late gestation in the SO group. ALA was the only source of n-3 PUFA in the SO diet, and the total amount of n-3 PUFA in the SO diet was also lower as compared to the FO diet, thus no detection of ALA at late gestation indicates that ALA is being all converted to longer chain n-3 PUFA in the SO group, specifically DHA to meet fetal requirement as gestation progressed.

The delivery and accretion of PUFA in the fetus is through the mother via placental transfer (Innis, 2005). Cell culture models (Tobin *et al.*, 2009), perfused placenta (Haggarty *et al*., 1997), as well as *in vivo* studies using radio-labelled DHA (Gil-Sánchez *et al*., 2010) revealed that placenta preferentially transfers essential fatty acids such as DHA into fetal circulation during pregnancy via passive diffusion and by specific placental membrane fatty acid transporters (Duttaroy, 2009). Hence, abnormal placental function involving impaired transfer of longer chain n-3 PUFA has been implicated in the pathogenesis of adverse pregnancy outcomes such as intrauterine growth restriction (Gauster *et al*., 2007; Larque *et al*., 2011).

Fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36), as well as FABPpm have been identified as key membrane proteins involved in fatty acid transport across the placenta (Duttaroy, 2009). Among these membrane proteins, FABPpm functionally exhibits a high affinity for longer chain n-3 PUFAs, suggesting that the transfer of DHA across the placenta is most probably mediated by this protein (Campbell *et al*., 1997). Our data revealed that the expression of FABPpm was significantly higher in the FO diet, compared to the SO diet. The role of FABPpm in the transport of DHA from maternal pool to fetal circulation has been linked to its exclusive location in the microvillous membrane of the placenta, as well as its binding specificity for longer chain n-3 PUFAs (Campbell *et al*., 1998). In our study, FAT/CD36 was only expressed on day 18.5, and it revealed no

significant difference between the two dietary groups, suggesting that FABPpm is the major DHA transporter in the placenta.

DHA is essential for brain development during pregnancy (Innis, 2007; Neuringer *et al.*, 1988). Growth and development of fetal brain is rapid during second trimester of pregnancy (Coletta *et al*., 2010), emphasizing that this stage of pregnancy is perhaps the most critical period for DHA supplementation. However, DHA accretion in fetal brain has been shown to be very rapid at near term and during the first year of birth in human (Clandinin *et al.*, 1980; Martínez & Mougan, 1998); fetal brain accrues about 70 mg DHA per day in the third trimester of pregnancy (Martínez & Mougan, 1998). Our findings using C57BL/6 mice show a significant increase in the amount of longer chain n-3 PUFA, especially DHA in fetal brain as gestation progressed from mid-gestation (day 12.5) to late-gestation (day 18.5) in both dietary groups.

DHA increased as gestation progressed to day 18.5 in both dietary groups, which is consistent with human studies demonstrating increase in DHA accretion at late gestation (Clandinin *et al*., 1980; Martínez & Mougan, 1998). However, we acknowledge that there are differences in fetal brain maturity at late gestation between mice and human. We also found that DHA as well as total n-3 PUFA in the fetal brain was consistently higher in the FO group compared to the SO group, signifying that maternal dietary intake of DHA is critical for DHA accretion in fetal brain during pregnancy. The incorporation of DHA into different brain regions, containing specific classes of lipids, has important implication in brain function; this will be explored in the future.

Studies have shown that an increase in the relative concentration of phospholipids DHA in cord serum by about 1% increased the duration of pregnancy by 1.5 days (Grandjean *et al*., 2001). Longer chain n-3 PUFAs perhaps regulates gestation length by decreasing the levels of inflammatory cytokines at near term. Elevated levels of pro-inflammatory cytokines

are suggested to trigger premature activation of labour in humans (Pandey *et al*., 2017). Inflammatory cytokines play vital roles in coordinating several processes leading to parturition such as cervical ripening, fetal membrane rupture and myometrial contraction during labour (Dimitriadis *et al*., 2005; Mendelson, 2009; Paulesu *et al*., 2010; Singh *et al*., 2011). In the current study, the most significant effect of the FO diet containing longer chain n-3 PUFA was seen at late gestation when pro-inflammatory cytokines such as IFN-γ, TNFα, MCP-1, IL-6, and IL-12 decreased significantly in both maternal plasma and placenta, compared to the SO group. The first trimester of pregnancy is primarily characterized by increased production of pro-inflammatory cytokines as they are required for embryo reception, successful implantation, and co-ordination of feto-maternal cross-talk (Dimitriadis *et al*., 2005; Paulesu *et al*., 2010).

IL-6 plays a key role in regulating the viability of the implantation sites at early gestation (De *et al*., 1993); impaired production at this stage may delay or prolong pregnancy establishment. IFN-γ has been shown to initiate uterine vasculature remodelling in mice (Ashkar *et al*., 2000), while a network of IL-6 and TNFα has been identified in the chorio-decidual environment, thereby indicating their role in membrane rupture and uterine contraction during labour (Paulesu *et al*., 2010). However, elevated levels of pro-inflammatory cytokines such as IL-6 and TNFα has been implicated in adverse pregnancy establishment and outcomes such as impaired implantation in mice and human (Chaouat *et al*., 1990; Chaouat *et al*., 2004), embryo rejection and spontaneous abortion in mice (Kiger *et al*., 1985; Zenclussen *et al*., 2003).

Although functions of IL-12 and MCP-1 during pregnancy are yet to be clearly defined; elevated levels of IL-12 has also been reported in women with history of miscarriage (Wilson *et al*., 2004), while MCP-1 has been suggested to potentially play an important role in the induction of inflammatory responses leading to preterm labour in mice (Diamond *et al*.,

2007). Our findings showed sites of fetal resorption in the SO group that had significantly higher levels of placental IL-6, TNFα and MCP-1 at day 12.5 and 18.5, compared to the FO group. We found a significant increase in maternal plasma concentrations of IL-6, IL-12, TNFα and IFN-γ as pregnancy progressed from mid- to late gestation; interestingly fetal resorption sites also increased as pregnancy progressed. We have previously reported a decrease in fetal numbers in the SO group, as gestation progressed from early- to mid-gestation, leading to smaller litter size at day 18.5 (Akerele & Cheema, 2017). Thus, a balance of pro- and anti-inflammatory cytokines at different stages of pregnancy may be important for pregnancy sustainability.

Cytokines profile has been reported to shift towards less inflammatory/anti-inflammatory cytokines, especially IL-10 as pregnancy progressed towards mid-gestation (Paulesu *et al*., 2010). IL-10 primarily exerts its anti-inflammatory effect by inhibiting a wide range of pro-inflammatory cytokines during pregnancy, thereby eliciting the required balance during critical stages of pregnancy (Thaxton & Sharma, 2010). As such, IL-10 plays a key role in inflammation resolution during mid-pregnancy (Chatterjee *et al*., 2014; Paulesu *et al*., 2010). We observed a significant increase in the concentration of IL-10 in maternal plasma at mid-gestation in the FO group, while there was no change in the SO group. IL-10 has also been shown to peak at mid-gestation during pregnancy which is equivalent of day 12.5 in mice (Lin *et al*., 1993); this is consistent with our finding where IL-10 peaked at day 12.5 in maternal plasma. Others have shown that fetuses exhibit growth retardation when IL-10 was inhibited in the mothers during pregnancy (Rijhsinghani *et al*., 1997), while administration of exogenous IL-10 prevent fetal resorption in pregnant mice (Chaouat *et al*., 1995). Our data suggest that the fetal resorption sites observed in the SO group could be due to lower concentration of IL-10 in maternal plasma at mid-gestation, compared to the FO group.

Moreover, higher concentration of IL-10 observed in maternal plasma at mid-gestation in the high n-3 PUFA (FO) group is consistent with other studies showing that n-3 PUFA increases the production of IL-10 (Foitzik *et al*., 2002) at the expense of pro-inflammatory cytokines (Calder, 2013). As such, the implication of anti-inflammatory cytokines in mediating positive pregnancy outcomes cannot be over-emphasized.

The anti-inflammatory property of n-3 PUFA is exemplified partly by suppressing the downstream metabolism of n-6 PUFA to produce pro-inflammatory cytokines (Schmitz & Ecker, 2008), and directly by downregulating the gene expression of pro-inflammatory cytokines (Yamashita *et a*l., 2013). We found that the levels of both EPA and DHA were significantly higher in the placental samples from high n-3 PUFA (FO) groups at both mid- and late gestation, which coincided with lower levels of placental TNFα as compared to the low n-3 PUFA (SO) group. On the other hand, higher levels of pro-inflammatory cytokines in the low n-3 PUFA (SO) group could be mediated by the predominance of n-6 PUFA in the maternal placental. Metabolism of AA to downstream eicosanoids has been linked to increased production of TNFα and IL-6 (Patterson *et al*., 2012). AA was significantly higher in both uterine and placental samples obtained from the low n-3 PUFA (SO) group, compared to the high n-3 PUFA (FO) group; this explains higher levels of placental TNFα and IL-6 in the low n-3 (SO) PUFA group.

3.6 CONCLUSION

Our findings provide evidence that a maternal diet enriched in longer chain n-3 PUFA from fish oil caused accretion of EPA and DHA in reproductive tissues. The accretion of longer chain fatty acids coincided with a significant reduction in the concentration of pro-inflammatory cytokines at late gestation in both maternal plasma and placenta, with a resultant increase in the levels of anti-inflammatory cytokines. Furthermore, the SO diet revealed sites of resorption, while there were no resorption sites observed in the FO group. We have previously shown that the FO diet sustained higher number of fetuses, compared to the SO diet. Our current findings suggest that a diet enriched in longer chain n-3 PUFA may improve fetal sustainability via altering cytokine levels. The breeding chow diets used in our study varied in both the quantity and the quality of fat; it is possible that the amount of fat during pregnancy may also be important. Furthermore, fetuses used in our study comprised of both sexes; future studies will investigate sex-specific effects.

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CHAPTER FOUR

Maternal omega-3 fatty acids prevented gestational dyslipidemia, maintained a balance of inflammatory cytokines and improved pregnancy outcomes of C57BL/6 mice

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4.1 ABSTRACT

Omega (n)-3 polyunsaturated fatty acids (PUFA) are known to regulate lipid metabolism and inflammation; however, the regulation of maternal lipid metabolism and cytokines profile by n-3 PUFA during different gestation stages, and its impact on fetal sustainability is not known. We investigated the effects of maternal diet varying in n-3 PUFA prior to, and during gestation, on maternal metabolic profile, placental inflammatory cytokines, and fetal outcomes. Female C57BL/6 mice were fed either a high, low or very low (9, 3 or 1% w/w n-3 PUFA) diet, containing n-6:n-3 PUFA of 5:1, 20:1 and 40:1, respectively for 2 weeks before mating, and throughout pregnancy. Animals were sacrificed prior to mating (NP), and during pregnancy at gestation days 6.5, 12.5 and 18.5. Maternal metabolic profile, placental cytokines and fetal outcomes were determined. Our results show for the first time that a maternal diet high in n-3 PUFA prevented dyslipidemia in NP mice and maintained the expected lipid profile during pregnancy. However, females fed the very low n-3 PUFA diet became hyperlipidemic prior to pregnancy and carried this profile into pregnancy. Maternal diet high in n-3 PUFA maintained maternal plasma progesterone and placental pro-inflammatory cytokines profile, and sustained fetal numbers throughout pregnancy, while females fed the low and very-low n-3 PUFA diet had fewer fetuses. Our findings demonstrate the importance of maternal diet before, and during pregnancy, to maintain maternal metabolic profile and fetus sustainability. These findings are important when designing dietary strategies to optimize maternal metabolism during pregnancy for successful pregnancy outcome.

4.2 INTRODUCTION

Maternal nutrition during pregnancy has a profound impact on the developmental and metabolic outcomes of the offspring at early or later life (Laker *et al*., 2013; Perera & Herbstman, 2011). Moreover, maternal nutrition during pregnancy has been shown to influence the initiation (Fleming *et al*., 2011), progression (Hiersch & Yogev, 2017), and the outcomes of pregnancy (Veena *et al*., 2016). Pregnancy is a dynamic state involving several physiological changes, with a concomitant alteration in maternal metabolic profile (Lain & Catalano, 2007). Metabolic regulation is carefully controlled at each stage of pregnancy; this allows mothers to support fetal growth and development as pregnancy progresses. For example, the fetus relies on the supply of lipids, and specific fatty acids, for proper growth and development (Herrera & Ortega-Senovilla, 2010; Zeng *et al*., 2017), thus establishing the importance of lipids and lipoprotein metabolism during pregnancy.

During pregnancy, alterations in maternal lipid metabolism could be divided into two distinct phases: anabolic and catabolic phases (Grimes & Wild, 2018). The first trimester of pregnancy is typified by increased lipid synthesis and storage (anabolic phase), in order to meet fetal lipid and energy requirement at later stage of pregnancy (Zeng *et al*., 2017). Interestingly, *de novo* lipogenesis at early gestation in humans is, in part, regulated by increased insulin sensitivity (Wilcox, 2005). A similar study in rats attributed fat accumulation during early pregnancy to enhanced insulin responsiveness (Ramos *et al*., 2003). Knockout mouse models of the rate-limiting enzymes for *de novo* lipid synthesis (*ACACA* and *FAS*) showed embryonic death, demonstrating the importance of lipogenesis during pregnancy (Abu-Elheiga *et al*., 2005; Chirala *et al*., 2003). Besides *ACACA* and *FAS*, *DGAT2* also plays a key role in hepatic lipogenesis by catalysing the final reaction for the formation of TG; TG plays a key role in fetal growth and development by carrying essential fatty acids to the placental interface (Zammit, 2013).

In mice, plasma TG levels increase gradually during gestation, while TC decreases (Nikolova *et al*., 2017). Maternally derived cholesterol has been shown to cross the placenta during early gestation to support fetal growth, and also serves as a precursor for the synthesis of sex steroid hormones, particularly progesterone and estradiol, which are essential for a successful pregnancy (Grimes & Wild, 2018; Lindegaard *et al.*, 2005). S*tAR* mediates cellular cholesterol delivery, as well as intracellular processing and utilization for biosynthesis of estradiol and progesterone (Hu *et al*., 2010). Of interest is the establishment of direct relationship between changes in sex steroid hormones during pregnancy and lipogenesis (Goldberg & Hegele, 2012). For instance, increased circulating TG levels during pregnancy has been found to be driven by rise in the levels of progesterone during pregnancy (Grimes & Wild, 2018).

Although hepatic lipogenesis increases in early pregnancy to supply lipids to the fetus, levels return to preconception levels in late pregnancy (Grimes & Wild, 2018; Nikolova *et al*., 2017). Failure to maintain the levels of circulating lipids (within the normal range during pregnancy) results in maternal dyslipidemia, which is characterized by elevated lipid levels. Elevated lipid levels during pregnancy is known to elicit adverse pregnancy outcomes, which includes GDM, hypertensive complications such as preeclampsia, preterm birth, and other complications during delivery (Hadden & McLaughlin, 2009; Vrijkotte *et al*., 2012). Maternal dyslipidemia has also been shown to impair placental function, with extended consequences on pregnancy and perinatal health outcomes (Louwagie *et al*., 2018). Most adverse pregnancy outcomes can trace their origin to placental inflammation (Redline, 2004).

We have previously shown that a maternal diet high in omega (n)-3 PUFA increased the incorporation of n-3 PUFA by the placenta (Akerele & Cheema, 2020). N-3 PUFAs have been shown to cause significant changes in the fatty acid composition of membranes lipids,

with a resultant anti-inflammatory response in pregnant mice (Yamashita *et al*., 2013), whilst n-6 PUFA are generally pro-inflammatory in nature (Simopoulos *et al*., 1999). *GATA-3* has been implicated in the maintenance of anti-inflammatory cytokine levels such as interleukin IL-10 (Lee *et al*., 2000; Zheng & Flavell, 1997), which regulate the levels of corresponding pro-inflammatory cytokines such as IL-6, TNF-α, IFN-γ, and MCP-1 in the placental interface (Akerele & Cheema, 2016; Thaxton & Sharma, 2010). An imbalance in the levels of anti- and pro-inflammatory cytokines has been reported to cause aberrant inflammatory response during pregnancy, resulting in a number of adverse pregnancy outcomes (Cotechini & Graham, 2015; Renaud *et al.*, 2011). Interestingly, a maternal diet high in n-3 PUFA has been shown to ameliorate several adverse pregnancy outcomes such as low birth weight, preterm birth and perinatal death by regulating inflammatory cytokines (Albert *et al*., 2017; Imhoff-Kunsch *et al.*, 2012).

The effects of n-3 PUFA on the regulation of lipid metabolism and immune response are well known; however, no study to date has investigated the effects of maternal n-3 PUFA on maternal metabolic and inflammatory profile during different stages of gestation, and its impact on pregnancy outcome. We investigated the effects of maternal diets varying in the amount of n-3 PUFA on the regulation of maternal lipid metabolism, placental inflammatory response, and the pregnancy outcomes. We hypothesized that a maternal diet high in n-3 PUFA will maintain optimum maternal plasma and hepatic lipid profiles, maintain a balance of pro- and anti-inflammatory cytokines on the placental interface, and improve fetal outcomes. The specific objectives of this study were to investigate the effects of maternal diets varying in the amount of n-3 PUFA prior to and during gestation on: 1) the RBC and hepatic fatty acid composition, and the mRNA expression of genes involved in lipid synthesis (*FAS*, *ACACA*, and *DGAT2*; 2) maternal plasma and hepatic lipid profiles, and the levels of sex steroid hormones; 3) placental inflammatory cytokines, and the mRNA expression of

GATA-3; and 4) fetal numbers as an indicator of pregnancy outcomes. Our findings show for the first time that a maternal diet containing high n-3 PUFA (9% w/w; n-6/n-3 5:1) maintained maternal lipid and progesterone profiles, prior to and during pregnancy, reduced the concentration of inflammatory cytokines on the placental interface, and sustained higher number of fetuses.

4.3 MATERIALS AND METHODS

4.3.1 Experimental Diets

A semi-purified base diet was purchased without fat to allow the control of fat level at 20% w/w (MP Biomedicals, USA). Safflower oil, extra-virgin olive oil, lard, and Menhaden fish oil were used as sources of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n-6 PUFA, and n-3 PUFA, respectively, as per our previous studies (Akerele & Cheema, 2020; Balogun *et al*., 2014). Three different oil mixtures were prepared to contain approximately 9% (High), 3% (Low) and 1% (Very low) n-3 PUFA of the total dietary fat using a mathematical model (Appendix IV), while keeping the total amounts of SFA and MUFA constant as per our previous publication (Akerele & Cheema, 2020). The high n-3 PUFA diet contained n-6:n-3 PUFA of 5:1, which has been recommended for optimal body homeostasis (Gómez Candela *et al*., 2011). The low n-3 PUFA diet was designed to contain n-6:n-3 PUFA of 20:1, which represents the current n-6:n-3 PUFA in a typical North American diet (Simopoulos, 2016). The very low n-3 PUFA diet contains n-6:n-3 PUFA of 40:1, which has been characterized in several vegetarian communities, especially in India (Urban) (Mani & Kurpad, 2016; Simopoulos, 2016). The amount of dietary fat in each experimental diet represents approximately 40% calories from fat (FAO, 2010). The fatty acid composition of the experimental diets has been previously published (Akerele & Cheema, 2020), and is given in Table 5.2 of Chapter 5.

4.3.2 Animals and experimental design

All experimental procedures were carried out in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by Memorial University's Animal Care Committee (approval no: 18-11-SC). The 3 Rs for animal ethics were duly followed. Seven weeks old C57BL/6 mice (male and female) were purchased from Charles Rivers Laboratories, and were housed in separate cages under controlled temperature (21 ± 1°C) and humidity (35 ± 5%) conditions with a 12-h light/12-h darkness period cycle. All animals were kept on rodent chow (Prolab RMH 3000) (PMI Nutrition, MO, USA) for one-week acclimatization period. After this period, female mice were randomly divided into three (3) groups, and each group was assigned one of the three experimental diets that differ in their n-3 PUFA composition (High, Low and Very-low) for two weeks before mating (Figure 4.1). The nutritional and fatty acid composition of the experimental diets are provided in table 4.1 and 4.2, respectively.

Estrous cycle of the female mice was determined by vaginal physical examination, and mating was carried out. Female mice were checked by 6:00 am the following morning for vaginal plug formation to confirm pregnancy, representing gestation day 0.5 (Croy *et al*., 2015). Mice with confirmed pregnancy were continued on the assigned diets throughout gestation; fresh food and water was provided *ad-libitum* every other day. Daily food intake and body weight was recorded every other day; no significant difference in food intake was observed, and there was no difference in maternal weight across the dietary groups at each stage of pregnancy (Appendix V).

Table 4.1: Composition of the experimental diets with 20% (w/w) fat level

Ingredients	Experimental Diet (g/kg)
Casein	200
DL-methionine	3
Sucrose	305
Corn starch	190
Alphacel non-nutritive bulk	50
Vitamin mix[¥]	12
Mineral mix*	40
Fat	200

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

[¥]**Vitamin Mix:** 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 4.2: Fatty acid composition of the experimental diets

Fatty acids (%)	High n-3	Low n-3	Very-low n-3
C14:0	2.42	1.06	0.66
C16:0	11.94	11.89	11.70
C18:0	4.36	4.75	5.16
Σ SFA	18.72	17.70	17.53
C16:1n7	3.70	1.49	0.94
C18:1	22.80	24.16	25.32
C20:1n9	0.35	0.11	0.04
Σ MUFA	26.85	25.76	26.30
C18:2n6	45.23	53.77	54.81
C20:4n6	0.42	0.10	ND
Σ Omega-6	45.65	53.87	54.81
C18:3n3	0.81	0.80	0.82
C18:4n3	0.56	0.14	0.26
C20:5n3	4.13	0.88	0.16
C22:5n3	0.66	0.32	0.12
C22:6n3	2.59	0.52	ND
Σ Omega-3	8.75	2.66	1.36
n-6/n-3	5.2	20.1	40.1

Data are expressed as relative weight percentage of the total extracted fatty acids. Σ SFA = sum of saturated fatty acids; Σ MUFA = sum of monounsaturated fatty acids; Σ Omega-6 = sum of omega-6 polyunsaturated fatty acids; Σ Omega-3 = sum of omega-3 polyunsaturated fatty acids.

Mice (non-fasted) were sacrificed before pregnancy (non-pregnant; NP), at early gestation (day 6.5), mid-gestation (day 12.5) and late gestation (day 18.5) using isoflurane by inhalation. Blood was collected by cardiac puncture in tubes containing EDTA (4.5mM, pH 7.4) and was separated immediately into plasma and red blood cells (RBC). Maternal tissues were removed and weighed at the time of sacrifice. Maternal liver was carefully removed, freeze-clamped immediately in liquid nitrogen and stored at −80 °C until further analyses. Number of fetuses at day 12.5 and 18.5 were recorded; pictures of embryonic resorption sites were taken at gestation day 12.5 and 18.5 using Canon camera (SX500 IS).

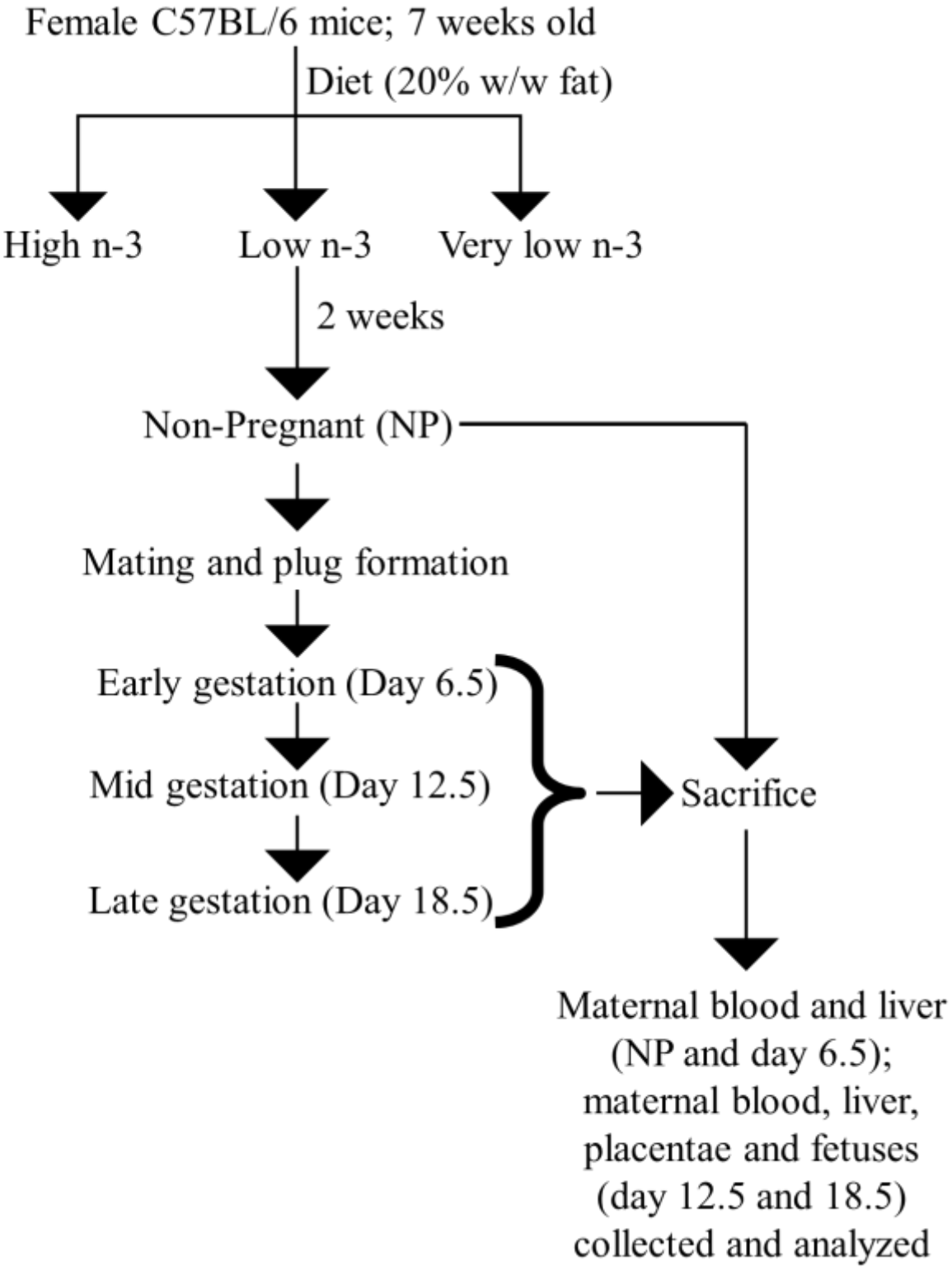

Figure 4.1: Experimental design: Each group of mice were fed one of the three experimental diets that differed in their n-3 PUFA amount, and designated as ‘‘High’’, ‘‘Low’’ and ‘‘Very-low’’, for two weeks before mating, and throughout gestation. Mice were sacrificed before

pregnancy (non-pregnant; NP), at early gestation (day 6.5), mid-gestation (day 12.5), and late gestation (day 18.5). Maternal blood and liver samples were collected at NP and day 6.5, while maternal blood, liver, placentae and fetuses were collected at day 12.5 and 18.5. N-3 PUFA: omega-3 polyunsaturated fatty acids.

4.3.3 Analyses of biochemical parameters

Lipids were extracted from the diets, RBC and liver samples according to the method of Folch *et al*. (Folch, Lees & Sloane, 1957) as per our previous publication (Chechi *et al*., 2010). Plasma biochemical parameters were quantified using commercially available kits according to the manufacturers' instructions: plasma and liver TG kit #236-17 (Genzyme Diagnostics, PEI, Canada); TC kit #234-60 (Genzyme Diagnostics, PEI, Canada); plasma glucose kit #10009582 (Cayman Chemical); insulin ELISA kit #KA3812 (Abnova Corporation, Taiwan); and non-esterified fatty acids (NEFA) kit #993-35191. Plasma progesterone concentration was determined using Architect Systems (#B7K770). The fatty acid composition of the extracted total lipids from diets, RBC and liver samples was determined using gas chromatography–flame ionization detection according to our previously published method (Chechi *et al*., 2010).

4.3.4 RNA extraction and real-time qPCR

Total RNA was extracted from placental and liver samples using Trizol method (Chomczynski & Sacchi, 1987). Genomic DNA contamination was removed by treating with DNase enzyme (Promega). The concentration of the extracted RNA was determined using Nano Drop 2000 (Thermo Scientific), and RNA integrity was assessed using 1.2% agarose gel. Complementary DNA (cDNA) was synthesized from the extracted RNA using reverse transcription as per our previous publication (Balogun & Cheema, 2014). All primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) (Accessed on 09/10/2019) and obtained from IDT technologies (IA, USA); primer sequences and efficiency are given in Table 4.1. Amplification was performed using iQ SYBR Green Supermix (Bio-rad). The reactions were run at a reaction volume of 10 μL and 50 ng cDNA per reaction.

Table 4.1: Primer sequences and efficiency

Gene (s)	Primers sequence (5′–3′)	Primer Efficiency (%)
Acetyl-CoA carboxylase 1; *ACACA* (Forward) Acetyl-CoA carboxylase 1; *ACACA* (Reverse)	ggccagtgctatgctgagat agggtcaagtgctgctcca	89.2
Fatty acid synthase; *FAS* (Forward) Fatty acid synthase, *FAS* (Reverse)	ctgcggaaacttcaggaaatg ggttcggaatgctatccagg	104.7
Diacylglycerol acyltransferase-2; *DGAT-2* (Forward) Diacylglycerol acyltransferase-2; *DGAT-2* (Reverse)	ctagctagttaggctaggtttcac caggaggatatagcgccagag	95.2
Steroidogenic acute regulatory protein; *StAR* (Forward) Steroidogenic acute regulatory protein; *StAR* (Reverse)	tgcccatcatttcattcatcctt aaaagcggtttctcactctcc	94.8
Gata-3 gene; *Gata-3* (Forward) Gata-3 gene; *Gata-3* (Reverse)	ggaaactccgtcagggcta agagatccgtgcagcagag	90.8
Beta-Actin; *Actb* (Forward) Beta-Actin; *Actb* (Reverse)	cacgcagctcattgtagaagg atggtgggaatgggtcagaag	107.5

All primers were designed using NCBI primer blast and obtained from IDT technologies.

Samples were run using the CFX96TM Real-Time System, while data output was managed using the CFX Manager version-3.0. The delta Ct values were recorded for each of the gene of interest and normalized with Beta-Actin (*Actb*) as the reference gene. The mRNA expression of enzymes involved in lipid synthesis (*ACACA*, *FAS*, and *DGAT2*) was measured in the liver, while *StAR* and *GATA-3* was measured in the placenta. The expression levels between groups were compared using the Livak method (Livak & Schmittgen, 2001).

4.3.5 Cytokine assays

Cytokine concentrations in the maternal placental samples were determined using BD Cytometric Bead Array (CBA) mouse inflammation kit #552364 (BD Biosciences, Canada) according to the manufacturer's instruction, and as per our previous publication (Akerele & Cheema, 2018). The efficiency and linearity of the kit for placental cytokines have been previously established (Akerele & Cheema, 2018). Cytokine concentrations were determined using FACSAria flow cytometer #650110C8 (BD Bioscience, Canada). Data analysis was performed using FCAP (flow cytometry analysis program) version-3.0.

4.3.6 Statistical analysis

Data were analysed using GraphPad Prism Software version-8.0. Sample means were compared using two-way analysis of variance (ANOVA) to determine main effects of diet and gestation stage, and the interactions between diet and gestation. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Results are expressed as mean ± standard deviation (SD) for $n = 8$ in each experimental group. Differences were considered to be statistically significant if $p < 0.05$. Pearson's correlation was used to compare the relationship between plasma TG and NEFA, as well as plasma progesterone and *StAR* mRNA expression.

4.4 RESULTS

4.4.1 High n-3 PUFA diet increased the incorporation of longer chain n-3 PUFA into maternal RBC during gestation

Diet had a significant effect on myristic acid (C14:0), showing higher levels in the high n-3 PUFA group (before and during pregnancy), compared to the low and very low n-3 PUFA groups (Table 4.2; $p<0.05$). Gestation stage had no effect on myristic acid. Diet and gestation stage had no significant effect on palmitic acid (C16:0), stearic acid (C18:0), and total SFA ($p>0.05$). However, there was a significant interaction between diet and gestation stage on stearic acid ($p<0.05$), revealing a decrease as gestation progressed in the low and very low n-3 PUFA groups, compared to the high n-3 PUFA group. Diet and gestation stage also had no effect on palmitoleic acid (C16:1n7), oleic acid (C18:1) and total MUFA ($p>0.05$). Interestingly, there was an interaction between diet and gestation stage on palmitoleic acid in all groups, revealing a significant decrease as gestation progressed in all groups ($p<0.05$).

There was no effect of diet on linoleic acid (C18:2n6; LA), however, there was a significant effect of gestation stage on LA ($p<0.05$). NP animals fed the very low n-3 PUFA diet had higher levels of LA, compared to the high and low n-3 PUFA groups. The levels of LA decreased as gestation progressed from day 6.5 to 18.5 in all diet groups ($p<0.05$). There was also a significant effect of diet on arachidonic acid (C20:4n6; AA), adrenic acid (C22:4n6), and total n-6 PUFA. The levels of AA ($p<0.001$), adrenic acid ($p<0.05$), and total n-6 PUFA ($p<0.001$) were lower in the high n-3 PUFA diet at NP and at all stages of gestation compared to the low and very low n-3 PUFA groups, respectively.

Table 4.2: Fatty acid composition of maternal red blood cells (RBC)

Fatty Acids (%)	High n-3				Low n-3				Very low n-3				Main Effects		
	NP	Day 6.5	Day 12.5	Day 18.5	NP	Day 6.5	Day 12.5	Day 18.5	NP	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation	Diet* Gestation
C 14:0	0.64±0.09	0.59±0.13	0.51±0.07	0.42±0.07	0.25±0.06	0.23±0.02	0.25±0.02	0.28±0.07	0.31±0.01	0.37±0.07	0.31±0.04	0.27±0.05	NS	NS	NS
C 16:0	31.73±3.46	30.37±2.95	31.66±0.94	32.32±1.34	23.81±0.54	25.69±0.76	24.89±0.96	27.37±0.30	28.77±1.16	28.36±1.24	30.49±0.39	31.58±1.72	NS	NS	NS
C 18:0	17.50±0.59^{b}	17.21±1.73^{b}	16.10±0.69^{bc}	15.34±1.07^{c}	21.44±1.08^{a}	18.19±0.72^{b}	21.47±0.28^{a}	18.77±1.71^{b}	16.81±0.64^{bc}	17.45±1.08^{b}	16.46±0.77^{bc}	16.37±0.57^{bc}	NS	NS	p<0.05
Σ SFA	**49.87±3.36^{a}**	**48.17±1.67^{a}**	**48.27±0.94^{a}**	**48.08±0.49^{a}**	**45.5±1.33^{b}**	**44.11±3.66^{b}**	**46.61±2.96^{ab}**	**46.42±0.93^{ab}**	**45.89±0.63^{b}**	**46.18±1.38^{ab}**	**47.26±0.80^{a}**	**48.22±0.87^{a}**	**NS**	**NS**	**p<0.05**
C 16:1n7	0.78±0.15^{a}	0.72±0.14^{a}	0.70±0.04^{a}	0.56±0.13^{b}	0.53±0.22^{b}	0.34±0.03^{c}	0.38±0.08^{c}	0.31±0.19^{c}	0.52±0.11^{b}	0.38±0.14^{c}	0.34±0.05^{c}	0.31±0.07^{c}	NS	NS	p<0.05
C 18:1	14.00±1.63	12.31±0.84	12.06±0.82	11.92±0.48	12.07±1.27	11.93±0.38	11.75±0.30	11.17±0.09	12.17±0.59	12.55±0.75	12.86±0.24	12.40±0.43	NS	NS	NS
Σ MUFA	**15.15±1.63**	**13.35±0.84**	**12.98±0.81**	**12.73±0.52**	**12.6±1.14**	**12.27±1.66**	**12.13±0.86**	**11.48±0.44**	**13.01±0.60**	**13.3±1.51**	**13.5±0.20**	**13.01±0.18**	**NS**	**NS**	**NS**
C18:2n6	14.68±0.42^{b}	15.83±0.90^{a}	12.87±0.68^{c}	12.14±1.04^{c}	14.81±0.72^{b}	15.69±0.25^{a}	12.20±0.61^{c}	11.66±0.76^{c}	16.17±0.68^{a}	15.17±1.23^{a}	12.98±1.37^{c}	12.67±0.98^{c}	NS	p<0.05	NS
C20:4n6	8.34±2.03^{b}	9.78±1.58^{b}	9.86±0.75^{b}	9.98±0.84^{b}	17.09±0.46^{a}	18.03±2.23^{a}	17.13±0.19^{a}	17.47±0.70^{a}	17.18±0.90^{a}	16.80±1.20^{a}	17.98±0.70^{a}	17.58±0.43^{a}	p<0.001	NS	NS
C22:4n6	1.25±0.12^{c}	0.53±0.08^{d}	0.51±0.11^{d}	0.64±0.09^{d}	1.62±0.12^{c}	1.45±0.20^{c}	1.61±0.08^{c}	1.50±0.03^{c}	1.82±0.12^{b}	1.86±0.18^{b}	2.52±0.16^{a}	2.45±0.11^{a}	p<0.05	NS	p<0.05
Σ n6 PUFA	**24.27±1.31^{cd}**	**26.14±1.18^{c}**	**23.24±0.69^{d}**	**22.76±0.97^{d}**	**33.52±1.50^{ab}**	**35.17±2.63^{a}**	**30.94±1.61^{b}**	**30.63±1.80^{b}**	**35.17±0.69^{a}**	**33.83±2.19^{ab}**	**33.48±0.60^{ab}**	**32.7±0.53^{b}**	**p<0.001**	**NS**	**NS**
C20:5n3	2.33±0.46^{b}	4.09±0.42^{a}	3.97±0.96^{a}	3.58±0.70^{a}	0.86±0.10^{d}	0.91±0.12^{d}	1.60±0.23^{c}	1.04±0.08^{c}	0.21±0.02^{e}	0.21±0.01^{e}	0.20±0.07^{e}	0.19±0.06^{e}	p<0.0001	NS	NS
C22:5n3	2.21±0.45^{a}	1.72±0.30^{a}	1.95±0.11^{a}	1.88±0.08^{a}	0.89±0.06^{c}	0.69±0.29^{c}	0.75±0.50^{c}	1.10±0.05^{b}	0.52±0.04^{d}	0.48±0.05^{d}	0.47±0.04^{d}	0.45±0.04^{d}	p<0.01	NS	p<0.05
C22:6n3	5.55±0.66^{c}	7.48±1.72^{b}	9.13±0.63^{a}	10.77±0.86^{a}	7.01±0.27^{b}	6.44±1.54^{b}	7.54±0.44^{b}	9.42±0.93^{a}	4.95±0.52^{c}	4.96±0.45^{c}	4.65±0.25^{c}	5.14±0.62^{c}	p<0.0001	p<0.001	p<0.001
Σ n3 PUFA	**10.09±0.68^{c}**	**13.29±0.46^{b}**	**15.05±0.95^{a}**	**16.23±0.89^{a}**	**8.76±0.45^{c}**	**8.04±1.91^{c}**	**9.89±0.48^{c}**	**11.56±1.11^{b}**	**5.68±0.57^{d}**	**5.65±1.23^{d}**	**5.32±0.82^{d}**	**5.78±0.52^{d}**	**p<0.0001**	**p<0.001**	**p<0.001**

Data are expressed as nmol percentage of total fatty acids. Values are expressed as mean ± SD, n=8. Main effects and interactions were determined by two-way ANOVA. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c, d, and e) show significant difference at NP and during gestation for each group ($p<0.05$). Σ SFA: sum of saturated fatty acids; Σ MUFA, sum of

monounsaturated fatty acids; Σ n-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σ n-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not determined; NS, not significant.

Diet also had a significant effect on the levels of eicosapentaenoic acid (C20:5n3; EPA), docosapentaenoic acid (C22:5n3; DPA), docosahexaenoic acid (C22:6n3; DHA) and total n-3 PUFA. The high n-3 PUFA diet had higher levels of EPA ($p<0.0001$), DPA ($p<0.01$), DHA ($p<0.0001$) and total n-3 PUFA, at NP and during gestation ($p<0.0001$), compared to the low and very low n-3 PUFA groups. DHA ($p<0.001$) and total n-3 PUFA ($p<0.001$) increased progressively from NP stage to late gestation (day 18.5) in the high n-3 PUFA group, while it only increased from day 12.5 to 18.5 in the low n-3 PUFA group. There was no change in DHA and total n-3 PUFA level in the very low n-3 PUFA group during gestation. The effect of diet on DPA, DHA and total n-3 PUFA was dependent on the gestation stage in the high and low n-3 PUFA groups, while there was no interaction between diet and gestation stage in the very low n-3 PUFA group.

4.4.2 High n-3 PUFA diet increased maternal hepatic n-3 PUFA as gestation progressed.

Diet and gestation stage had no significant effect on myristic acid, palmitic acid, stearic acid, and total SFA (Table 4.3). However, there was a significant interaction between diet and gestation stage on palmitic acid and total SFA ($p<0.05$), revealing a significant increase in palmitic acid from NP stage to late gestation (day 18.5) in the high n-3 PUFA group only, while total SFA increased significantly from NP stage to late gestation in high n-3 and very low n-3 PUFA groups, compared to the low n-3 PUFA group. Diet had no effect on palmitoleic acid, oleic acid, eicosenoic acid (C20:1n9), and total MUFA. However, gestation stage had an independent significant effect on oleic acid, eicosenoic acid and total MUFA to reveal a significant decrease in oleic acid, eicosenoic acid and total MUFA from NP stage to day 18.5 in the low n-3 PUFA and very low n-3 PUFA groups, compared to the high n-3 PUFA group. There was a significant interaction between diet and gestation stage on oleic acid, eicosenoic acid and total MUFA ($p<0.05$). The very low n-3 PUFA group had the highest levels of oleic acid, eicosenoic acid and total MUFA, at NP stage, followed by the low n-3 and high n-3 PUFA, respectively.

Diet and gestation stage had no significant effect on LA, AA, adrenic acid and total n-6 PUFA ($p>0.05$). However, there was an interaction between diet and gestation stage, revealing lowest levels in the high n-3 PUFA group at NP stage and at day 6.5, compared to the low and very low n-3 PUFA groups. There was a significant independent effect of diet on n-3 PUFAs, revealing that the high n-3 PUFA diet had higher amount of EPA, DPA, DHA, and total n-3 PUFA at NP stage and during gestation ($p<0.001$), compared to other groups. Gestation stage also had an independent significant effect on ALA, EPA, DHA and total n-3 PUFA ($p<0.05$). There was no change in ALA in the high n-3 PUFA group as gestation progressed to day 18.5, while ALA decreased significantly as gestation progresses in the low n-3 PUFA group. Interestingly, ALA was not detected in the low n-3 PUFA group at day 18.5, while it was not detected at all gestation stages (day 6.5 to 18.5) in the very low n-3 PUFA group.

Table 4.3: Maternal hepatic fatty acid composition

Fatty Acids (%)	High n-3				Low n-3				Very low n-3				Main Effects		
	NP	Day 6.5	Day 12.5	Day 18.5	NP	Day 6.5	Day 12.5	Day 18.5	NP	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation	Diet* Gestation
C 14:0	0.30±0.04	0.47±0.09	0.38±0.09	0.45±0.14	0.27±0.12	0.51±0.12	0.25±0.03	0.23±0.06	0.51±0.11	0.41±0.08	0.37±0.04	0.40±0.12	NS	NS	NS
C 16:0	22.05±1.06^{b}	24.14±2.21^{ab}	24.58±1.04^{ab}	26.91±1.25^{a}	18.91±0.39^{c}	20.81±2.06^{bc}	19.97±0.51^{c}	19.57±0.76^{c}	21.54±1.15^{bc}	21.20±1.84^{bc}	24.29±0.41^{ab}	23.77±1.61^{ab}	NS	NS	p<0.05
C 18:0	14.92±0.66	13.07±1.25	14.53±1.67	15.65±1.72	14.30±1.44	11.49±1.42	13.96±1.44	13.16±0.75	12.32±0.47	12.91±2.35	14.75±1.35	14.45±1.83	NS	NS	NS
Σ SFA	**37.27±0.91^{b}**	**37.68±1.43^{b}**	**39.49±1.20^{ab}**	**43.01±2.23^{a}**	**33.48±1.56^{c}**	**32.81±0.85^{d}**	**34.18±1.14^{c}**	**32.96±1.23^{cb}**	**34.37±1.93^{c}**	**34.52±0.95^{c}**	**39.41±1.59^{ab}**	**38.62±1.53^{b}**	**NS**	**NS**	**p<0.05**
C 16:1n7	1.21±0.22	1.62±0.45	1.24±0.44	1.18±0.44	0.83±0.29	1.51±0.38	0.70±0.16	0.53±0.11	1.73±1.04	1.23±0.85	1.24±0.28	0.75±0.29	NS	NS	NS
C 18:1	14.35±1.12^{d}	20.52±2.13^{a}	13.03±1.76^{d}	12.32±1.36^{de}	16.03±2.40^{c}	21.76±3.73^{a}	15.78±1.70^{c}	11.54±1.07^{e}	22.37±1.34^{a}	19.81±1.61^{ab}	18.82±2.51^{b}	18.18±1.47^{b}	NS	p<0.05	P<0.05
C 20:1n9	0.15±0.07^{c}	0.16±0.07^{c}	0.12±0.06^{d}	0.14±0.03^{c}	0.23±0.07^{b}	0.28±0.01^{a}	0.13±0.03^{c}	0.15±0.00^{c}	0.33±0.06^{a}	0.23±0.05^{b}	0.14±0.02^{c}	0.21±0.05^{b}	NS	p<0.05	P<0.05
Σ MUFA	**15.71±1.33^{d}**	**22.3±2.16^{a}**	**14.39±1.02^{d}**	**13.64±1.70^{d}**	**17.09±1.92^{c}**	**23.55±3.37^{a}**	**16.61±1.22^{cd}**	**12.22±1.23^{e}**	**24.43±2.46^{a}**	**21.27±1.72^{a}**	**20.2±2.20^{ab}**	**19.14±1.45^{b}**	NS	p<0.05	P<0.05
C18:2n6	22.30±1.95^{bc}	20.80±1.65^{c}	21.28±0.80^{c}	25.00±1.78^{ab}	26.74±1.83^{a}	27.50±2.19^{a}	22.87±2.58^{bc}	22.25±2.53^{bc}	24.37±1.83^{b}	23.62±2.91^{b}	20.88±1.89^{c}	21.19±2.81^{c}	NS	NS	p<0.05
C20:4n6	8.67±0.78^{b}	6.85±0.90^{c}	8.05±0.89^{b}	10.17±1.22^{b}	12.11±1.57^{a}	9.10±1.22^{b}	11.37±1.67^{ab}	11.03±0.87^{ab}	11.30±2.20^{ab}	11.30±2.01^{ab}	12.32±1.28^{a}	13.52±2.29^{a}	NS	NS	p<0.001
C22:4n6	0.12±0.02^{d}	0.09±0.01^{e}	0.12±0.04^{d}	0.13±0.05^{d}	0.19±0.06^{c}	0.34±0.17^{b}	0.26±0.02^{c}	0.36±0.02^{b}	0.33±0.08^{b}	0.30±0.05^{b}	0.46±0.09^{a}	0.55±0.17^{a}	NS	NS	p<0.05
Σ N6 PUFA	**31.09±1.46^{c}**	**27.74±2.21^{e}**	**29.45±1.03^{d}**	**35.3±2.13^{b}**	**39.04±1.48^{a}**	**36.94±2.31^{b}**	**34.5±1.07^{b}**	**33.64±2.59^{bc}**	**36.00±1.78^{b}**	**35.22±3.61^{b}**	**33.66±1.53^{bc}**	**35.26±3.07^{b}**	**NS**	NS	p<0.05
C18:3n3	0.12±0.08	0.13±0.07	0.11±0.01	0.18±0.03	0.13±0.07	0.12±0.02	0.10±0.02	ND	0.13±0.04	ND	ND	ND	NS	p<0.05	p<0.05
C20:5n3	3.11±0.47^{a}	2.70±0.37^{ab}	1.92±0.48^{b}	2.30±0.29^{b}	0.53±0.09^{c}	0.42±0.07^{d}	0.17±0.03^{e}	0.20±0.04^{e}	0.16±0.07^{e}	0.14±0.07^{e}	0.09±0.00^{f}	0.06±0.00^{f}	p<0.001	p<0.001	NS
C22:5n3	1.30±0.09	0.89±0.19	1.01±0.09	1.15±0.40	0.45±0.05	0.39±0.05	0.32±0.02	0.59±0.05	0.65±0.19	0.45±0.18	0.77±0.11	0.56±0.17	p<0.001	NS	NS
C22:6n3	10.92±0.97^{c}	9.32±1.06^{c}	13.33±1.11^{b}	19.00±1.09^{a}	8.80±0.81^{c}	6.28±0.61^{d}	9.19±0.85^{c}	13.97±1.78^{b}	4.94±1.13^{e}	4.20±0.83^{e}	4.73±0.57^{e}	5.51±0.54^{e}	p<0.001	p<0.001	NS
Σ N3 PUFA	**15.45±1.75^{bc}**	**13.04±1.39^{c}**	**16.37±1.16^{b}**	**22.63±2.34^{a}**	**9.91±0.81^{d}**	**7.21±1.11^{e}**	**9.78±0.76^{d}**	**14.76±1.77^{c}**	**5.88±1.26^{f}**	**4.79±1.40^{f}**	**5.59±0.38^{f}**	**6.13±1.66^{fe}**	p<0.001	p<0.001	NS

Data are expressed as nmol percentage of total fatty acids. Values are expressed as mean ± SD, n=8. Main effects and interactions were determined by two-way ANOVA. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c, d, e, and f) show significant difference at NP and during gestation for each group ($p<0.05$). Σ SFA: sum of saturated fatty acids; Σ MUFA, sum of monounsaturated fatty acids; Σ n-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σ n-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not determined; NS, not significant.

EPA decreased from NP stage to day 18.5 in all groups. DHA and total n-3 PUFA increased significantly from NP stage to day 18.5 in the high n-3 and low n-3 PUFA groups ($p<0.0001$), while there was no change in the very low n-3 PUFA group.

4.4.3 High n-3 PUFA diet maintained maternal plasma metabolic profile during gestation

Diet had a significant effect on plasma NEFA, revealing lower maternal plasma NEFA in the high n-3 PUFA diet group at NP stage and during gestation, compared to the low n-3 PUFA and very low n-3 PUFA group ($p<0.0001$; Fig 4.2A). Gestation stage had no effect on plasma NEFA across all groups ($p>0.05$). However, there was a significant interaction between diet and gestation stage ($p=0.048$), revealing that maternal plasma NEFA increased from NP stage and peaked at mid-gestation in the high and low n-3 PUFA groups only, compared to the very low n-3 PUFA group. Intriguingly, maternal plasma NEFA returned to pre-pregnancy state (NP) at late gestation in all groups.

There was a significant effect of diet on plasma TG ($p<0.0001$; Fig. 4.2B). Maternal plasma TG level was two times higher in the very low n-3 PUFA group at NP stage, compared to the high n-3 and low n-3 PUFA groups. The high n-3 PUFA group had the lowest TG, followed by the low n-3 PUFA and very low n-3 PUFA group, respectively, at NP stage and during gestation. Gestation stage had significant effect on maternal plasma TG levels ($p<0.05$), revealing an increase from NP stage and peaked at mid-gestation (day 12.5) in the high n-3 and low n-3 PUFA groups only, while there was no change in the very low n-3 PUFA group. There was a significant interaction between diet and gestation stage ($p=0.002$); maternal plasma TG increased as gestation progressed and returned to NP level at day 18.5 in high n-3 PUFA group only.

Maternal plasma TG also increased during gestation in the low n-3 PUFA group but did not return to NP level at day 18.5, while maternal plasma TG remained high at NP stage and during gestation in the very low n-3 PUFA group. Plasma TG levels correlated positively

with plasma NEFA across all diet groups (Fig. 4.2F). Diet also had a significant effect on maternal plasma TC levels ($p<0.001$; Fig. 4.2C). Maternal plasma TC level was significantly higher in the very low and low n-3 PUFA group at NP stage, compared to the high n-3 PUFA group. The high n-3 PUFA group had the lowest TC levels, compared to the low n-3 PUFA and very low n-3 PUFA groups, respectively at NP and during gestation. Gestation stage had a significant effect on maternal plasma TC level ($p<0.001$), revealing a progressive decrease from NP stage to late gestation (day 18.5) in all groups. There was a 41% decrease in maternal plasma TC level from NP stage to day 6.5 in the very low n-3 PUFA group.

Diet had an independent effect on maternal plasma insulin ($p<0.0001$; Fig. 4.2D), revealing that high n-3 PUFA diet had lower maternal plasma insulin level, compared to the very low n-3 PUFA group at NP, and during gestation. Gestation stage had an independent significant effect on maternal plasma insulin that increased from NP stage and peaked at mid-gestation (day 12.5) in the high n-3 PUFA and low n-3 PUFA groups. Maternal plasma insulin increased from NP stage to day 6.5 in the very low n-3, while there was no change during gestation. There was no effect of either diet or gestation stage on plasma glucose at NP and during gestation (Fig. 4.2E).

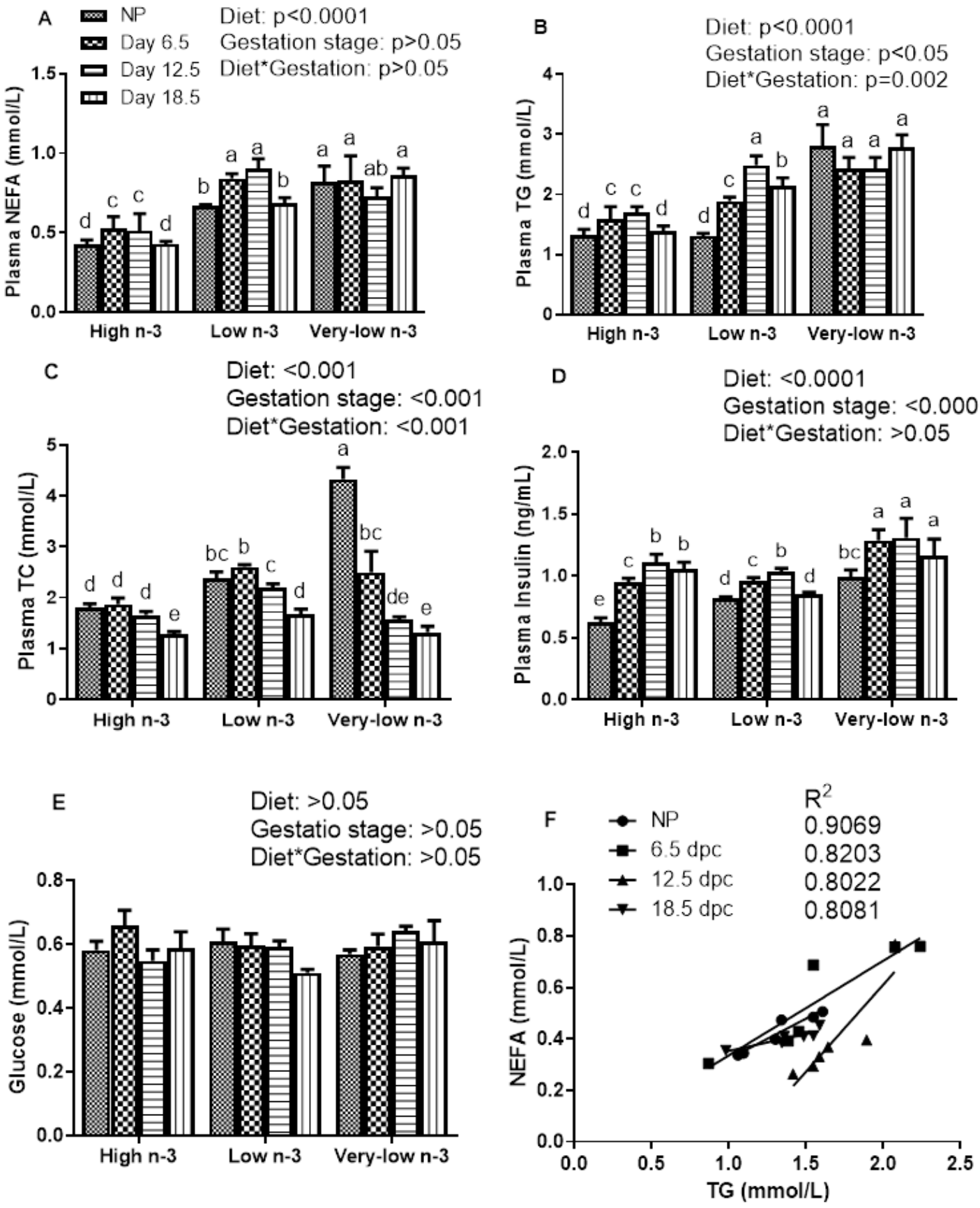

Figure 4.2: Effects of maternal diets varying in the amount of n-3 PUFA on plasma non-esterified fatty acid (NEFA; A), triacylglycerol (TG; B), total cholesterol (TC; C), insulin

(D), and glucose (E) was measured in non-pregnant (NP) females, and during gestation at day 6.5, 12.5 and 18.5 as explained in the materials and methods section. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Pearson's correlation analysis was carried out on plasma NEFA and TG (F). Data are presented as mean (n=8 at each gestation stage) ± SD; $p<0.05$ was considered significant. N-3 PUFA: omega-3 polyunsaturated fatty acids.

4.4.4 High n-3 PUFA diet maintained maternal hepatic lipid profile during gestation

Diet had a significant effect on maternal hepatic TG (Fig. 4.3A; $p<0.0001$), revealing lowest TG levels in the high n-3 PUFA group, followed by the low n-3 and very low n-3 PUFA groups, respectively at NP, and during gestation. Maternal liver TG level at NP stage was three-times higher in the very low n-3 PUFA and two-times higher in the low n-3 PUFA group, compared to the high n-3 PUFA group. Gestation stage also had an independent significant effect ($p<0.05$); hepatic TG increased from NP stage and peaked during gestation at day 12.5 in all diet groups. There was no interaction between diet and gestation stage ($p>0.05$).

Diet also had a significant effect on hepatic TC (Fig. 4.3B; $p<0.0001$), revealing lower TC levels in the high n-3 PUFA group, compared to the other groups at NP stage, and during gestation. Gestation stage had a significant effect on hepatic TC ($p<0.05$); lowest level of hepatic TC was observed on day 18.5 across all groups. There was an interaction between diet and gestation stage, revealing an increase from NP to day 6.5 in the high n-3 PUFA and low n-3 PUFA groups, while it decreased from NP to day 6.5 with no change during gestation in the very low n-3 PUFA group.

Diet had an independent significant effect on *ACACA* ($p<0.05$; Fig. 4.3C), *FAS* ($p<0.05$; Fig. 4.3D) and *DGAT2* ($p<0.05$; Fig. 4.3E), revealing lowest expressions in the high n-3 PUFA group, followed by the low n-3 and very low n-3 PUFA groups, respectively at NP stage, and during gestation. The mRNA expressions of *ACACA* and *FAS* at NP stage were two-times higher in the very low n-3 and low n-3 PUFA groups, compared to the high n-3 PUFA group. There was no effect of gestation stage on *ACACA* and *FAS* ($p>0.05$), while the mRNA expression of *DGAT2* increased as gestation progressed in the very low n-3 PUFA group only ($p< 0.05$).

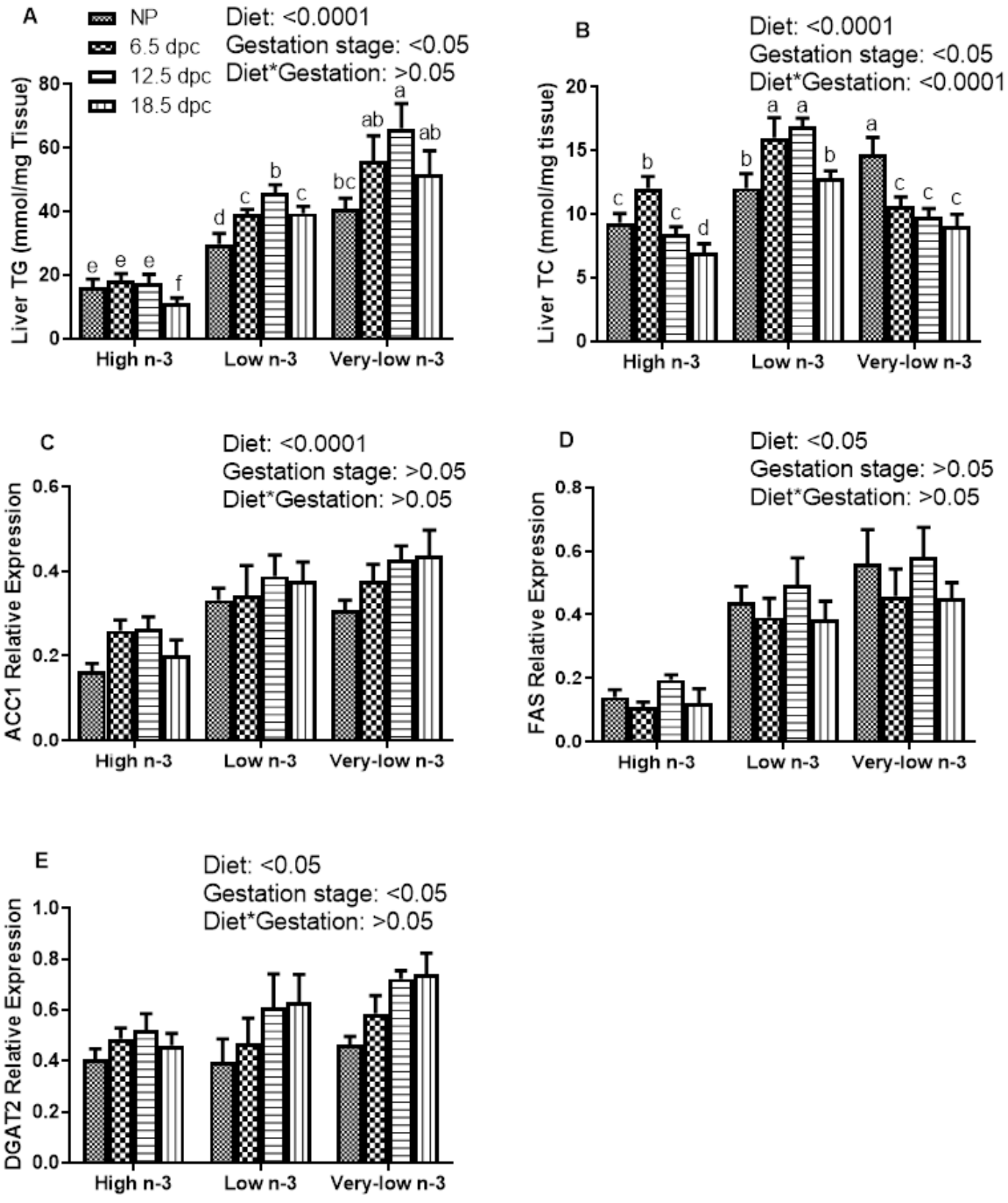

Figure 4.3: Effects of maternal diets varying in the amount of n-3 PUFA on hepatic triacylglycerol (TG; A), total cholesterol (TC; B), and the mRNA expressions of acetyl-CoA carboxylase 1 (ACACA; C), fatty acid synthase (FAS; D), and diacylglycerol

acetyltransferase 2 (DGAT2; E) was measured in non-pregnant (NP) females, and during gestation at day 6.5, 12.5 and 18.5 as explained in the materials and methods section. The mRNA expressions were normalized to β-actin (ActB) as the reference gene. Data were analyzed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Data are presented as mean (n=8 at each gestation stage) ± SD; $p<0.05$ was considered significant. N-3 PUFA: omega-3 polyunsaturated fatty acids.

4.4.5 High n-3 PUFA diet maintained maternal plasma progesterone and placental *StAR* mRNA expression during gestation

Diet and gestation stage had significant effects on plasma progesterone (Fig. 4.4A; $p<0.001$). The high n-3 PUFA group had higher levels of plasma progesterone in the NP females, compared to the low n-3 and very low n-3 PUFA groups. The low n-3 PUFA group had highest levels of plasma progesterone during gestation, followed by the high n-3 PUFA and very low n-3 PUFA group, respectively. Plasma progesterone levels peaked at mid-gestation (day 12.5) in all diet groups. There was an interaction between diet and gestation stage, revealing highest level of progesterone in the low n-3 PUFA group at both day 6.5 and 12.5, followed by the high n-3 PUFA group and then very low n-3 PUFA group. Similarly, *StAR* gene expression was highest in the low n-3 PUFA group, followed by high n-3, and then very low n-3 PUFA group (Fig. 4.4B; $p<0.0001$). Gestation stage also had an independent effect ($p<0.0001$), revealing that the mRNA expression of *StAR* decreased as gestation progressed from day 12.5 to 18.5 in all dietary groups. There was an interaction between diet and gestation stage ($p=0.02$); low n-3 PUFA group had the highest expression at both day 12.5 and 18.5, followed by high n-3 and very low n-3 PUFA group, respectively. The mRNA expression of *StAR* correlates positively with the plasma progesterone levels in all diet groups (Fig. 4.4C).

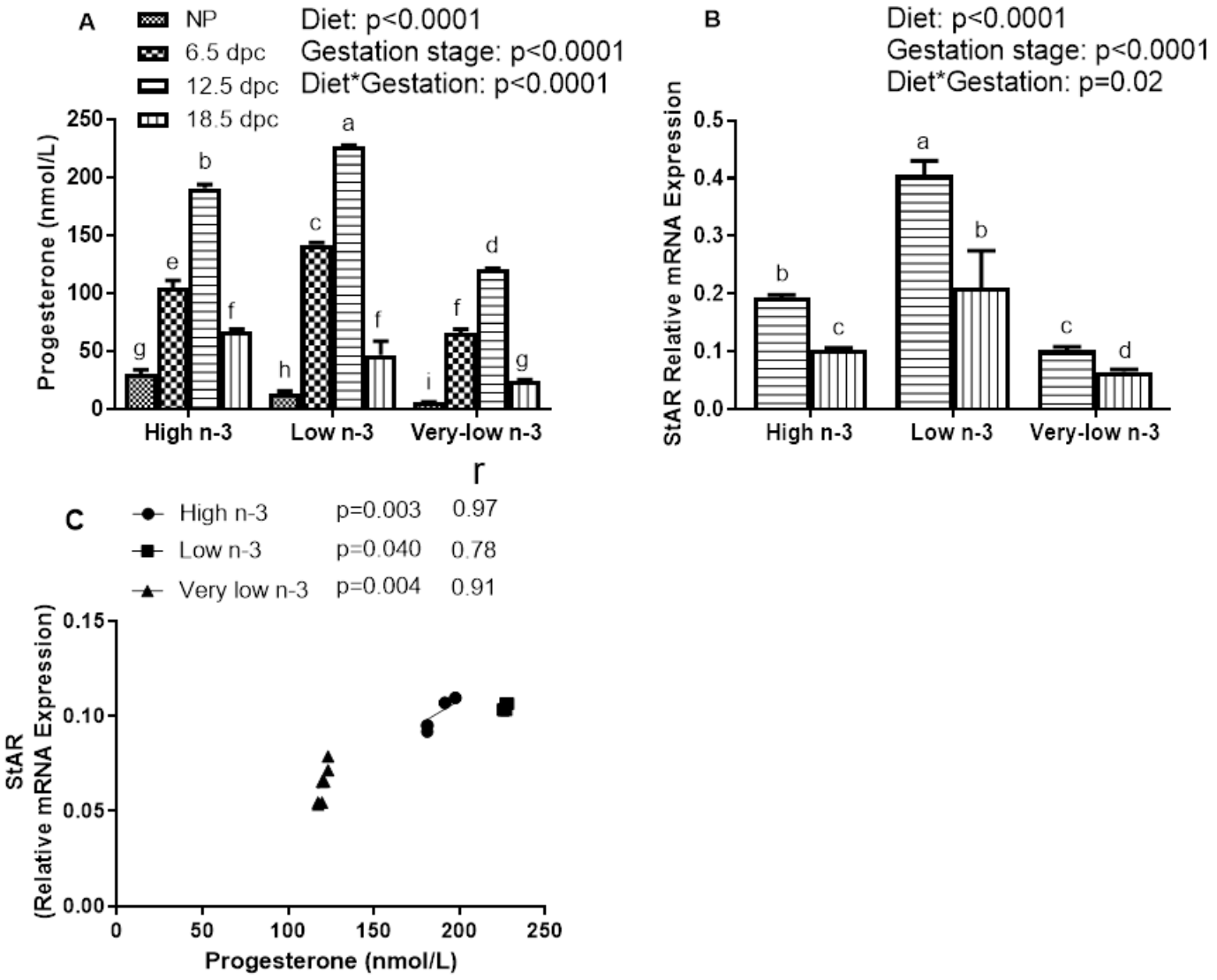

Figure 4.4: Effects of maternal diets varying in the amount of n-3 PUFA on plasma progesterone (A) and the mRNA expression of placental steroidogenic acute regulatory protein (*StAR*; B) was measured during different stages of gestation ((A: NP, day 6.5, 12.5 and 18.5; B: day 12.5 and 18.5) as explained in the materials and methods section. The mRNA expression was normalized to β-actin (ActB) as the reference gene. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Data are presented as mean (n=8 at each gestation stage) ± SD; $p<0.05$ was considered significant. Pearson's correlation analysis was carried

out on plasma progesterone and *StAR* mRNA expression (C). N-3 PUFA: omega-3 polyunsaturated fatty acids; NP: non-pregnant

4.4.6 High n-3 PUFA diet reduced placental inflammatory cytokines levels during gestation

Diet had a significant effect on TNF-α (Fig. 4.5A; $p<0.0001$), revealing the lowest level in the high n-3 PUFA group, compared to other diet groups. Gestation stage also had an independent significant effect ($p<0.0001$); TNF-α increased as pregnancy progressed from day 12.5 to 18.5 in all diet groups. High n-3 PUFA group had the lowest level of TNF-α at both day 12.5 and 18.5, compared to other groups. Diet had a significant effect on IFN-γ (Fig. 4.5B; $p=0.009$), revealing the lowest level in the high n-3 PUFA group, compared to other diet groups. Gestation stage also had an independent significant effect on IFN-γ ($p=0.008$); IFN-γ increased as pregnancy progressed from day 12.5 to 18.5 in the low n-3 PUFA diet group only, while there was no change in other groups. The high n-3 PUFA group had the lowest level of IFN-γ at day 18.5.

Diet had significant effect on IL-6 (Fig. 4.5C; $p<0.0001$), revealing highest level in the very low n-3 PUFA group, compared to other groups. There was also a significant effect of gestation stage on IL-6 levels ($p<0.0001$). A significant interaction was observed between diet and gestation stage, revealing that the levels of IL-6 increased from day 12.5 to 18.5 in the high and low n-3 PUFA groups only, while there was no change in the very low n-3 PUFA group. There was no significant difference in IL-6 levels at day 18.5 in all diet groups. Diet had a significant effect on MCP-1 (Fig. 4.5D; $p=0.0001$), revealing highest level in the low n-3 PUFA group, compared to the other groups; gestation stage had no effect on MCP-1 levels ($p>0.05$). There was an interaction between diet and gestation stage, revealing that the levels of MCP-1 increased from day 12.5 to 18.5 in the low n-3 PUFA, while it decreased in

the very low n-3 PUFA group. However, there was no change in MCP-1 levels the high n-3 PUFA group as gestation progressed from day 12.5 to 18.5.

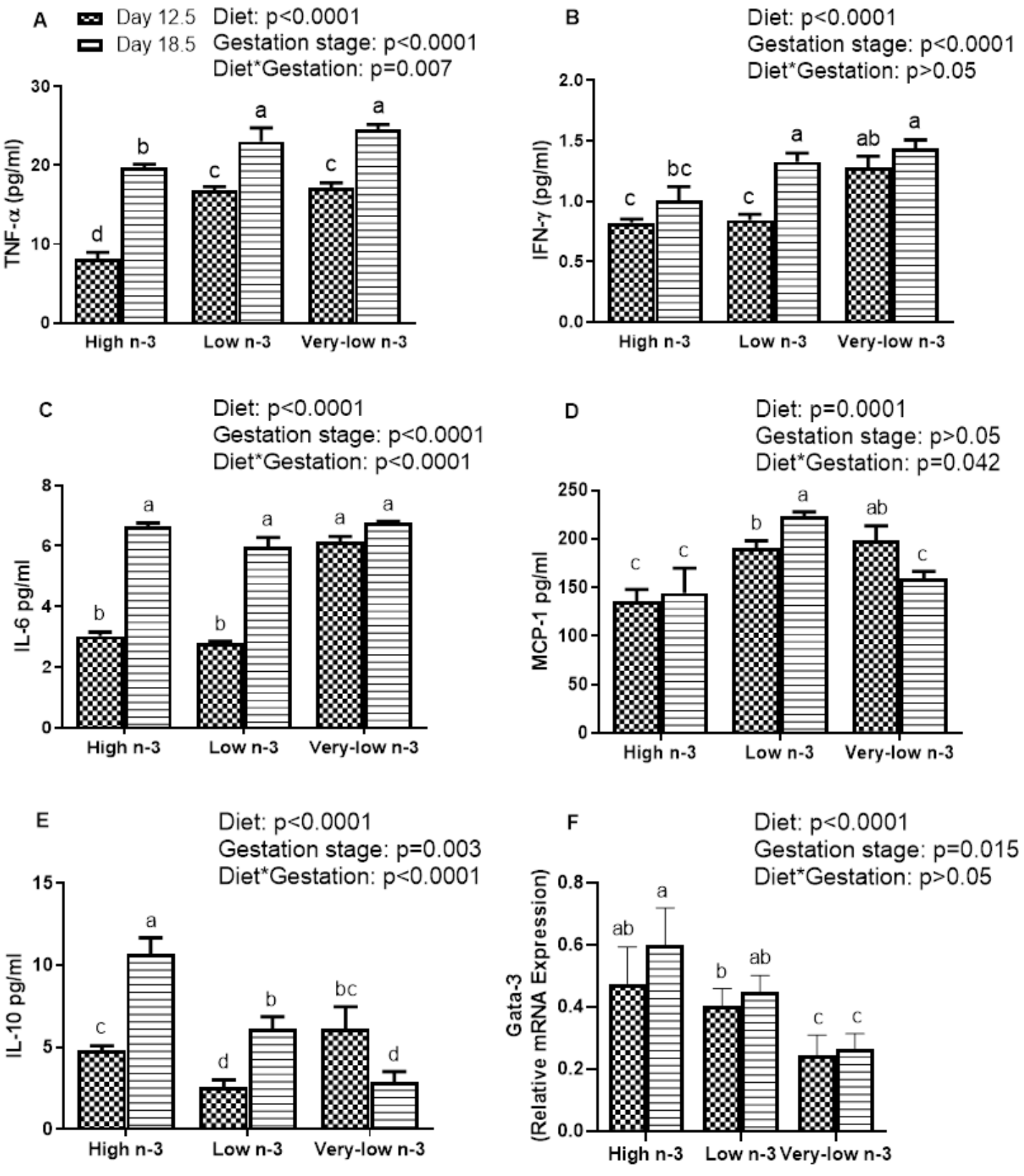

Figure 4.5: Effects of maternal diets varying in the amount of n-3 PUFA on placental tumor necrosis factor alpha (TNF-α; A), interferon gamma (IFN-γ; B), interleukin-6 (IL-6; C), monocyte chemotactic protein-1 (MCP-1; D), IL-10 (E), and the mRNA expression of *GATA-3* (F) was

measured during pregnancy at gestation day 12.5 and 18.5 as explained in the materials and methods section. The mRNA expression was normalized to β-actin (*ActB*) as the reference gene. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Data are presented as mean (n=8 at each gestation stage) ± SD; $p<0.05$ was considered significant. N-3 PUFA: omega-3 polyunsaturated fatty acids.

Diet had significant effect on IL-10 (Fig. 4.5E; $p<0.0001$), revealing highest level in the high n-3 PUFA group, compared to other groups. Gestation stage also had a significant effect on IL-10 levels ($p=0.003$). However, there was an interaction between diet and gestation stage ($p<0.0001$), revealing that the levels of IL-10 increased from day 12.5 to 18.5 in the high and low n-3 PUFA groups, while it decreased in the very low n-3 PUFA group. Diet had significant effect on the mRNA expression of *GATA-3* (Fig. 4.5F; $p<0.0001$), revealing highest level in the high n-3 PUFA group, followed by low n-3 PUFA group and very low n-3 PUFA group. There was no significant effect of gestation stage on the mRNA expression of *GATA-3* ($p>0.05$). There was an interaction between diet and gestation stage ($p=0.004$), revealing lower expression in the very low n-3 PUFA group, compared to the high n-3 and low n-3 PUFA groups.

4.4.7 High n-3 PUFA diet improves fetal sustainability during gestation

There was no significant difference in the pregnancy rates among all diet groups; however, it was also interesting to see that some animals delivered before day 18.5 in the low n-3 PUFA and very low n-3 PUFA groups, compared to the high n-3 PUFA group (Appendix VI). There was a significant independent effect of gestation stage on fetal number, revealing a decrease in fetal numbers as gestation progressed from day 6.5 to 18.5 in the low and very low n-3 PUFA group, while there was no change in fetal numbers as gestation progressed in the high n-3 PUFA group. Pictorial images revealed fewer fetuses at day 18.5 in the low n-3 and very low n-3 PUFA groups, with clear evidence of fetal resorption in the very low n-3 PUFA group at day 18.5, compared to the high n-3 PUFA group (Fig. 4.6). Diet had no effect on fetal weight, placental weight, liver weight and whole uterine weight ($p>0.05$) (Table 4.4). Gestation stage had an independent significant effect on fetal weight, placental weight, liver weight and whole uterine weight, revealing an increase in these parameters as gestation progressed from day 6.5 to day 18.5. There was no interaction between diet and gestation stage.

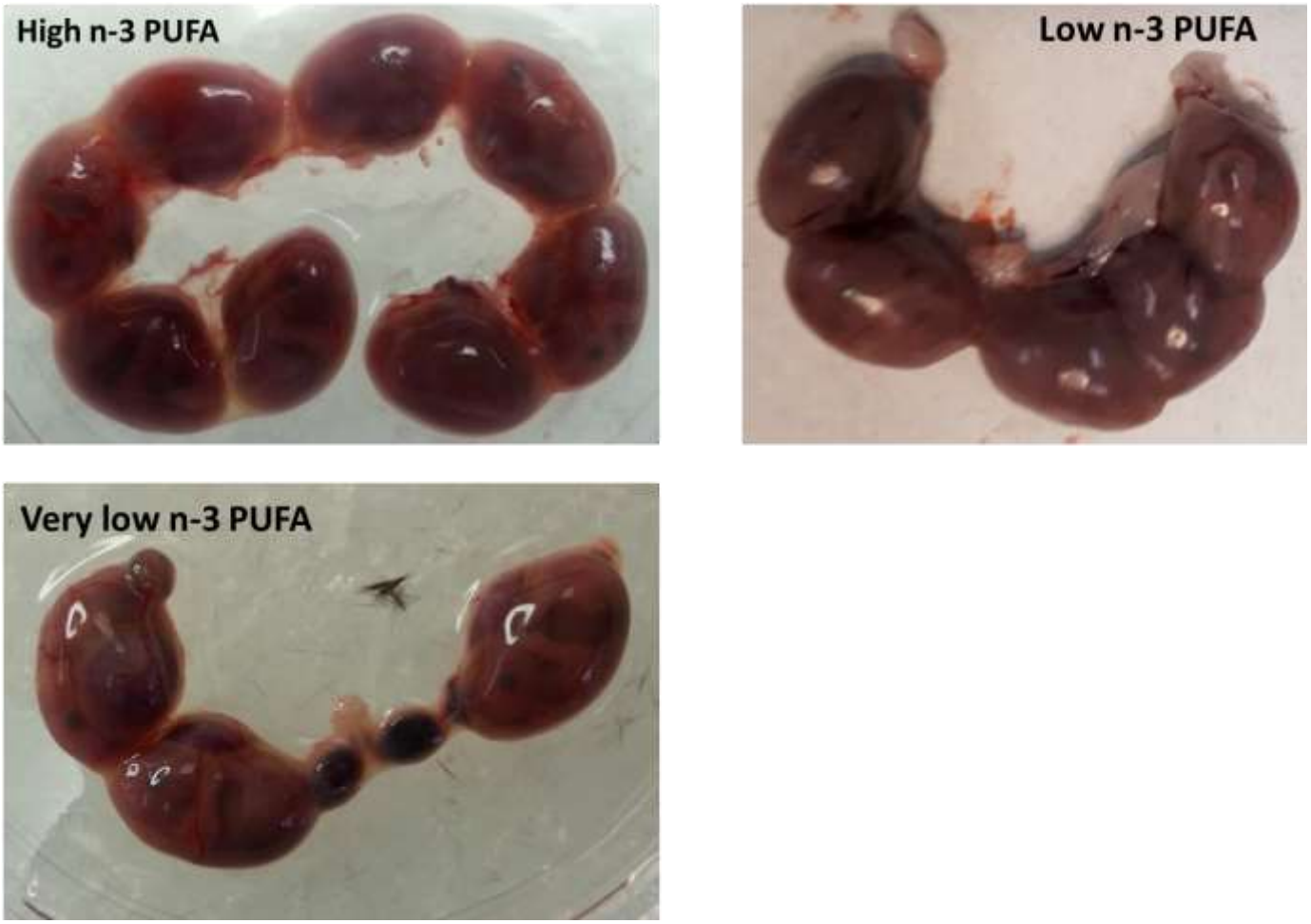

Figure 4.6: Representative images of fetuses at gestation day 18.5. The low n-3 and very low n-3 PUFA groups had fewer fetuses at gestation day 18.5, with clear evidence of fetal resorption (reddish-black spots) in the very low n-3 PUFA group at day 18.5, compared to the high n-3 PUFA group; n-3 PUFA: omega-3 polyunsaturated fatty acids.

Table 4.4: Pregnancy Outcomes

Outcomes	High n-3			Low n-3			Very low n-3			Main Effects		
	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Day 6.5	Day 12.5	Day 18.5	Diet	Gestation	Diet* Gestation
Fetal Number	9.0±1.07^{a}	8.3±1.11^{a}	8.0±1.41^{a}	8.4±0.92^{a}	7.9±1.70^{ab}	7.1±1.95^{b}	8.9±1.38^{a}	8.6±1.06^{a}	6.4±2.67^{b}	p>0.05	p=0.01	p>0.05
Fetal Weight (g)	N/A	0.11±0.03^{b}	1.15±0.16^{a}	N/A	0.13±0.04^{b}	1.10±0.14^{a}	N/A	0.11±0.01^{b}	1.02±0.12^{a}	p>0.05	p<0.001	p>0.05
Placental Weight (g)	N/A	0.07±0.01^{b}	0.11±0.01^{a}	N/A	0.08±0.01^{b}	0.11±0.02^{a}	N/A	0.08±0.01^{b}	0.09±0.01^{ab}	p>0.05	p<0.001	p>0.05
Liver Weight (g)	1.25±0.16^{b}	1.41±0.36^{ab}	1.64±0.24^{a}	1.22±0.18^{b}	1.40±0.11^{ab}	1.50±0.15^{a}	1.29±0.21^{b}	1.63±0.16^{a}	1.66±0.23^{a}	p>0.05	p<0.05	p>0.05
Whole Uterus (g)	0.16±0.04^{d}	3.44±0.16^{c}	11.10±0.84^{a}	0.14±0.02^{d}	3.19±0.09^{c}	9.21±0.14^{b}	0.15±0.04^{d}	3.04±0.25^{c}	9.52±2.58^{b}	p>0.05	p<0.0001	p>0.05

Values are presented as mean ± S.D., n = 8 females at each stage of pregnancy. Data were analysed using two-way ANOVA to determine the main effects and the interactions of diet and gestation stage. Pairwise comparison using Bonferroni correction was used to determine differences between groups. Letters (a, b, c, and d) represent significant difference between stages of pregnancy in each dietary group. $p < 0.05$ was considered significant; N/A: not available.

4.5 DISCUSSION

Dyslipidemia, as well as disrupted balance of cytokine profile in maternal placenta, has been implicated in the pathophysiology of several adverse pregnancy outcomes (Ilekis *et al.*, 2016; Nasioudis *et al.*, 2019). N-3 PUFA are well known to regulate lipid metabolism and the levels of cytokines; however, the effects of maternal diet varying in the amount of n-3 PUFA (and/or n-6:n-3) on maternal lipid metabolism and placental cytokines, and how it impacts pregnancy outcomes is not known. The key finding of this study was that maternal diet enriched in n-3 PUFA maintained maternal lipid metabolism prior to pregnancy, and as gestation progresses from early to late gestation, compared to the low and very low n-3 PUFA diet. Furthermore, a maternal diet high in n-3 PUFA increased the mRNA expression of *GATA-3*, a transcription factor involved in the induction of anti-inflammatory cytokine synthesis in the placenta. We show for the first time that these metabolic regulations by n-3 PUFA had a positive effect on fetal sustainability during pregnancy in C57BL/6 mice.

We found that females fed the high n-3 PUFA diet have higher levels of EPA, DPA and DHA, as well as total n-3 PUFA in RBC and liver, prior to pregnancy, and the levels further increased as gestation progressed, compared to other diet groups. Interestingly, there was no difference in hepatic ALA at NP stage for all dietary groups. However, hepatic ALA was not detected at day 18.5 in the low n-3 PUFA group, while ALA was not detected during any stage of gestation in the very low n-3 PUFA group. These findings indicate that ALA is being metabolized to longer chain n-3 PUFA in the low and very-low n-3 PUFA groups as pregnancy progressed.

The conversion of ALA to DHA is upregulated up to about 9% during pregnancy (Burdge & Wootton, 2002; Childs *et al*., 2011); no detection of ALA in the low and very-low n-3 PUFA groups in our study suggests that ALA is being converted to DHA to provide DHA for proper fetal growth and development. This is likely responsible for the observed increase

in the levels of DHA as gestation progressed to day 18.5. In contrast, the high n-3 PUFA group revealed no change in ALA before pregnancy and as gestation progressed from day 6.5 to 18.5. It has been reported that dietary DHA and EPA downregulate the conversion of ALA to EPA and DHA by up to 70% (Arterburn *et al*., 2006; Pawlosky *et al.*, 2003); this would explain why there was no change in ALA during gestation in the high n-3 PUFA group. DHA is crucial for fetal brain development (Innis, 2007); accretion of DHA in fetal brain has been shown to occur more rapidly during late gestation (Clandinin *et al*., 1980), demonstrating the significance of maternal DHA status on fetal brain development. We have previously shown that a maternal diet high in n-3 PUFA increased the mRNA expression of DHA transporters in maternal placental and fetal brain, which correlated with an increase in DHA accretion in the fetal brain (Akerele & Cheema, 2020). More so, we have previously reported that a diet high in n-3 PUFA increased neurotrophin signalling in fetal-brain as gestation progressed, demonstrating the importance of n-3 PUFA on fetal brain development (Akerele & Cheema, 2020).

Elevated lipid levels during pregnancy has deleterious impact on pregnancy (Wild *et al*., 2016); studies have revealed negative effects of maternal hypertriglyceridemia on pregnancy outcomes (Hadden & McLaughlin, 2009; Vrijkotte *et al*., 2012). Our findings revealed that the TG levels in the NP females fed the low and very low n-3 PUFA diet were significantly higher, compared to NP females fed the high n-3 PUFA diet. In fact, plasma TG levels in the NP females fed a very low n-3 PUFA diet was two-times higher than those of NP females in the high n-3 PUFA group. Thus, hyperlipidemia in NP females fed the very-low n-3 PUFA group may have caused further complications during pregnancy to impact fetal sustainability/pregnancy outcome. Apparently, females fed the very low n-3 PUFA diet entered pregnancy being hypertriglyceridemic.

Hypertriglyceridemia at early pregnancy has been associated with an increased risk of pregnancy-induced hypertension, pre-eclampsia and induced preterm delivery (Cortés-Vásquez *et al*., 2018; Vrijkotte *et al.*, 2012). During pregnancy, an increase in maternal TG contributes to proper fetal development by serving as a carrier for essential fatty acids, which are later released and transported across the placenta into fetal circulation (Catov *et al.*, 2007)**.** We found a significant increase in plasma TG at both early and mid-gestation in both high and low n-3 PUFA groups, while there was no further increase from NP as gestation progressed in the very low n-3 PUFA group. Plasma TG higher than 1.79 mmol/L has been suggested to be hypertriglyceridemic in mice (Nikolova *et al.*, 2017), revealing an optimum maternal plasma TG levels in the high n-3 PUFA group during pregnancy. However, the low n-3 PUFA group tends towards hypertriglyceridemia at early gestation, and became hypertriglyceridemic at mid-gestation., while the very low n-3 PUFA group was clearly hypertriglyceridemic prior to mating (NP stage), and TG levels remained high throughout pregnancy and did not return to normal levels at the end of pregnancy.

Women with high TG at early pregnancy were associated with 2.8-fold increase in the risk of spontaneous preterm birth (Catov *et al.*, 2007). Maternal dyslipidemia was also significantly associated with increased odds of premature membrane rupture during pregnancy (Smith *et al*., 2018). As such, any abnormality in the regulation of lipid metabolism during pregnancy could elicit deleterious fetal/pregnancy outcomes. Our result showed that females fed with low and very low n-3 PUFA had fewer fetuses at late gestation, compared to the female mice fed a diet high in n-3 PUFA. Pictorial image taken at day 18.5 clearly showed evidence of fetal resorption in the very low n-3 PUFA group. This corroborates a previous report showing that hypertriglyceridemia during pregnancy elicits fetal death at neonatal period in mice (Weinstock *et al*., 1995). More so, hypertriglyceridemia

has also been shown to impact embryonic viability and consequently neonatal lethality in mice (Ehrhardt *et al*., 2014).

De novo lipogenesis is an established mechanism responsible for increased plasma TG during pregnancy (Grimes & Wild, 2018). The increase in plasma and hepatic TG during gestation coincided with an increase in the mRNA expression of hepatic lipogenic genes; *ACACA, FAS* and *DGAT2*. Maternal plasma insulin levels increased during pregnancy and peaked at mid-gestation in the high and low n-3 PUFA groups. Maternal plasma insulin levels were higher in the very low n-3 PUFA group during gestation, and this corresponds with the plasma TG levels, which corroborates previous evidence that insulin plays a key role in *de novo* lipid synthesis during pregnancy (Kersten, 2001). Maternal lipids serves as the primary source of energy during pregnancy, thus sparing glucose and amino acid (Ghio *et al.*, 2011); this could explain no change in maternal plasma glucose during gestation in all groups (as shown in Figure 4.2E).

The very low n-3 PUFA group had two-fold higher TC levels at NP stage, compared to the high n-3 PUFA group, indicating maternal hypercholesterolemia prior to pregnancy. Pre-pregnancy hypercholesterolemia has been implicated in adverse pregnancy progression, as well as impaired fetal development in rodents (Miller, 1998). However, during pregnancy, maternally derived cholesterol is a major source of fetal cholesterol as it plays a key role in cell membrane formation and fetal growth (Bartels & O'Donoghue, 2011; Krause & Regen, 2014). Plasma and hepatic TC decreased progressively in all groups from NP stage to late gestation, suggesting a gestation-dependent delivery of cholesterol to fetal circulation.

It is well known that cholesterol is also used for the synthesis of sex steroid hormones (progesterone and estradiol) (Grimes & Wild, 2018), which are necessary for a suitable uterine environment for pregnancy establishment and progression (Miller, 1998). Dysregulation in progesterone synthesis during pregnancy has been greatly associated with a

number of adverse pregnancy outcomes, such as preterm birth in both humans and mice (Blanks & Brosens, 2012; Mendelson, 2009). Progesterone also plays a key role in maintaining uterine quiescence and preventing early contraction during pregnancy (Wira *et al*., 2010). During pregnancy, placentae become the primary site for sex steroid hormone synthesis (Yivgi-Ohana *et al*., 2009), and *StAR* predominantly mediates the rate-limiting step in the pathway (Stocco & Clark, 1996). We found that the mRNA expression of *StAR* correlates with the level of progesterone at both day 12.5 and 18.5 in all diet groups.

Progesterone peaked at mid-gestation in all dietary groups but the very low n-3 PUFA group had the lowest levels, which correlates with *StAR* mRNA expression. Earlier studies have also shown similar plasma progesterone levels, which peaked at mid-gestation (Bell & Dawson, 1983; Holinka *et al*., 1979). As such, our results revealed that plasma progesterone level was higher than optimum level (Bell & Dawson, 1983; Holinka *et al*., 1979) in the low n-3 PUFA group, while it was lower in the very low n-3 PUFA level. Although the effect of excess progesterone has not been fully determined, however, evidence suggests that excess progesterone could impair endometrial function during pregnancy (Liang *et al*., 2018). Mice with progesterone levels similar to the high n-3 PUFA group maintained viable embryos and reduced resorption frequency throughout pregnancy (Holinka *et al*., 1979). Interestingly, more fetuses were sustained in the high n-3 PUFA group. Progesterone has been shown to be potent in treating pregnancies threatened by abortion/miscarriage, in part by suppressing the expression of inflammatory cytokines in the placental interface (Blanks & Brosens, 2012; Kumar & Magon, 2012).

The high n-3 PUFA group had the lowest levels of pro-inflammatory cytokines (TNF-α, IFN-γ and MCP-1) in the placenta, especially at mid-gestation, which could further explain why more fetuses were sustained in the high n-3 PUFA group, while fetuses decreased significantly in the low n-3 and very low n-3 PUFA groups, respectively. N-3

PUFAs are generally anti-inflammatory, while n-6 PUFA propagates pro-inflammatory signals (Simopoulos, 2016). We have previously shown that a maternal diet high in n-3 PUFA increased the incorporation of n-3 PUFA into the placenta (Akerele & Cheema, 2020). Although pro-inflammatory cytokines play key roles during parturition by regulating cervical ripening, membrane rupture and myometrial contraction (Paulesu *et al*., 2010; Singh *et al*., 2011); however, elevated levels have been implicated in the pathogenesis of several adverse pregnancy outcomes such as preterm labour in humans (Pandey *et al.*, 2017).

In this study, the levels of pro-inflammatory cytokines such as TNFα, IFN-γ, IL-6 and MCP-1 were significantly higher in the placentae of animals fed the low and very low n-3 PUFA diets, compared to the high n-3 PUFA group. Studies have shown that high levels of TNFα, IFN-γ and IL-6 in the placenta mediate spontaneous fetal resorption by up to 50% in mice (Ali *et al*., 2014; Carpentier *et al*., 2011; Prins *et al.*, 2012). Although the functions of MCP-1 are yet be fully understood during pregnancy, MCP-1 has been suggested to be involved in the initiation of fetal growth restriction in rats (Robb *et al*., 2017). Our findings show evidence of fetal resorption in the low and very low n-3 PUFA groups, which had higher levels of pro-inflammatory cytokines, leading to significantly lower number of fetuses in these groups at late gestation, compared to the high n-3 PUFA group.

On the other hand, IL-10 is an anti-inflammatory cytokine which has been shown to inhibit the activity of several inflammatory cytokines (Paulesu *et al*., 2010), thereby establishing a required cytokine balance at critical stages of pregnancy (Thaxton & Sharma, 2010). We observed that the level of IL-10 was significantly higher in the high n-3 group during gestation, compared to other groups. IL-10 decreased significantly as gestation progressed from day 12.5 to 18.5 in the very low n-3 PUFA group. Fetal growth retardation has been reported when IL-10 was inhibited during pregnancy (Rijhsinghani *et al*., 1997), while fetal resorption was prevented when exogenous IL-10 was administered in mice

(Chaouat *et al*., 1995). Our data suggest higher fetal resorption when IL-10 decreased as gestation progressed from day 12.5 to 18.5 in the very low n-3 PUFA group. Other studies have also showed that n-3 PUFA increases the production of IL-10 (Foitzik *et al*., 2002), with a concomitant reduction in inflammatory cytokines (Calder, 2013). We also found a significant increase in the expression of *GATA-3* which plays a major role in the synthesis of IL-10 (Lee *et al*., 2000; Zheng & Flavell, 1997). Thus, the implication of IL-10 in mediating fetal sustainability during pregnancy cannot be over-emphasized.

Literature reports are inconsistent on the effects of n-3 PUFA on fetal number/litter size in mice, and other animal models; some reported increase, while others reported a decrease or no effects (Anderson *et al*., 2014; Eastwood *et al*., 2014; Fountain *et al*., 2008; Rebollar *et al*., 2014; Smit *et al*., 2015; Smits *et al*., 2011; Yi *et al*., 2012). Nonetheless, a diet high in n-6 PUFA during gestation was not necessarily associated with an increase in litter size (Fattahi *et al*., 2018; Shahnazi *et al*., 2018), but was found to cause intrauterine growth restriction (Reyes-Hernández & Ramiro-Cortijo., 2018). *Fat-1* transgenic mice that are engineered to endogenously synthesize n-3 PUFA, and yield 1:1 tissue ratio of n-6:n-3 PUFA, show increased pregnancy rates and shorter time to pregnancy (Hohos *et al*., 2018). Our result showing that more fetuses were sustained till late gestation in mice fed a diet high in n-3 PUFA is consistent with other reports (Kasture *et al*., 2019; Yan *et al*., 2013).

4.6 CONCLUSION

Overall, our results show for the first time that a maternal diet high in n-3 PUFA prevented dyslipidemia in NP mice, while very low n-3 PUFA diet caused hyperlipidemia prior to pregnancy. Females with elevated lipids before pregnancy carried this profile into pregnancy and lacked metabolic regulation during pregnancy. As such, maternal diet before, and during pregnancy, is very important to ensure that mothers enter pregnancy with the metabolic profile required to establish pregnancy successfully, as well as to maintain

pregnancy; Fig. 4.7 proposes an overview of the effects of n-3 PUFA on maternal metabolic regulation, and its effects on fetal sustainability. Furthermore, maternal diet high in n-3 PUFA maintained the maternal plasma progesterone and placental pro-inflammatory cytokines profile thereby sustaining fetal numbers. These novel findings may be important when designing dietary strategies to optimize maternal metabolism during pregnancy and to elicit fetal sustainability.

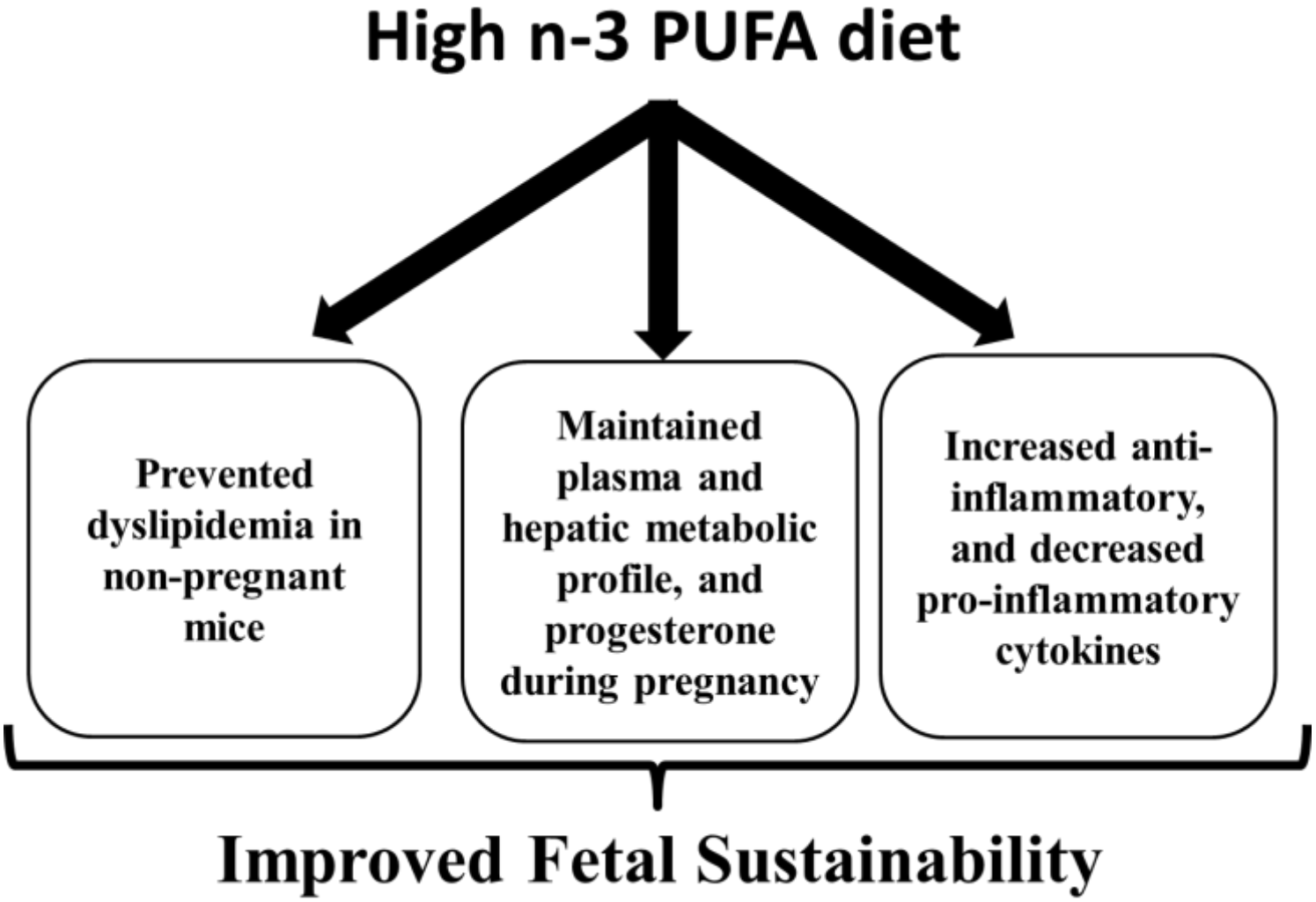

Figure 4.7: High n-3 PUFA diet prevented maternal dyslipidemia prior to pregnancy, maintained metabolic and inflammatory profile during pregnancy, and improved pregnancy outcomes.

4.7 REFERENCES

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CHAPTER FIVE

Maternal diet high in omega-3 fatty acids upregulate genes involved in neurotrophin signalling in fetal brain during pregnancy in C57BL/6 mice

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5.1 ABSTRACT

Neurotrophins play a critical role in the development, maintenance, and proper function of the brain. We investigated the effects of maternal diet high in omega (n)-3 polyunsaturated fatty acids (PUFA) on fatty acids composition and the gene expression of neurotrophins in fetal brain at different gestation stages. Female C57BL/6 mice (7 weeks old, n=8/group) were fed a diet containing high, low or very low n-3 PUFA (9, 3 or 1% w/w, respectively), with an n-6:n-3 PUFA of 5:1, 20:1 and 40:1, respectively, for two weeks before mating and throughout pregnancy. Animals were sacrificed during pregnancy at gestation day 12.5 and 18.5 to determine placental and fetal-brain fatty acids composition. The gene expressions of endothelial lipase (EL) and plasma membrane fatty acid-binding protein (FABPpm) were measured in the placenta, while major facilitator superfamily domain-containing protein-2 (Mfsd2a), brain-derived neurotrophic factor (BDNF), tropomyosin-receptor kinase (TrK)-B, and cAMP response element-binding protein (CREB) were measured in fetal-brain, using qPCR. The protein expression of phosphorylated CREB (pCREB) was determined using ELISA. The high n-3 PUFA diet increased the mRNA expression of EL, FABPpm, and Mfsd2a at both gestation days, compared to other groups. Docosahexaenoic acid (DHA) and total n-3 PUFA were significantly higher in the high n-3 PUFA group, compared to the other groups at both gestation days. The high n-3 PUFA diet also increased the mRNA expressions of BDNF, TrKB and CREB, as well as the protein concentration of pCREB as gestation progressed, compared to the other groups. Our findings show for the first time that maternal diet high in n-3 PUFA increased the mRNA expression of Mfsd2a, which correlated with an increase in DHA accretion in the fetal-brain. A diet high in n-3 PUFA increased neurotrophin signalling in fetal-brain as gestation progressed, demonstrating the importance of n-3 PUFA during brain development.

5.2 INTRODUCTION

Mental and neurological disorders are rising at an alarming rate, with a global burden surpassing cardiovascular diseases and cancer (Collins *et al*., 2011; Whiteford *et al.*, 2015). Currently, mental and neurological disorders affect more than 1 billion people globally (Rehm & Shield, 2019). The causes of most neurological disorders are vaguely understood and are defined by numerous factors. Neurodevelopmental hypotheses have been suggested to explain the pathogenesis of a number of neurological disorders (Murray *et al*., 2017; Owen *et al*., 2011). These hypotheses identify disrupted developmental processes occurring in the brain, such as impaired synaptogenesis, aberrant genes, as well neuronal hazards (involving abnormal maturation and differentiation of neuronal cells) as the major players in the development of neurological problems. Gestational environment / early life insult has been identified as a risk factor for the development of neurodegenerative diseases at late-life (Barlow *et al*., 2007; Miller & O'Callaghan, 2008) as the composition and numbers of neurons are determined early in development (Oppenheim, 1991).

Approximately 60% of the brain's structural component is lipid (Chang *et al*., 2009). The accumulation of PUFA in the brain is critical during development; PUFA represents about 35% and 30% of brain lipids and dry weight of the brain, respectively (Hallahan & Garland, 2005; Liu *et al*., 2015). The brain is highly enriched in arachidonic acid (AA; omega (n)-6 PUFA) and docosahexaenoic acid (DHA; n-3 PUFA); these fatty acids make up about 90% of brain PUFAs (Lauritzen *et al*., 2001; Singh, 2005). DHA constitute about 10-15% of total fatty acids in the brain, representing more than 97% of total n-3 PUFA (Makrides *et al*., 1994; O'Brien *et al*., 1964). Both n-6 and n-3 PUFA are regarded as essential fatty acids because the body cannot synthesize these endogenously; hence, the developing fetus relies solely on the mother to meet their requirement (Crawford *et al.*, 1976; Devarshi *et al.*, 2019). A recent global survey revealed a significant decline in the consumption of n-3 PUFA due to

Westernized dietary habits (Stark *et al*., 2016), with a concomitant increase in the burden of neurological disorders across the globe (Collins *et al*., 2011).

Different international organisations have made dietary recommendations of n-6 to n-3 PUFA ratio of approximately 5:1 as an optimal ratio for whole-body homeostasis (Gómez Candela *et al*., 2011). However, a typical Western diet contains a high ratio of n-6 to n-3 PUFA of between 20-30:1 (Gómez Candela *et al*., 2011; Simopoulos, 2016). Accretion of DHA in the brain is most rapid during the third trimester of pregnancy and the first year after birth (Clandinin *et al*., 1980; Martínez & Mougan, 1998). Developing fetus accrues up to 70 mg DHA per day during the last trimester, specifically in the brain (Innis, 2005), demonstrating the significance of maternal DHA status on fetal brain development at this critical stage. Maternal DHA level is low during the last trimester of pregnancy, which perhaps explains a higher rate of transfer of DHA to the fetus (Montgomery *et al*., 2003). Nonetheless, insufficient intake of n-3 PUFA during pregnancy results in impaired cognitive and physiological functions in infants (Catalan *et al*., 2002), which has been suggested to be irreversible by postnatal supplementation (Nesheim & Yaktine, 2007).

During gestation, the placenta uptakes the maternal circulating free fatty acids released by maternal LPL and EL (Gil-Sánchez *et al*., 2012). EL hydrolyses both phospholipids and TGs (McCoy *et al*., 2002); studies have shown that increased EL expression contributes majorly to placental fatty acid uptake (Lindegaard *et al*., 2005). Fatty acids are then transported across the placenta through passive diffusion and majorly by membrane carrier proteins (Gil-Sánchez *et al*., 2012). These membrane carrier proteins include FAT/CD36, FATP and FABPpm. However, FABPpm is the main transporter for longer chain PUFAs (AA and DHA) across the placenta into fetal circulation (Campbell *et al*., 1998). A member of the major facilitator superfamily domain containing 2a (Mfsd2a) is

required for the uptake of DHA into the brain (Nguyen *et al*., 2014), where DHA affects brain growth and development by regulating neurotrophins (Sona *et al*., 2018).

BDNF has been extensively studied and characterized as an important neurotrophin in the central nervous system, due to its pivotal role in the development and maintenance of normal brain function (Bathina & Das, 2015). The mRNA expression of BDNF has been shown to fluctuate at different stages of development, indicating different regulatory roles at different stages of life (Maisonpierre *et al.*, 1990). For instance, BDNF promotes neuronal development and survival, and prevent the death of peripheral sensory neurons at early postnatal period in mice (Ernfors *et al*., 1994), while it modulates synaptic plasticity to enhance learning and long-term memory in adult mice (Egan *et al.*, 2003). BDNF binds to its high-affinity receptor, TrKB, and signals the downstream activation of the transcription factor CREB (Bhatia *et al*., 2011). In the developing brain, CREB regulates crucial cell stages such as proliferation, differentiation, and survival of neurons, as well as neuronal plasticity (Ortega-Martínez, 2015).

We have previously shown that DHA differentially regulates BDNF and its target receptor at weaning and 16-weeks post-weaning in mice (Balogun & Cheema, 2014). A plethora of recent evidence from the literature have shown that n-3 PUFA regulates BDNF in adult human (Ferreira *et al*., 2014; Pawełczyk *et al*., 2019); however, vast majority of neurons are formed prenatally in the brain. Thus, we investigated the effects of maternal diets high or low in n-3 PUFA on DHA accretion in fetal brain and the regulation of gene expression of BDNF, its receptor TrkB, and CREB during different stages of gestation. We hypothesized that maternal diet high in n-3 PUFA would cause an accretion of DHA in the fetal brain during gestation, and consequently increase the mRNA expression of BDNF, TrKB, and CREB as gestation progresses.

The specific objectives of this study were to investigate the effects of maternal diets varying in the amount of n-3 PUFA during different stages of gestation on: 1) placental fatty acid composition, and the mRNA expressions of EL and FABPpm, 2) the mRNA expression of Mfsd2a and incorporation of n-3 PUFA into fetal brain, and 3) the regulation of mRNA expression of BDNF, TrKB, and CREB. Our findings show for the first time that maternal diets high in n-3 PUFA cause increased mRNA expression of Mfsd2a, which correlates with increased accretion of DHA in the fetal brain, with a concomitant increase in the mRNA expression of neurotrophins and their target receptors as gestation progressed.

5.3 MATERIALS AND METHODS

5.3.1 Ethics statement

All experimental protocols involving animal handling and surgeries were approved by Memorial University's Animal Care Committee (approval number: 18-11-SC) in accordance with the principles and guidelines of the Canadian Council on Animal Care, and following the 3 Rs for animal ethics.

5.3.2 Diets

A custom semi-synthetic base diet was purchased without fat (MP Biomedicals, USA) to allow the control of fat level at 20% w/w. Four different oils (menhaden fish oil, safflower oil, extra-virgin olive oil, and lard) were used as sources of fatty acids (saturated fatty acids, SFA; monounsaturated fatty acids, MUFA; n-3 PUFA; n-6 PUFA) (Table 4.1). These oils were used to make three different diet mixtures: high n-3, low n-3, and very low n-3, containing approximately 9, 3, and 1% (w/w fatty acids) n-3 PUFA, respectively. The amount

of total SFA and MUFA in all experimental diets was kept constant using a mathematical model as per our previous publication (Balogun & Cheema, 2014). The high n-3 PUFA diet contained n-6:n-3 PUFA of 5:1; this ratio has been suggested to be adequate for optimum body homeostasis (Gómez Candela *et al*., 2011). The low n-3 PUFA diet was designed to contain n-6:n-3 PUFA of 20:1, which represents a typical North American diet (Simopoulos, 2016); while the very low n-3 PUFA diet contains n-6:n-3 PUFA ratio of 40:1, which has been characterised in vegetarian communities, especially in current India (Urban) (Mani & Kurpad, 2016; Simopoulos, 2016). The fatty acid composition of all the experimental diets is given in Table 4.2.

5.3.3 Experimental design

Seven-week old male and female C57BL/6 mice were purchased from Charles River Laboratories (MA, USA). Female mice were housed in separate cages with regulated environmental conditions (temperature, 21 ± 1^0C; humidity, 35 ± 5%; 12 hours light / 12 hours dark period cycle). All mice were fed the standard rodent chow (Prolab RMH 3000; PMI nutrition, USA) during one week of acclimatization period. Females were then randomly divided into three (3) groups (Figure 5.1) and each group was fed with one of the three experimental diets (high n-3, low n-3 and very low n-3 PUFA diets, respectively), for two weeks prior to mating. Animals were then mated, and females were checked for plug formation by 6:00 am the following morning. Animals with plugs were recorded with gestation day 0.5 (Croy *et al*., 2015).

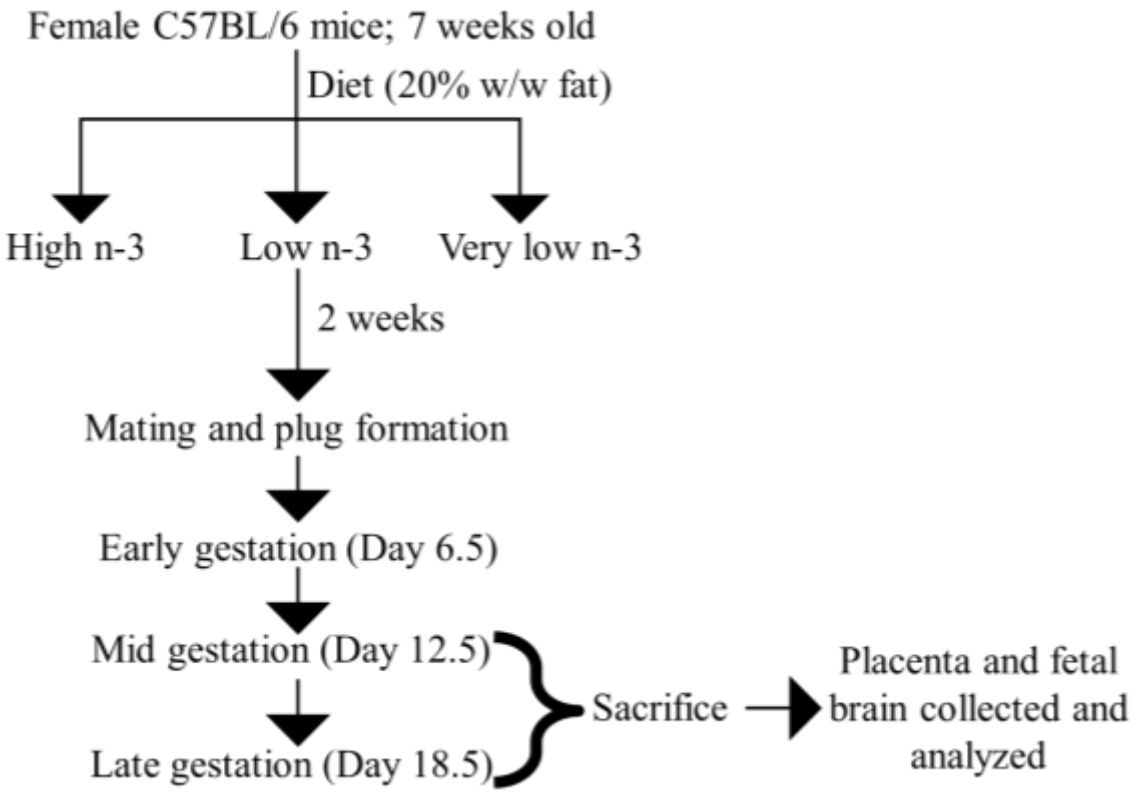

Figure 5.1: Experimental design. Each group was fed one of the three experimental diets that differed in their n-3 PUFA amount, and designated as ‘‘High n-3’’, ‘‘Low n-3’’ and ‘‘Very-low n-3’’ PUFA diets, for two weeks before mating; n-3 PUFA: omega-3 polyunsaturated fatty acids.

Female mice confirmed for pregnancy were continued on the assigned diets throughout gestation. All animals were fed with fresh food and water *ad-libitum* every other day. Food intake and body weight were recorded every day; there was no significant difference across all the dietary groups when adjusted for fetal weight at each gestation stage. Mice were sacrificed at gestation day 12.5 and 18.5 using 2.5% isoflurane. Blood was collected in tubes containing EDTA (4.5 mM, pH 7.4) via cardiac puncture and was immediately centrifuged to obtain plasma and red blood cells. Maternal and fetal tissues were collected and weighed at the time of sacrifice. All fetuses were carefully separated from the uterus. Placenta attached to each fetus was carefully separated and washed free of maternal blood in ice-cold phosphate-buffered saline. Each fetus was dissected, and the brain was collected. Collected tissue samples were snap-frozen in liquid nitrogen and stored at −80 °C until further analyses.

5.3.4 Fatty acids analyses

Total lipids were extracted from the maternal placenta and fetal brains (Folch, Lees & Sloane, 1957), and trans-methylated as per our previous publications (Akerele & Cheema, 2018; Chechi *et al*., 2010). The fatty acids composition was then determined using gas chromatography according to our previous publications (Akerele & Cheema, 2018; Chechi *et al*., 2010); heptadecanoic acid (C17:0) was used as an internal standard. Placenta and fetal brain samples were analysed individually, and results were averaged per litter for each dam; data were expressed as nmol%. The amount of DHA per mg brain tissue was calculated for each fetus, and results were averaged for each dam.

5.3.5 RNA extraction and real-time qPCR

Trizol method was used to extract total RNA from the placenta and fetal brain samples (Chomczynski & Sacchi, 1987). DNase enzyme (#M610A, Promega, USA) treatment was used to eliminate genomic DNA contamination in the RNA samples. The concentration of the extracted RNA samples was then determined using NanoDrop 2000 (Thermo Scientific, USA). Agarose gel (1.2%) was used to confirm the integrity of each RNA sample. Complementary DNA (cDNA) was synthesized from the extracted RNA samples using reverse-transcription method as per our previous publications (Akerele & Cheema, 2018; Balogun & Cheema, 2014). Real-time qPCR primers were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) (accessed on 02/05/2019) and obtained from integrated DNA technologies (IDT) (IA, USA). The forward and reverse sequences for each primer pair are given in Table 5.3. SYBR Green Supermix (#1708880, Bio-rad, USA) was used to initiate amplification, and samples were run using the CFX96TM Real-Time System. Data output was managed using the CFX Manager^{TM} Software 3.0. The cycle threshold (Ct values) of each reaction was determined. The delta Ct values were calculated for each of the genes of interest, corrected for amplification efficiency, and normalized with the reference gene (β-actin; ActB). The expression levels of each gene between groups were compared using the Livak method (Livak & Schmittgen, 2001).

Table 5.3: Sequences of primers used for qPCR

Gene (s)		Primers (5' - 3')	Efficiency (%)
Brain-derived neurotrophic factor (BDNF)	Forward	tacttcggttgcatgaaggcg	97.6
	Reverse	gtcagacctctcgaacctgcc	
Tropomyosin receptor kinase B (TrKB)	Forward	cggcacataaatttcacacg	98.4
	Reverse	ttacccgtcaggatcaggtc	
CAMP response element binding protein (CREB)	Forward	acaatggtacggatggggta	100.5
	Reverse	ctgctgtccatcagtggtc	
Plasma membrane fatty acid-binding protein (FABPpm)	Forward	agcggctgaccaaggagtt	97.1
	Reverse	gacccctgccacggagat	
Endothelial lipase (EL)	Forward	acgcacattctttgcatctg	91.9
	Reverse	acccaaggtggaagtcacag	
Major facilitator superfamily domain-containing protein 2a (Mfsd2a)	Forward	aaagacacgcaaaatgcttacct	90.4
	Reverse	aatgaaggcacagaggacgtaga	
Beta-Actin (ActB)	Forward	cacgcagctcattgtagaagg	107.5
	Reverse	atggtgggaatgggtcagaag	

All primers were designed using NCBI primer blast and obtained from IDT technologies.

5.3.6 Measurement of CREB protein concentration

Fetal brain samples were homogenized in an extraction buffer as per our previous publication (Balogun & Cheema, 2014). Bicinchoninic acids (BCA) protein assay method was used to determine the total protein concentration of the lysate, using bovine serum albumin (BSA) as standards. Phosphorylated-CREB (pCREB) and total CREB protein concentrations were determined using ELISA kits (# KHO0241 and # KHO0231, Invitrogen, USA) according to the manufacturer's instructions. PowerWave XS microplate reader (Biotek, USA) was used to measure the intensity of the coloured product at 450 nm. The total amount of pCREB derived from the phosphorylation of 80 pg of CREB by protein kinase A is equivalent to one unit of the standard. Values of pCREB were normalized for total CREB, and the results are presented as pCREB/total CREB.

5.3.7 Statistical analyses

Group means were compared using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Results are expressed as mean ± SD (standard deviation); n=8 dams in each experimental group. Pearson's correlation was used to compare the relationship between gene or protein expression levels and fetal brain DHA composition. Placenta and brain samples for all fetuses in each litter were individually analysed, and results were averaged per litter for each dam. Differences were statistically significant if $p<0.05$. All data were analysed using GraphPad Prism 8.0.

5.4 RESULTS

5.4.1 High n-3 PUFA diet increased the incorporation of longer chain n-3 PUFA into the placenta during gestation

Diet had no significant effect on total saturated fatty acids (SFA). However, gestation time had a significant independent effect on myristic acid (C14:0), stearic acid (C18:0), and total SFA (Table 5.4). Myristic acid decreased significantly ($p<0.05$), while stearic acid increased from day 12.5 to 18.5 in all diet groups ($p<0.0001$). Interestingly, gestation time had no effect on palmitic acid in all diet groups. Furthermore, gestation time had no effect on total SFA in the high n-3 PUFA group, compared to the low and very-low n-3 PUFA groups; total SFA increased at day 18.5 in the low and very-low n-3 PUFA groups ($p=0.002$). Diet and gestation time had no significant effect on palmitoleic acid (C16:1n7), oleic acid (C18:1), eicosenoic acid (C20:1n9), and total MUFA. However, there was a significant interaction between diet and gestation stage on palmitoleic acid and total MUFA ($p<0.05$), revealing a gestation dependent decrease in palmitoleic acid and total MUFA in the low n-3 PUFA group only.

Diet and gestation stage had no significant effect on linoleic acid (C 18:2n6; LA); however, the diet had a significant effect on arachidonic acid (C20:4n6; AA), adrenic acid and total n-6 PUFA. The high n-3 PUFA diet had lower levels of AA ($p<0.0001$), adrenic acid ($p<0.0001$) and, total n-6 PUFA ($p<0.0001$), compared to the low and very low n-3 PUFA groups, respectively. Total n-6 PUFA and adrenic acid decreased from day 12.5 to 18.5 in all diet groups ($p<0.0001$).

Table 5.4: Fatty acid composition of maternal placenta

Fatty Acids (nmol%)	**High n-3**		**Low n-3**		**Very low n-3**		**Main Effects**		
	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Diet	Gestation	Diet* Gestation
C14:0	0.55±0.06	0.42±0.02	0.52±0.09	0.31±0.03	0.44±0.03	0.36±0.07	NS	p=0.049	NS
C16:0	19.28±0.43	19.08±0.59	18.70±.040	18.49±0.31	18.09±0.21	18.91±.018	NS	NS	NS
C18:0	23.93±0.45	25.25±0.59	22.54±2.08	24.82±0.52	24.05±0.31	26.25±0.83	NS	p<0.0001	p=0.01
Σ SFA	43.76±0.61	44.74±0.46	41.75±1.92	43.62±0.77	42.58±0.35	45.51±0.76	NS	p<0.0001	p=0.0024
C16:1n7	0.79±0.25	0.67±0.17	0.84±0.34	0.54±0.05	0.63±0.04	0.50±0.07	NS	NS	p=0.017
C18:1	10.18±0.68	9.35±0.23	12.31±2.26	10.15±0.29	10.81±0.27	10.38±0.66	NS	NS	NS
C20:1n9	0.24±0.05	0.22±0.08	0.32±0.06	0.25±0.06	0.32±0.06	0.39±0.13	NS	NS	NS
Σ MUFA	11.22±0.88	10.16±0.36	13.71±2.91	10.84±0.40	11.77±0.28	11.04±0.86	NS	NS	p=0.041
C18:2n6	13.90±0.75	12.87±0.39	15.46±1.54	14.04±0.40	14.40±1.02	13.43±0.36	NS	NS	p=0.0013
C20:4n6	12.00 ± 0.96^{c}	11.71 ± 0.78^{c}	15.50 ± 1.17^{b}	15.44 ± 0.30^{b}	19.08 ± 0.90^{a}	18.77 ± 0.62^{a}	p<0.0001	NS	NS
C22:4n6	1.97 ± 0.24^{c}	1.19 ± 0.17^{d}	3.38 ± 0.30^{b}	2.32 ± 0.12^{c}	5.12 ± 0.31^{a}	3.45 ± 0.04^{b}	p<0.0001	p<0.0001	p=0.0004
Σ n-6 PUFA	27.86 ± 0.58^{d}	25.77 ± 0.76^{e}	34.33 ± 0.49^{b}	31.80 ± 0.18^{c}	38.59 ± 0.60^{a}	35.65 ± 0.42^{b}	p<0.0001	p<0.0001	p<0.05
C20:5n3	2.19 ± 0.44^{a}	2.28 ± 0.14^{a}	0.50 ± 0.13^{b}	0.49 ± 0.03^{b}	0.56 ± 0.14^{b}	0.56 ± 0.02^{b}	p<0.0001	NS	NS
C22:5n3	3.34 ± 0.11^{a}	2.44 ± 0.08^{b}	0.99 ± 0.19^{c}	1.02 ± 0.03^{c}	0.51 ± 0.04^{d}	0.49 ± 0.04^{d}	p<0.05	NS	NS
C22:6n3	11.63 ± 0.49^{b}	14.62 ± 0.46^{a}	8.66 ± 0.95^{c}	11.85 ± 0.36^{b}	6.00 ± 0.41^{d}	6.58 ± 0.19^{d}	p<0.0001	p<0.0001	p<0.0001
Σ n-3 PUFA	17.16 ± 0.96^{b}	19.33 ± 0.56^{a}	10.21 ± 1.11^{d}	13.74 ± 0.37^{c}	7.06 ± 0.42^{e}	7.80 ± 0.25^{e}	p<0.0001	p<0.0001	p<0.0001

Data are expressed as nmol percentage of total fatty acids. Values are expressed as mean ± SD, n=8. Main effects and interactions were determined by two-way ANOVA. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean

values within a row with unlike superscript letters (a, b, c, d, and e) show significant difference during gestation for each group ($p<0.05$). Σ SFA: sum of saturated fatty acids; Σ MUFA, sum of monounsaturated fatty acids; Σ n-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σ n-6 PUFA, sum of omega-6 polyunsaturated fatty acids; NS, not significant.

Diet also had a significant effect on n-3 PUFAs; the amount of eicosapentaenoic acid (C20:5n3; EPA), docosapentaenoic acid (C22:5n3; DPA), DHA (C22:6n3) and total n-3 PUFA was significantly higher in the high n-3 PUFA group, followed by the low n-3 and very low n-3 PUFA group, respectively ($p<0.0001$). DHA and total n-3 PUFA increased from day 12.5 to 18.5 in the high n-3 PUFA ($p<0.0001$) and low n-3 PUFA groups ($p<0.0001$), while there was no change in the very low n-3 PUFA group. The effect of diet on DHA and total n-3 PUFA in the high and low n-3 PUFA groups was dependent on the gestation time ($p<0.0001$).

5.4.2 High n-3 PUFA diet increased the mRNA expression of endothelial lipase and plasma membrane fatty acid-binding protein in the placenta during gestation

Diet had a significant effect on the mRNA expression of EL in the placenta; high n-3 PUFA group had higher mRNA expression at both days 12.5 and 18.5 ($p<0.0001$; Figure 5.2A), followed by the low and very low n-3 PUFA group, respectively. The gestation stage had no effect on the mRNA expression of EL in all the diet groups. Similarly, the diet had an independent significant effect on the mRNA expression of plasma membrane fatty acid-binding protein (FABPpm) in the placenta, revealing a higher expression in the high n-3 PUFA group ($p=0.03$; Figure 5.2B) during pregnancy, compared to other groups. Gestation stage also had an independent effect on the mRNA expression of FABPpm ($p<0.001$), and there was no interaction between diet and gestation stage. There was a significant positive correlation between FABPpm mRNA expression and the fetal brain DHA in the high n-3 PUFA group ($p=0.002$; Figure 5.2E), while the correlation was not significant in the low and very low n-3 PUFA groups ($p>0.05$). Both diet and gestation stage had no effect on the mRNA expression of FAT/CD36 (Appendix VII).

5.4.3 High n-3 PUFA diet increased the mRNA expression of major facilitator superfamily domain-containing protein 2 in fetal brain during gestation

Diet had an independent significant effect on the mRNA expression of Mfsd2a in fetal brain ($p<0.0001$; Figure 5.2C); the high n-3 PUFA group had higher expression at both gestation days 12.5 and 18.5, compared to the low and very low n-3 PUFA groups. There was no effect of gestation stage on the mRNA expression of Mfsd2a. However, there was a significant interaction between diet and gestation stage ($p<0.05$), revealing a gestation-dependent increase in the low n-3 PUFA group only. There was a significant effect of diet on fetal brain DHA composition ($p<0.0001$; Figure 5.2D). The high n-3 PUFA group had higher DHA levels at both day 12.5 and 18.5, compared to the low and very low n-3 PUFA groups. Gestation stage also had a significant effect on fetal brain DHA levels ($p<0.001$), showing an increase from day 12.5 to 18.5 in all diet groups. There was also a significant positive correlation between Mfsd2a mRNA expressions and fetal brain DHA in the high n-3 PUFA and low n-3 PUFA groups ($p=0.004$ and 0.030 respectively; Figure 5.2F), while the correlation was not significant in the very low n-3 PUFA group ($p>0.05$).

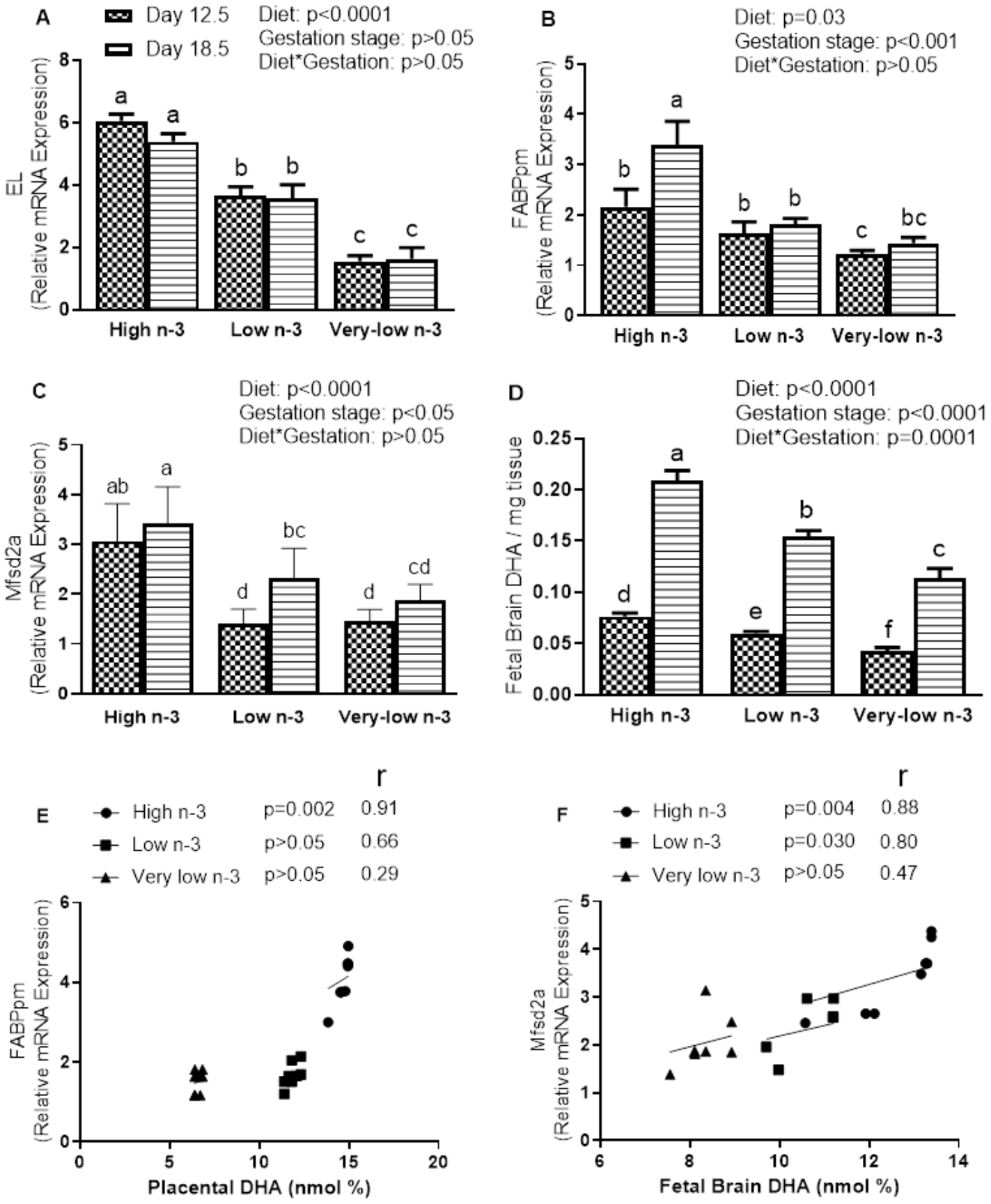

Figure 5.2: Effects of maternal diets varying in the amount of n-3 PUFA on the placental mRNA expression of endothelial lipase (EL; A), plasma membrane fatty acid-binding protein

(FABPpm; B), fetal brain mRNA expression of major facilitator superfamily domain-containing protein 2 (Mfsd2a; C) and fetal brain DHA levels (D) was measured at gestation day 12.5 and 18.5 as explained in the materials and methods section. The mRNA expressions were normalized to β-actin (ActB) as the reference gene. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups. $p<0.05$ was considered significant. Data are presented as mean (n=8 at each gestation stage) ± SD; DHA: docosahexaenoic acid; n-3 PUFA: omega-3 polyunsaturated fatty acids.

5.4.4 High n-3 PUFA diet increased the accretion of longer chain n-3 PUFA into fetal brain during gestation

Diet and gestation stage had no significant effect on myristic acid, palmitic acid, stearic acid, and total SFA (Table 5.5). However, there was an independent effect of gestation stage on stearic acid and total SFA ($p<0.0001$), revealing an increase from day 12.5 to 18.5 in the low and very low n-3 PUFA groups, respectively. There was no effect of diet on palmitoleic acid, oleic acid and total MUFA, while gestation stage had a significant effect to reveal a significant decrease in total MUFA from day 12.5 to 18.5 in all the diet groups. Diet had no effect on LA and AA, while LA decreased as gestation progressed in all diet groups ($p<0.05$). Diet had a gestation dependent effect on adrenic acid and total n-6 PUFA ($p<0.05$), revealing lower levels at day 18.5 in the high n-3 PUFA group, compared to the low and very low n-3 PUFA groups.

There was a significant effect of diet on n-3 PUFAs, revealing that the high n-3 PUFA diet had higher amount of EPA, DPA, DHA, and total n-3 PUFA in fetal brain at both gestation days, compared to the low and very low n-3 PUFA groups ($p<0.05$). Gestation time also had a significant effect on PUFAs; DHA and total n-3 PUFA increased from day 12.5 to day 18.5 in all diet groups ($p<0.0001$). EPA was not detected in the low and very low n-3 PUFA groups at all stages of pregnancy, while DPA was not detected at both day 12.5 and 18.5 in the very low n-3 PUFA group only.

Table 5.5: Fetal brain fatty acid composition

Fatty Acids (%)	High n-3		Low n-3		Very low n-3		Main Effects		
	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Day 12.5	Day 18.5	Diet	Gestation	Diet* Gestation
C14:0	2.02±0.11	1.98±0.13	1.90±0.10	1.96±0.09	1.99±0.21	2.05±0.07	NS	NS	NS
C16:0	29.66±0.88	29.20±0.95	29.85±.1.18	30.16±0.24	29.57±1.55	30.71±0.19	NS	NS	NS
C18:0	14.69±0.70	17.11±0.42	15.35±0.80	17.81±0.17	15.09±1.51	18.01±0.33	NS	p<0.0001	NS
Σ SFA	46.38±1.55	48.29±1.31	47.09±0.60	49.93±0.34	46.66±3.07	50.771±0.34	NS	P<0.0001	NS
C16:1n7	3.93±0.10	2.64±0.09	3.60±0.08	2.56±0.10	3.84±0.17	2.74±0.05	NS	p=0.0001	p<0.05
C18:1	23.21±1.12	18.64±0.86	23.11±0.22	18.10±0.36	23.39±1.37	18.63±0.41	NS	p<0.0001	NS
Σ MUFA	27.14±1.15^{a}	21.28±0.77^{b}	26.71±0.26^{a}	20.66±0.38^{b}	27.33±1.47^{a}	21.37±0.39^{b}	NS	p<0.0001	NS
C18:2n6	3.79±0.35^{a}	0.87±0.12^{b}	3.99±0.17^{a}	1.01±0.13^{b}	3.54±0.10^{a}	1.02±0.12^{b}	NS	p<0.05	NS
C20:4n6	11.04±0.17	10.79±0.97	13.36±0.49	12.24±0.32	13.38±1.72	13.69±0.32	NS	NS	p<0.05
C22:4n6	2.12±0.06	2.06±0.16	3.39±0.15	2.97±0.11	3.54±0.50	3.44±0.21	p<0.05	NS	p<0.05
Σ n-6 PUFA	16.95±0.94^{c}	13.72±1.55^{d}	20.74±0.57^{a}	16.22±0.41^{c}	21.43±0.48^{a}	18.15±0.50^{b}	p<0.05	p<0.05	p<0.05
C20:5n3	0.26±0.07	0.21±0.03	ND	ND	ND	ND	p<0.05	NS	NS
C22:5n3	3.63±0.82	0.86±0.05	0.01±0.00	0.48±0.17	ND	ND	p<0.05	NS	NS
C22:6n3	6.71±0.37^{d}	14.10±0.94^{a}	5.31±0.12^{d}	12.66±0.35^{b}	3.68±0.49^{e}	9.63±0.47^{c}	p<0.0001	p<0.0001	p=0.007
Σ n-3 PUFA	10.60±0.87^{c}	15.17±2.09^{a}	5.32±0.24^{d}	13.14±0.28^{b}	3.68±0.49^{e}	9.63±0.47^{c}	p<0.0001	p<0.0001	p<0.05

Data are expressed as nmol percentage of total fatty acids. Values are expressed as mean ± SD, n=8. Main effects and interactions were determined by two-way ANOVA. Pairwise comparison using Bonferroni correction was used to determine differences among the groups. Mean values within a row with unlike superscript letters (a, b, c, d and e) show significant difference during gestation for each group (p<0.05). Σ SFA:

sum of saturated fatty acids; Σ MUFA, sum of monounsaturated fatty acids; Σ n-3 PUFA, sum of omega-3 polyunsaturated fatty acids; Σ n-6 PUFA, sum of omega-6 polyunsaturated fatty acids; ND, not determined; NS, not significant.

5.4.5 High n-3 PUFA diet increased the mRNA expressions of neurotrophins during gestation

There was a significant effect of diet ($p<0.0001$) and gestation stage ($p<0.0001$) on the mRNA expression of BDNF. The mRNA expression of BDNF increased significantly from day 12.5 to 18.5 in all diet groups ($p<0.001$; Figure 5.3A). However, a diet high in n-3 PUFA had significantly higher mRNA expression of BDNF at all gestation stages, compared to the low and very low n-3 PUFA diet. Furthermore, there was a significant positive correlation between BDNF mRNA expression and fetal brain DHA composition in the high n-3 and low n-3 PUFA group; however, no significant correlation was observed in the very low n-3 PUFA group (Figure 5.3B).

Diet and gestation stage had a significant effect on the mRNA expression of TrKB ($p<0.0001$; Figure 5.3C). More so, a significant interaction was observed between diet and gestation stage on the mRNA expression of TrKB. The mRNA expression of TrKB was not different across all groups at day 12.5; however, there was a significant increase as gestation progressed from day 12.5 to 18.5 in all diet groups. Diet high in n-3 PUFA had higher mRNA expression of TrKB at day 18.5, compared to the low and very low n-3 PUFA diets. Furthermore, there was a positive correlation between TrKB mRNA expression and fetal brain DHA composition, which was only significant in the high n-3 diet group ($r=0.77$; $p=0.03$; Figure 5.3D).

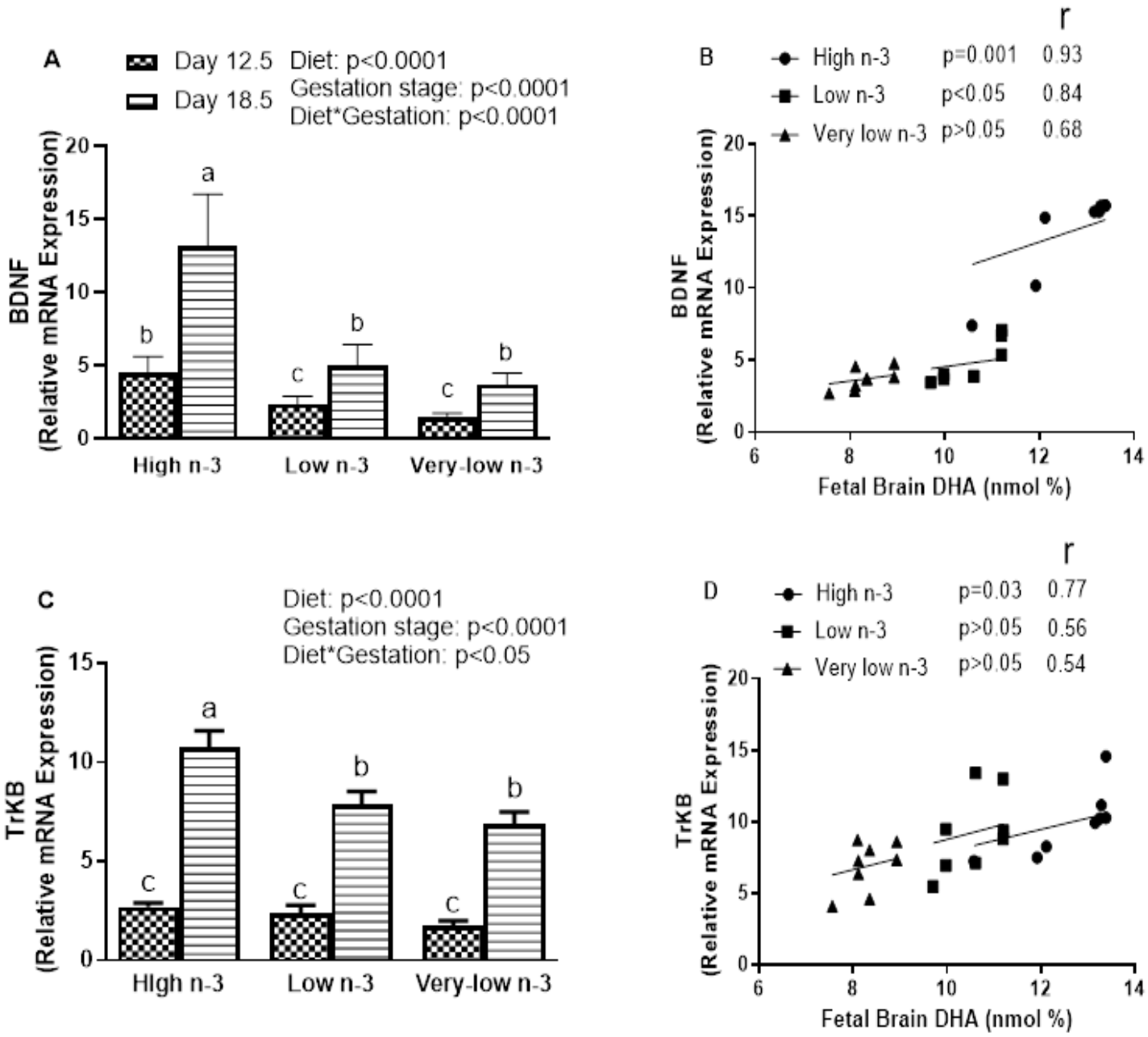

Figure 5.3: Effects of maternal diets varying in the amount n-3 PUFA on the mRNA expression of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrKB) in fetal brain at different gestation days: The data represent fetal brain mRNA expressions of BDNF (A) and TrKB (B) normalized to β-actin (ActB) as the reference gene. Pearson's correlation analyses were performed between fetal brain DHA and the mRNA expressions of BDNF (C) and TrKB (D) at gestation day 12.5 and 18.5. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using

Bonferroni correction was used to determine differences among the groups. $p<0.05$ was considered significant. Data are presented as mean (n=8 at each gestation stage) ± SD; n-3 PUFA: omega-3 polyunsaturated fatty acids.

There was a significant effect of diet and gestation stage on the mRNA expression of CREB ($p=0.003$; Figure 5.4A). There was no difference in the mRNA expression of CREB at day 12.5 across all diet groups. However, the mRNA expression of CREB increased significantly during gestation from day 12.5 to 18.5 in the high n-3 PUFA group only, while there was no change in the mRNA expression of CREB as gestation progressed from day 12.5 to 18.5 in the low n-3 and very low n-3 groups. There was a significant positive correlation between CREB mRNA expression and fetal brain DHA in the high n-3 diet group only ($r=0.91$; $p=0.002$; Figure 5.4B), compared to the other groups. CREB has been shown to be activated by phosphorylation; thus, the protein expressions of total CREB and CREB phosphorylated at Ser-133 (pCREB) were measured. There was a significant effect of diet on the relative expression of pCREB to total CREB (pCREB/total CREB) ($p<0.0001$; Figure 5.4C). Phosphorylated CREB/total CREB increased significantly as gestation progressed in all groups ($p<0.001$); however, phosphorylated CREB/total CREB was significantly higher in the high n-3 PUFA group at both day 12.5 and 18.5, compared to the low n-3 and very low n-3 PUFA groups. There was also a significant positive correlation between pCREB/total CREB and fetal brain DHA in the high n-3 PUFA and low n-3 PUFA groups ($p=0.026$ and 0.047 respectively; Figure 5.4D), while the correlation was not significant in the very low n-3 PUFA group ($p>0.05$).

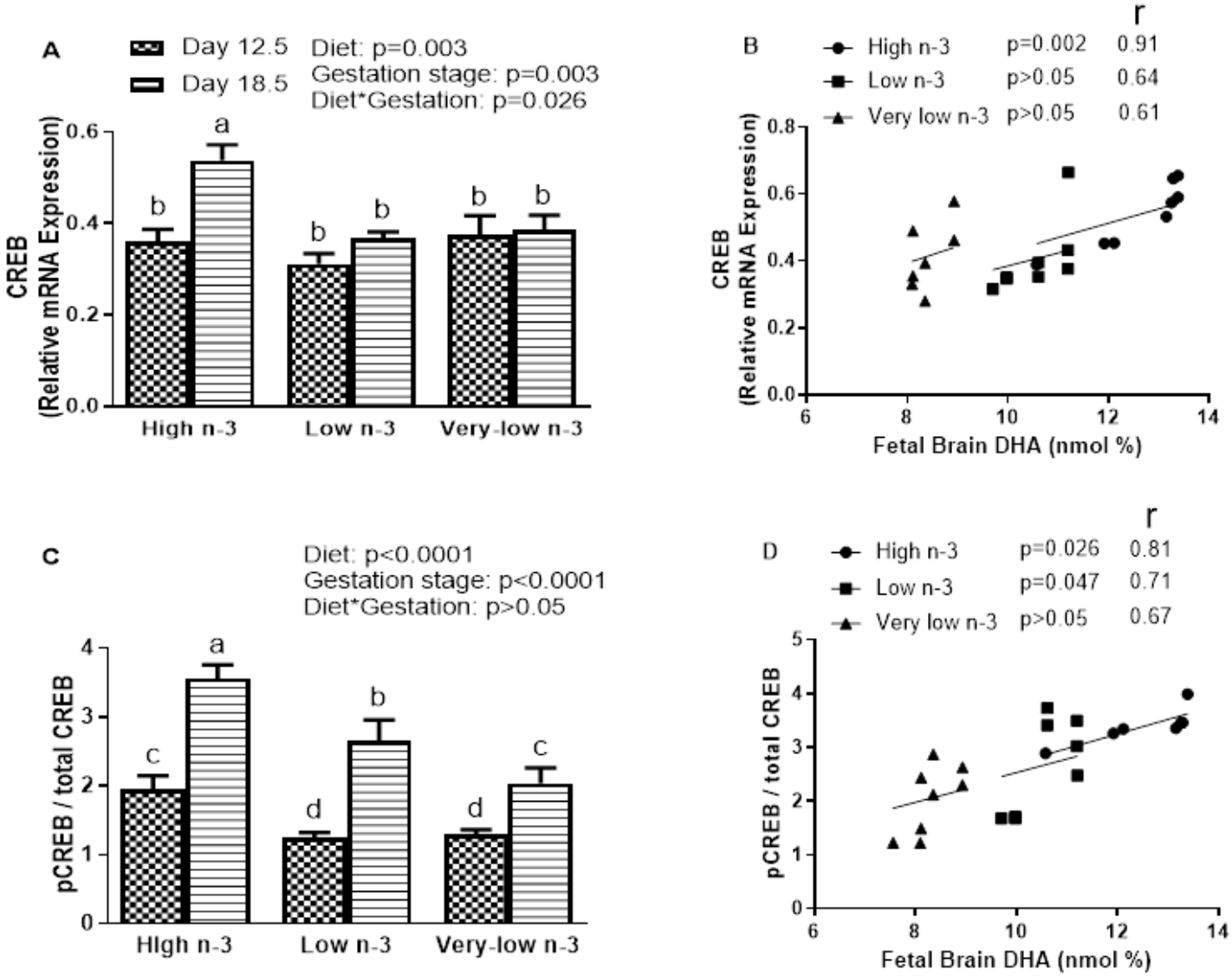

Figure 5.4: Effects of maternal diets varying in the amount n-3 PUFA on the expression of cAMP response element-binding protein (CREB) in fetal brain at different gestation days: The data represent the mRNA expression of CREB normalized to β-actin (ActB) as the reference gene at gestation day 12.5 and 18.5 (A), phosphorylated CREB (pCREB) protein concentration normalized for total CREB (pCREB/total CREB) at day 12.5 and 18.5 (C), and Pearson's correlation analyses between the fetal brain DHA composition and CREB mRNA expression (B) and pCREB/total CREB (D) during gestation. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; Pairwise comparison using Bonferroni correction was used to determine differences among the groups.

$p<0.05$ was considered significant. Data are presented as mean (n=8 at each gestation stage) ± SD; n-3 PUFA: omega-3 polyunsaturated fatty acids.

5.5 DISCUSSION

Fetal brain develops rapidly during the last trimester of pregnancy and the early postnatal period in humans (Wainwright, 2002), and this correlates with increased accretion of DHA in the brain. (Dyall, 2015; McNamara & Carlson, 2006). Deficiency in n-3 PUFA during pregnancy has been implicated in altered brain lipid composition in fetuses and induces spatial memory deficits in adult mice (Labrousse *et al*., 2018). N-3 PUFA has been shown to have positive effects on the brain function by regulating the expression of neurotrophins, such as BDNF and its target receptor (Balogun & Cheema, 2014; Bhatia *et al*., 2011; Sable *et al*., 2012).

The placenta uptakes the maternal circulating non-esterified fatty acids (NEFAs) released by maternal LPL and EL (Gil-Sánchez *et al*., 2012). We found a significantly higher mRNA expression of EL in the placenta of mice fed a high n-3 PUFA diet at both gestation days, compared to the low and very low n-3 PUFA groups. EL hydrolyses both phospholipids and TGs (McCoy *et al*., 2002); studies have shown that increased EL expression contributes greatly to placental fatty acid uptake (Lindegaard *et al*., 2005). The NEFAs released by the EL are then transported across the placenta through passive diffusion and majorly by membrane carrier proteins (Gil-Sánchez *et al*., 2012). These membrane carrier proteins include FAT/CD36, FATP and FABPpm. We found that a diet high in n-3 PUFA significantly increased the mRNA expression of FABPpm in the placenta at both gestation days. The FABPpm is the main transporter for longer chain PUFA, such as DHA, across the placenta interface into fetal circulation (Campbell *et al*., 1998). We found a significant correlation between the mRNA expression of placental FABPpm and accretion of DHA into the placenta in mice fed the high n-3 PUFA diet (Figure 5.2E). We did not find a significant effect of diet or gestation stage on the mRNA expression of FAT/CD36; these findings are similar to our previously published observations (Akerele and Cheema, 2018). We further found that a diet high in n-3 PUFA

increased the mRNA expression of Mfsd2a, which correlated with an increased accretion of DHA in the high n-3 PUFA group, compared to the low n-3 and very low n-3 PUFA groups.

Mfsd2a has been identified as the major transporter for DHA uptake into the brain (Nguyen *et al*., 2014). Mfsd2a-knockout mice showed a drastic reduction in the levels of DHA in the brain, with a concomitant loss of neuronal cells, as well as cognitive deficits (Nguyen *et al*., 2014). EPA was only detected in the high n-3 PUFA diet group, while it was not detectable in the low n-3 and very low n-3 PUFA group at both day 12.5 and 18.5. However, the levels of EPA were relatively very low in the brain, compared to DHA. Increased EPA metabolism via beta-oxidation or rapid conversion to DHA is possible explanations for lower EPA levels in the fetal brain (Chen *et al*., 2013). The high n-3 PUFA diet showed lower amounts of total n-6 PUFA, compared to the low and very low n-3 PUFA groups. We have previously reported similar findings in mice offspring fed a high n-3 PUFA diet at weaning and at 16-weeks post-weaning (Balogun & Cheema, 2014; Feltham *et al*., 2019). Linoleic acid (LA), an n-6 PUFA, decreased as gestation progressed in all diet groups; however, the levels were not different across all dietary groups at day 18.5. A similar observation was reported in neonatal rats that were fed diets varying in n-3 PUFA, where no difference was observed in brain LA composition (Suganuma *et al*., 2010). Interestingly, there was no significant change in AA levels in all diet groups.

Desaturation and elongation of LA to AA has been shown to greatly reduce in mouse brain (Bourre *et al*., 1990; Cook, 1991). It has been shown that brain elongation of LA is a negligible source of the AA in the brain of rats (DeMar *et al*., 2006). More so, it has been suggested that LA that enters the brain is largely beta-oxidized and is not a major source of AA in the brain (DeMar *et al*., 2006). However, the effect of n-3 PUFA on the oxidation of LA in fetal brain is not known and requires further investigation. Furthermore, reduced incorporation of AA into the brain has also been suggested to be due to an increase in the expression of

phospholipase-A2 (Bosetti & Weerasinghe, 2003). Our findings show a gestation-dependent decrease in LA and no changes in AA, suggesting a gestation-dependent increase in beta-oxidation of LA, or reduced elongation and desaturation of LA to AA. Reduction in brain n-6 PUFA has also be suggested to be a compensatory mechanism for increased incorporation of DHA into the brain (Wainwright *et al*., 1991).

Although the brain *de novo* synthesizes SFA and MUFA (Edmond *et al*., 1998; Marbois *et al*., 1992), diets varying in the amount of n-3 PUFA were found to influence brain SFA and MUFA. There was a significant interaction between diet and gestation stage, revealing an increase in SFA, while MUFA decreased from gestation day 12.5 to 18.5. A human study that examined gestational age-dependent changes in fetal brain fatty acids also reported a significant gestational-dependent decease in MUFA (Kuipers *et al*., 2012). We have recently shown that MUFA increases significantly from weaning to 16 weeks post-weaning in mice offspring fed similar diets (Feltham *et al*., 2019). MUFA has been shown to play a key role in myelin sheath formation during development (Velasco *et al*., 2003). Evidence from the literature suggests that myelination in fetal brain begins around mid-gestation and continues progressively up to 60 days post-natal periods in mice (Baumann & Pham-Dinh, 2001; Luse, 1956). In humans, cortical myelination peaks during the first year of life, but continues into early adulthood (Fields, 2008). Our findings revealing a gestation-dependent decrease in MUFA suggests that the increase in brain MUFA perhaps only occurs after parturition.

Brain neurotrophins, especially BDNF, promote synaptic plasticity and survival of nerve cells by playing a major role in the growth, maturation (differentiation), and maintenance of neuronal cells (Huang & Reichardt, 2001). We have previously shown that a maternal diet high in n-3 PUFA increased the mRNA expression of BDNF in mice offspring at weaning and at 16 weeks post-weaning (Balogun & Cheema, 2014). Recent evidence has also shown that n-3 PUFA regulates BDNF in adult humans (Ferreira *et al*., 2014; Pawełczyk *et al*., 2019). However,

the effect of a maternal diet high in n-3 PUFA on BDNF mRNA expression in fetal brain during different stages of gestation is not known. We found that a diet high in n-3 PUFA increased the mRNA expression of BDNF, compared to the low and very low n-3 PUFA groups, at both day 12.5 and 18.5. Gestation stage also had a significant effect on the mRNA expression of BDNF, revealing a significant increase from day 12.5 to 18.5 across all the diet groups. We also found a positive correlation between BDNF mRNA expression and fetal brain DHA composition.

BDNF modulates neurotransmitters and participates in neuronal plasticity, which is essential for learning and memory by binding and activating its high-affinity receptor TrKB (Huang & Reichardt, 2001). The binding of BDNF to TrkB leads to the phosphorylation of TrKB, with concomitant activation of the downstream signal transduction pathway critical for BDNF activities in the brain (Numakawa *et al*., 2010). We found that the mRNA expression of TrKB increased significantly as gestation progressed from day 12.5 to 18.5 in all dietary groups. Although there was no difference in the mRNA expression of TrKB at mid-gestation (day 12.5), the expression at day 18.5 was higher in the high n-3 PUFA group, compared to the low and very low n-3 PUFA groups. Furthermore, the mRNA expression of TrKB at day 18.5 was consistent with the mRNA expression of BDNF, and there was a positive correlation between TrKB mRNA expression and fetal brain DHA levels. Our findings suggest a potential role of DHA in regulating BDNF and TrKB mRNA expression during gestation. We have previously shown that maternal diets high in n-3 PUFA increased the mRNA expression of offspring BDNF and TrKB in mice (Balogun & Cheema, 2014). A similar study has also shown that n-3 PUFA increased the protein expression of BDNF and TrKB in rats offspring (Bhatia *et al*., 2011).

The binding of BDNF to TrkB activates CREB via phosphorylation at serine 133 (S133), thereby stimulating intracellular signalling critical for neuronal survival and differentiation (Landeira *et al.*, 2016), neuronal plasticity and protection (Sakamoto *et al*., 2011), learning, memory formation and long-term potentiation (Kida, 2012). Similar to our observation with

BDNF and TrKB, a diet high in n-3 PUFA increased the mRNA expression of CREB in the fetal brain at both mid- and late gestation, which positively correlated with the levels of DHA in the fetal brain. CREB-binding sequences have been identified in the BDNF gene (Yossifoff *et al*., 2008); thus, most of CREB's functions in the central nervous system are mediated through positive feedback activation of BDNF as a major target gene.

CREB is post-translationally regulated (Wang *et al*., 2017); we found that a diet high in n-3 PUFA significantly increased the protein concentration of phosphorylated CREB, thereby increasing the pCREB/total CREB protein at both day 12.5 and 18.5, compared to the low and very low n-3 PUFA groups. We have previously reported the effects of n-3 PUFA on post-translational regulation of CREB in adult mice (Balogun & Cheema, 2014); however, this is the first study to report that n-3 PUFA regulates CREB at both transcription and post-translational level in the fetal brain during pregnancy. Moreover, we found that pCREB/total CREB protein correlated positively with the levels of DHA in the fetal brain, indicating a relationship between n-3 PUFA and CREB phosphorylation. The phosphorylation of CREB via TrKB activation could be a potential mechanism through which DHA regulates neurotrophin signalling. We are proposing a mechanism by which n-3 PUFA affects transport and accretion of DHA into the brain and the regulation of neurotrophins in the brain.

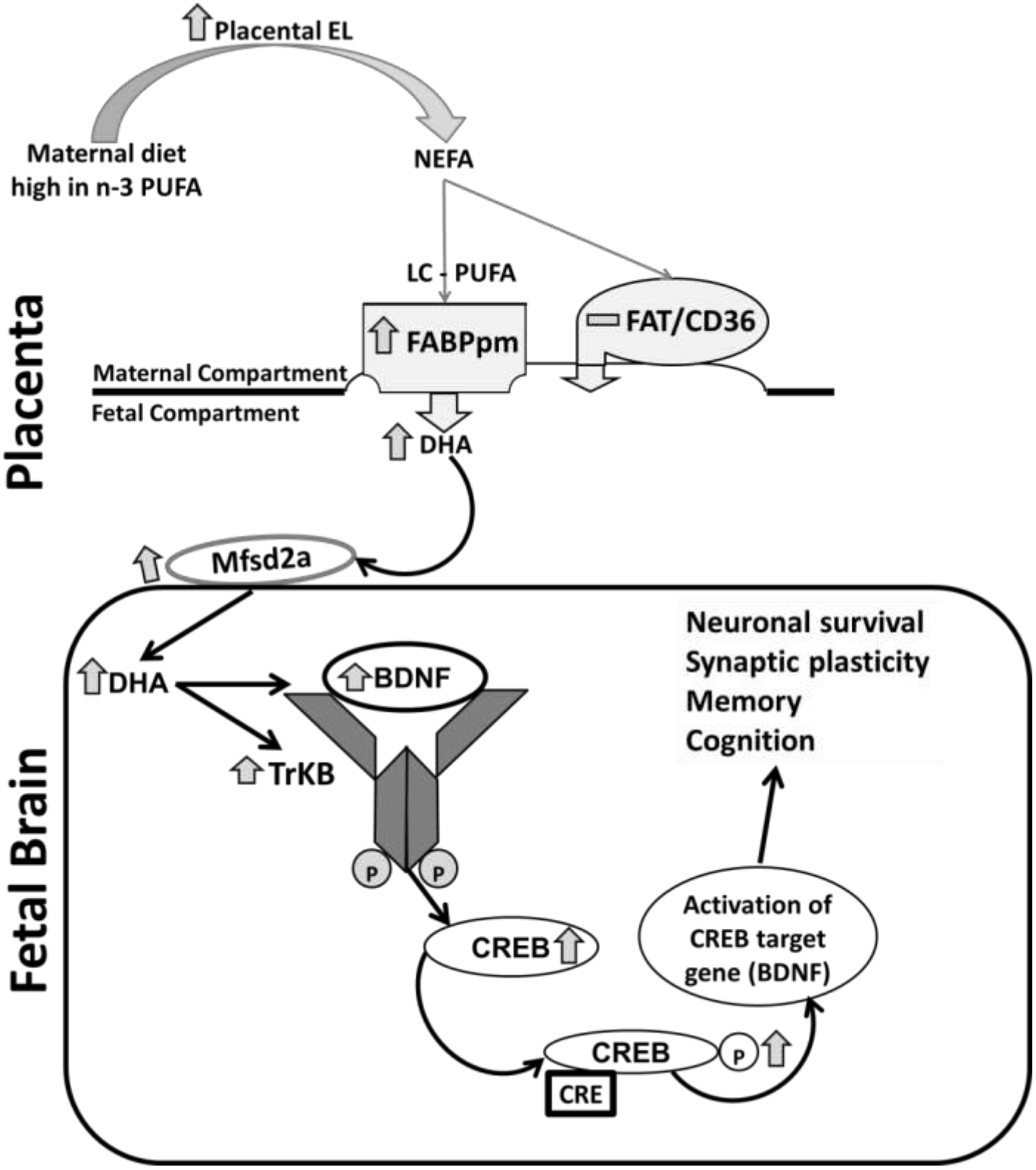

Figure 5.5: Proposed pathway on the effects of maternal diet high in n-3 PUFA on brain fatty acids and neurotrophins during fetal brain development. N-3 PUFA increases EL gene expression, likely releasing DHA, which is then selectively transported across the placental

interface to fetal circulation by FABPpm. N-3 PUFA also increase the mRNA expression of Mfsd2a, thereby increasing the accretion of DHA in fetal brain, which then upregulates the mRNA expression of BDNF and TrKB, with a concomitant increase in CREB phosphorylation. Phosphorylated CREB then activates BDNF as a target gene via positive feedback loop to regulate brain development, synaptic plasticity, memory and cognition. BDNF: Brain-derived neurotrophic factor; CREB: cAMP response element-binding protein; DHA: Docosahexaenoic acid; EL: Endothelial lipase; FABPpm: Plasma membrane fatty acid-binding protein; FAT/CD36: fatty acid translocase; LC-PUFA: long chain polyunsaturated fatty acids; Mfsd2a: Major facilitator superfamily domain-containing 2a; TrkB: Tropomyosin receptor kinase B; n-3 PUFA: omega-3 polyunsaturated fatty acids; up arrow represent an increase; flat line represent no change.

5.6 CONCLUSION

In conclusion, we report for the first time that a maternal diet high in n-3 PUFA increased the mRNA expression of placental EL and placental fatty acid transporter (FABPpm), with a concomitant increase in placental DHA. Subsequently, the high n-3 PUFA diet influenced the accretion of DHA into the fetal brain as gestation progressed by regulating the mRNA expression of mfsd2a during pregnancy. Our findings further revealed that n-3 PUFA increases the expression of BDNF and TrKB as gestation progresses, and that n-3 PUFA regulates CREB at both transcription and post-translational levels. Impaired BDNF expression has been widely implicated in neuropsychiatric disorders (Autry & Monteggia, 2012), thus maintaining the levels of BDNF and other neurotrophins during critical stages of brain development may be important in preventing neurological problems later in life. Therefore, an adequate dietary intake of n-3 PUFA during pregnancy is of critical importance.

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CHAPTER SIX

Summary and Conclusions

6.1 Summary and conclusions

Maternal nutritional status before and during pregnancy is a crucial factor in healthy prenatal development and fetal health outcomes (Laker *et al*., 2013). Maternal diet is also vital for a successful pregnancy establishment and progression (Grieger & Clifton, 2015; Marangoni *et al.*, 2016). The quantity, as well as the quality of dietary fats, consumed during pregnancy have profound health implication on both maternal and fetal health during and after pregnancy (Coletta *et al.*, 2010; Schwab *et al*., 2014). Our laboratory has previously shown that offspring born to C57BL/6 females fed a diet high in n-3 PUFA during gestation and lactation, and continuing on their maternal diet, had lower levels of lipids, had higher accretion of n-3 PUFA in offspring brain, along with an increase in the expression of neurotrophins. During these studies, it was observed that females fed a diet low in n-3 PUFA had fewer fetuses. The effects of n-3 PUFA on pregnancy/fetal outcomes are controversial, suggesting an increase (Rebollar *et al*., 2014; Smits *et al.*, 2011), decrease (Fountain *et al.*, 2008; Smit *et a*l., 2015) or no change (Estienne *et al*., 2006; Perez Rigau *et al*., 1995) in fetal numbers. This discrepancy is likely due to differences in the amount and/or source of n-3 PUFA used in these studies.

In my first study, I exposed C57BL/6 female mice were exposed to two different breeding chow diets with varied quantity (5% vs. 11% w/w), and the quality (providing n-3 PUFA from fish oil vs. soybean oil) of fat. Females were fed the specific diets for 2 weeks prior to mating, and throughout gestation. I examined the *in-utero* effects at different stages of gestation (day 6.5, 12.5 and 18.5). As expected, perinatal exposure of mice to breeding chow diet containing n-3 PUFA from fish oil caused higher accretion of n-3 PUFA into maternal uterus, placenta, and reduced inflammatory cytokines in the placenta and plasma (Chapter 3). Diet containing n-3 PUFA from fish oil also increased the levels of plasma estradiol and progesterone, two important hormones involved in pregnancy establishment and maintenance, as well as fetal development, compared to the diet containing n-3 PUFA from soybean oil. The low

fat diet containing n-3 PUFA from fish oil was found to increase maternal plasma TG as gestation progressed, which coincided with higher mRNA expressions of rate-limiting lipogenic genes. There was also an increase in cholesterol efflux, which likely occurred to meet fetal demand for cholesterol during pregnancy. The diet containing n-3 PUFA from fish oil had a lower fetal resorption rate and higher fetal numbers sustained at day 18.5, compared to the diet containing n-3 PUFA from soybean. These findings suggested that n-3 PUFA from fish oil likely created a favourable *in utero* environment, with a concomitant improvement in fetal sustainability.

A limitation to this study however was that the breeding chow diets varied in both the quantity (5% vs 11% w/w fat), and the quality (n-3 PUFA from fish oil at 8% vs soybean oil at 3% w/w, respectively) of fat. However, this study allowed us to investigate the effect of maternal diet before and during gestation on maternal lipid profile, pregnancy-related sex steroid hormones, placental fatty acids composition, and the mRNA expressions of key transporters, plasma and placental cytokines profile. My findings from this study clearly revealed that the quantity and the quality of dietary fat is important in regulating maternal metabolic profile during pregnancy, and impact pregnancy/fetal outcome (Chapters 2 and 3).

The previous study led me to study fish oil more in depth. As such, I designed a follow up study where I kept the amount of fat consistent to the levels consumed by a typical North American population (Simopoulos, 2016), and investigated the effects on maternal metabolic profile and pregnancy outcomes. This study was designed using semi-purified diets (20% fat w/w) and the amount of n-3 PUFA (from fish oil) was controlled to give a high (9%), low (3%) and very low (1%) n-3 PUFA respectively, for 2 weeks before mating and throughout pregnancy. The effects of maternal diets varying in the amount of n-3 PUFA were investigated on the regulation of maternal lipid and lipoprotein metabolism, transporters involved in fatty acids

transport to the fetus and fetal brain, placental and blood cytokines, and accretion of n-3 PUFA in fetal brain and the gene expression of neurotrophins (Chapters 4 and 5).

The main objective of the current thesis was to investigate the effects of the quality, and the quantity of fat, on maternal metabolic profile prior to pregnancy, and at different stages of pregnancy, with particular focus on the regulation of maternal lipid metabolism, plasma and placental cytokines profile, and fetal sustainability. The propensity of n-3 PUFA to potentially ameliorate adverse pregnancy outcomes by maintaining optimum maternal lipids profile prior to pregnancy and at different stages of pregnancy was explored in this thesis. The effect of high n-3 PUFA diet on the mRNA expression of placental endothelial lipase and fatty acid transporters, accretion of longer chain n-3 PUFA in fetal brain and how it regulates signalling of neurotrophin during different stages of pregnancy were also studied.

The findings from the current thesis revealed that maternal diet high in n-3 PUFA has a gestational-dependent effect on maternal lipids metabolism, and further showed a novel regulatory pathway through which n-3 PUFA could prevent placental inflammation and maternal dyslipidemia before and during pregnancy. Furthermore, findings from this thesis demonstrated the importance of maternal diet enriched in n-3 PUFA during pregnancy on the accretion of DHA in fetal brain, and the regulation of neurotrophin. Finally, the findings from this thesis revealed the potential mechanism through which high n-3 PUFA diet could improve fetal sustainability during pregnancy through a common pathway.

6.1.1 *Key observations*

My first study shows that a low fat breeding chow diet containing n-3 PUFA from fish oil alters maternal plasma, hepatic and placental lipid metabolism, likely to meet fetal demand at different stages of gestation. In addition, the low fat breeding chow diet maintained a balance of pro- and anti-inflammatory cytokines, and sex steroid hormone profiles to improve fetal sustainability during gestation (Figure 6.1). However, I was not able to pinpoint that the effects

of the diets are specifically due to n-3 PUFA from fish oil in the breeding chow diet. I thus designed my second study to specifically investigate the effects of n-3 PUFA, from fish oil, using a semi-purified diet containing varying amounts of n-3 PUFA. This study revealed that a maternal diet containing high n-3 PUFA prevented maternal dyslipidemia prior to pregnancy and maintained maternal plasma and hepatic metabolic profile, and progesterone levels during different stages of gestation. The high n-3 PUFA diet also increased anti-inflammatory cytokines and decreased pro-inflammatory cytokines. In addition, the high n-3 PUFA diet increased placental transport and incorporation of DHA in fetal brain, and upregulated neurotrophin signalling pathway in fetal. My second study confirmed that the amount of n-3 PUFA (from fish oil) has important implications in regulating maternal metabolism during gestation thereby affecting fetus sustainability (Figure 6.2).

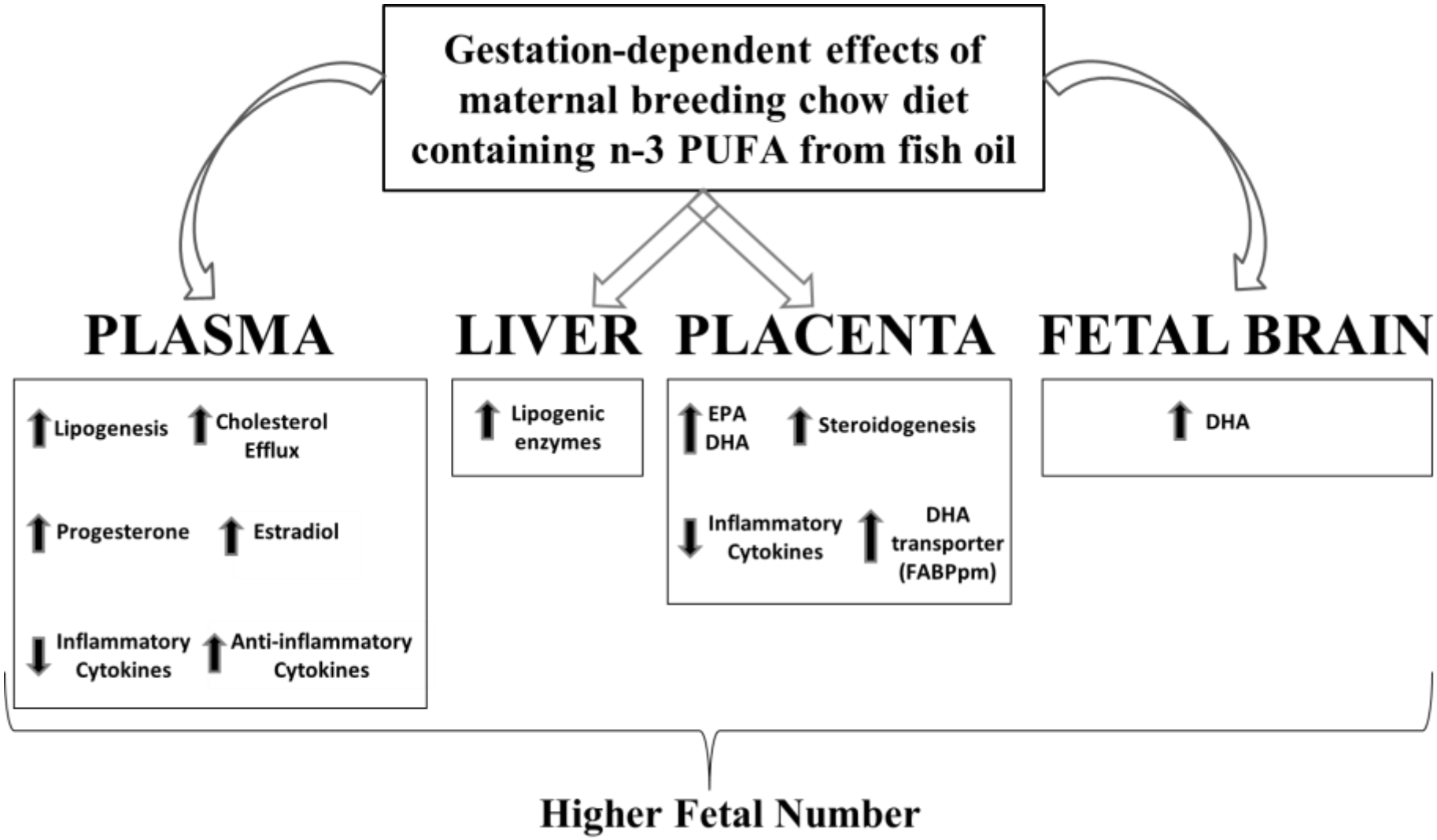

Figure 6.1: Breeding chow diet containing n-3 PUFA from fish oil altered maternal metabolic profile, cytokine levels, and sustained more fetuses during pregnancy. N-3: Omega-3 polyunsaturated fatty acid; DHA: Docosahexaenoic acid; EPA: Eicosapentaenoic acid.

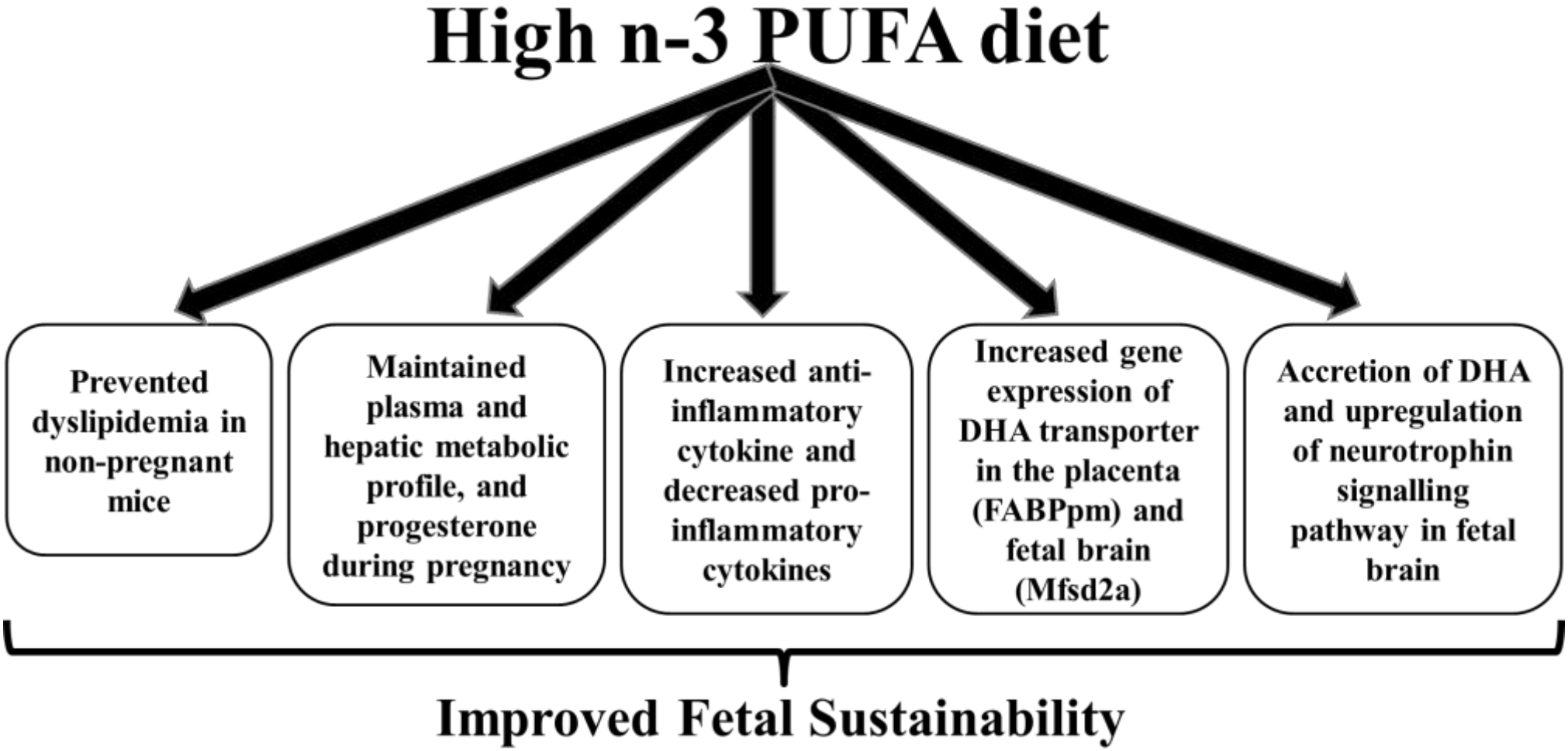

Figure 6.2: A diet high in n-3 PUFA (9%) prevented dyslipidemia prior to pregnancy, and improved fetal sustainability. N-3: Omega-3 polyunsaturated fatty acid; DHA: Docosahexaenoic acid.

6.2 Implications and future directions

During pregnancy, several physiological, anatomical and metabolic changes occur in the mother to permit adequate fetal development, and to prepare the body for parturition. These changes are initiated at early pregnancy and progresses till late gestation. The series of studies presented in this thesis show the effects of longer chain n-3 PUFA in the prevention of maternal dyslipidemia, which is a known risk factor for several adverse pregnancy outcomes in rodents (de Assis *et al*., 2003; Nikolova *et al*., 2017; Reijnders *et al.*, 2019) and in humans (Grimes & Wild, 2018; Jiang *et al*., 2017; Jin *et al*., 2016; Wild *et al*., 2016).

Judging by the effects of breeding chow diet containing n-3 PUFA from fish oil (8% w/w) on maternal lipid metabolism during pregnancy, as presented in chapter 2, a low fat maternal diet containing n-3 PUFA from fish oil increased maternal endogenous lipid synthesis, along with increasing cholesterol efflux, likely to meet fetal lipid demand during pregnancy. Interestingly, macrophage cholesterol efflux increased as gestation progressed in response to plasma from the diet containing n-3 PUFA from fish oil. As such, I can speculate that n-3 PUFA from fish oil increases cholesterol efflux in a gestation-dependent fashion during pregnancy; however, I cannot unequivocally say that the observed increase in cholesterol efflux was due to direct regulation of HDL function by n-3 PUFA, owing to the fact that we used plasma samples as our acceptor. Increased HDL-mediated cholesterol efflux during pregnancy has been suggested as a rescue mechanism to prevent adverse pregnancy outcomes, such as preeclampsia in women with high risk pregnancies by removing cholesterol from cells to limit peroxidation (Mistry *et al*., 2017).

Plasma is known to contain other proteins and lipoproteins capable of influencing the efficiency of cholesterol efflux; the particle size of HDL and the concentration of apolipoprotein (APO)-A1 could influence cholesterol efflux capacity (Sacks & Jensen, 2018). Interestingly, high n-3 PUFA has no significant effect on maternal plasma concentration of HDL-c before and during gestation (Appendix VIII). Although I studied cholesterol efflux in my first study, I did not study

this in my second study due to sample limitation. Cholesterol efflux assay would have further corroborated our previous observation that n-3 PUFA increases cholesterol efflux in a gestation-dependent fashion during pregnancy. Future studies could investigate the direct effect of n-3 PUFA on cholesterol efflux capacity at different time-points during pregnancy by isolating HDL from plasma samples. This will give direct information on the influence of n-3 PUFA on HDL function during pregnancy.

Longer chain n-3 PUFA are known to regulate inflammatory markers, such as pro-inflammatory cytokines and eicosanoids (Calder, 2013). My data, presented in chapter 3 and 4 showed that n-3 PUFA from fish oil reduced the level of pro-inflammatory cytokines in maternal plasma, as well as in the placenta. The propensity of n-3 PUFA to influence inflammation is usually mediated in part by changing the fatty acid composition of the corresponding cell membrane. Our findings provide evidence that a maternal diet containing n-3 PUFA from fish oil caused accretion of EPA and DHA in reproductive tissues, particularly the uterus and the placenta. The accretion of longer chain fatty acids coincided with a significant reduction in the concentration of pro-inflammatory cytokines at late gestation in both maternal plasma and placenta, with a resultant increase in the levels of anti-inflammatory cytokines.

Furthermore, the diet containing n-3 PUFA from fish oil showed no fetal resorption, compared to the breeding chow diet containing n-3 PUFA from soybean which showed clear fetal resorption sites. Recent evidence revealed that spontaneous fetal resorption starts with endogenous apoptosis of the embryo; apoptotic embryo is aborted into the uterine lumen, and then rapidly resorbed (Drews *et al*., 2020). The initiation of the apoptotic process has in part been associated to innate inflammation in the embryonic cells (Drews *et al*., 2020). Inflammation during pregnancy clearly influences fetal development. Eicosanoids are lipid mediators derived from PUFAs which can influence inflammation. Future studies could examine keys eicosanoids, particularly prostaglandin E_2, which is known to contribute to the upregulation of pro-

inflammatory cytokines, and consequently spontaneous abortion (Ricciotti & FitzGerald, 2011). More so, future studies could collect resorption sites to classify and characterize these sites based on the morphology.

Our findings on the effect of maternal semi-purified diet high in n-3 PUFA on maternal lipid metabolism revealed that a high n-3 diet (9% w/w) prevented dyslipidemia in non-pregnant mice, while very low n-3 PUFA diet (1% w/w) caused hyperlipidemia prior to pregnancy. Dams with elevated lipids before pregnancy carried this profile into pregnancy and lacked lipid regulation during pregnancy. As such, I can safely say that maternal diet before pregnancy is also very important to ensure that dams enter pregnancy with the metabolic profile required to establish pregnancy successfully, as well as to maintain pregnancy. Future studies could investigate the effects of maternal hyperlipidemia on offspring lipid metabolism at early and later life. More so, the effects of a different post-weaning diet on the regulation of lipid metabolism in the offspring could also be investigated.

We studied fetuses at gestation day 6.5, 12.5 and 18.5; these gestation stages correspond to first, second and third trimesters of pregnancy in mice. Our findings demonstrate for the first time that a high n-3 PUFA diet maintained maternal plasma concentration of maternal sex-steroid hormones during pregnancy, which may be responsible higher fetal sustainability till late gestation. Dietary n-3 PUFA has been suggested to affect offspring sex-ratio (Fountain *et al*., 2008). Fetuses used in our study comprised of both sexes; future studies should investigate sex-specific effects in fetal sustainability; this will give insight into whether there are more males, compared to females, or vice versa in terms of fetal resorption and survival.

A number of studies have documented the neuroprotective effects of n-3 PUFA in rodents (Firląg *et al*., 2013; Lopes *et al.*, 2017) and humans (Yanai, 2017); however, it is not known if maternal diets containing high n-3 PUFA cause DHA accretion in fetal brain at different stages of gestation, and regulate the expression of neurotrophins. I investigated the gestation-dependent

effect of n-3 PUFA on the expressions of key proteins involved in neurotrophin signalling. I observed an increase in BDNF and TrKB gene expression in response to maternal diet high in n-3 PUFA, as presented in chapter 5. Our findings suggest that increased mRNA expression of BDNF as gestation progressed from mid gestation to late gestation was due to increased CREB phosphorylation by n-3 PUFA. This finding is novel; however, nerve growth factor (NGF) also contributes to brain development by promoting neuronal survival and preventing neuronal apoptosis (Chen *et al*., 1997).

High n-3 PUFA increased the mRNA expression of NGF at day 12.5, compared to other diets, while there was no change at day 18.5 across all dietary groups (Appendix IX). This is a fascinating observation, as the mRNA and protein expressions of NGF has been previously shown to fluctuate at different stages of development in rats (Maisonpierre *et al*., 1990). As such, future studies could investigate the effect of high n-3 PUFA diet on the signalling pathway of NGF in fetal brain at both mid- and late gestation. To further appreciate the significance of maternal diet enriched in high n-3 PUFA during pregnancy on neurotrophin signalling, it may be pertinent to explore the effect of these diets on the fetal brain's lipidomic profile.

Finally, this current thesis showed that maternal diet varying in the quantity and quality of dietary fat has a gestational-dependent effect on maternal lipids metabolism, and further showed that breeding chow diet containing high n-3 PUFA from fish oil regulates maternal lipid and cytokine profile to elicit positive pregnancy outcomes. My findings further demonstrated that a maternal semi-purified diet high in n-3 PUFA (9% w/w) increased the incorporation of DHA into the placenta, and subsequently influenced the accretion of DHA into the fetal brain as gestation progressed. We have identified novel mechanisms on the neuroprotective potentials of maternal diet high in n-3 PUFA. Specifically, our findings revealed that n-3 PUFA increases the expression of BDNF and TrKB as gestation progresses, and that n-3 PUFA regulates CREB at both transcription and post-translational levels; thus maintaining the levels of BDNF and other

neurotrophins during critical stages of brain development may be important in preventing neurological problems later in life.

As the understanding of positive effects of n-3 PUFA on high risk pregnancies continues to grow, our data also report that a high n-3 PUFA diet maintained maternal metabolic profile and prevented maternal dyslipidemia, prior to and during pregnancy, to elicit positive pregnancy outcomes. Therefore, dietary intake of high n-3 PUFA during pregnancy is of critical importance. Overall, dietary intervention remains the safest strategy to prevent adverse pregnancy outcomes; thus, intake of high n-3 PUFA diet (9% w/w) could serve as a promising approach to prevent maternal dyslipidemia and improve fetal sustainability.

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APPENDICES

Appendix I

Body weight and average weekly food intake before pregnancy and at different stages of gestation (Chapter 2)

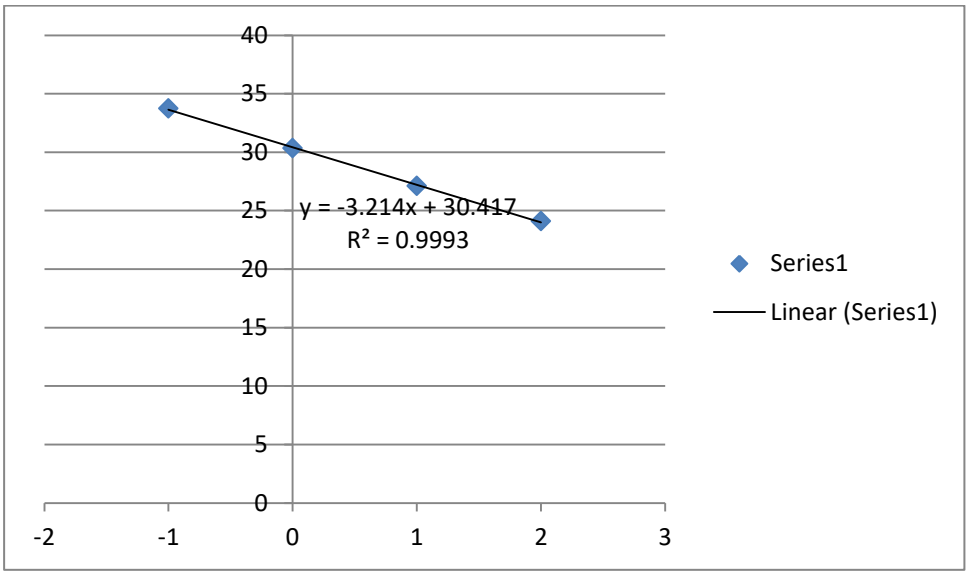
Body Weight (g)	**5% Fat Diet**	**11% Fat Diet**
NP	17.93 ± 0.22	17.90 ± 0.30
Day 6.5	19.31 ± 0.22	19.16 ± 0.14
Day 12.5	25.57 ± 0.92	25.40 ± 1.04
Day 18.5	29.14 ± 0.81	28.88 ± 0.32
Food Intake (g/week)	22.99 ± 0.50	22.82 ± 0.88

Values are expressed as means ± SD, n=8. Data were analysed using one-way ANOVA. NP: Non-pregnant mice.

Appendix II

Ct Values (FAS)			
cDNA Conc. (ng/ul)	Rep1	Rep2	Ave. Ct
100	23.98	23.91	23.945
10	26.79	26.74	26.765
1	30.26	30.4	30.33
0.1	33.66	33.79	33.725

Conc. (ng/ul)	Log cDNA	Ave. Ct
100	2	24.09
10	1	27.095
1	0	30.33
0.1	-1	33.725



Slope = -3.214

Efficiency = 10^(-1/slope)-1*100

= 10^(-1/-3.214)-1*100

= 104.7

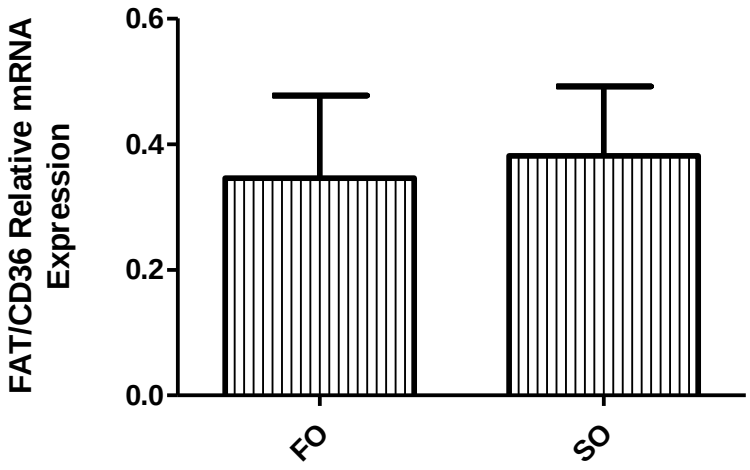
Gene Expression Calculation

*ΔCt = Avg. FAS Ct – Avg. *Actb* Ct

**$\Delta\Delta C$t = Avg. ΔCt - Avg. $\Delta C\text{t}_{\text{Liver}}$

***Normalized FAS expression = $2^{-\Delta\Delta C\text{t}}$

Appendix III



Effect of dietary omega-3 polyunsaturated fatty acids on maternal mRNA expression of fatty acid translocase (FAT/CD36) was determined during gestation at day 18.5 as explained in the Material and Methods section. Values are presented as mean ± SD, n=8. The mRNA expressions were normalized with *Actb* as the reference gene. Data analyzed using student's t-test. *Actb*, beta actin; FO, fish oil based diet; SO, soybean based diet (Chapter 3).

Appendix IV

Mathematical model for diet formulation

FORMULA FOR 5:1

x(3.28-(5*31.56))+y(73.27-(5*0.23))+z(15.54-(5*0.72))+w(17.38-(5*0.76))=0	x	0.99489
x(28.19-32)+y(16.96-32)+z(66.58-32)+w(49.74-32)=0	y	1.8751
x(25.48-19)+y(9.27-19)+z(16.81-19)+w(31.70-19)=0	z	0.41213
	w	1

FORMULAR FOR 20:1

x(3.28-(20*31.56))+y(73.27-(20*0.23))+z(15.54-(20*0.72))+w(17.38-(20*0.76))=0	x	0.22582
x(28.19-32)+y(16.96-32)+z(66.58-32)+w(49.74-32)=0	y	1.4259
x(25.48-19)+y(9.27-19)+z(16.81-19)+w(31.70-19)=0	z	0.13205
	w	1

FORMULAR FOR 40:1

x(3.28-(40*31.56))+y(73.27-(40*0.23))+z(15.54-(40*0.72))+w(17.38-(40*0.76))=0	x	0.056442
x(28.19-32)+y(16.96-32)+z(66.58-32)+w(49.74-32)=0	y	1.327
x(25.48-19)+y(9.27-19)+z(16.81-19)+w(31.70-19)=0	z	0.07036
	w	1

NB: X, Y, Z and W represent variables for n-3 PUFA, n-6 PUFA, MUFA and SFA respectively. N: omega; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids.

Appendix V

Body weight and average weekly food intake of mice fed with maternal diet varying in the amount of n-3 PUFA (Chapter 4).

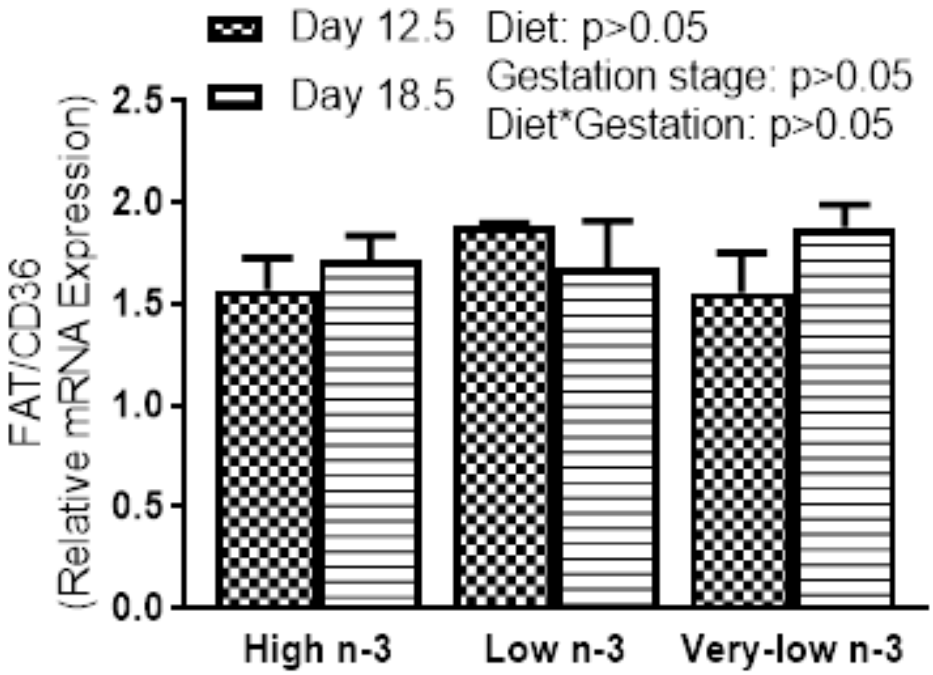
Gestation Stage	High n-3	Low n-3	Very low n-3
Body Weight (g)			
NP	18.16 ± 0.32	18.25 ± 0.90	18.31 ± 0.93
Day 6.5	19.22 ± 0.42	19.11 ± 0.64	19.28 ± 0.78
Day 12.5	24.64 ± 0.88	24.78 ± 0.45	24.83 ± 0.32
Day 18.5	28.19 ± 0.50	28.41 ± 0.41	28.88 ± 0.63
Food Intake (g/week)	22.05 ± 0.49	23.10 ± 0.71	22.40 ± 0.62

Values are expressed as means ± SD, n=8. Data were analysed using one-way ANOVA. NP: Non pregnant; n-3 PUFA: omega-3 polyunsaturated fatty acids.

Appendix VI

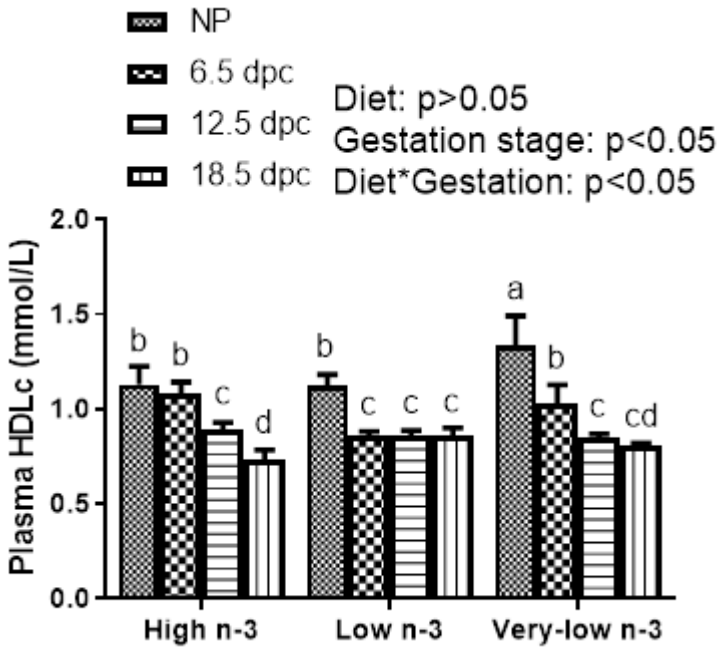
	Total number of female mice bred	Confirmed pregnancy	Delivery before day 18.5
High n-3 PUFA	12	10	0
Low n-3 PUFA	12	9	1
Very low n-3 PUFA	12	10	2

Appendix VII



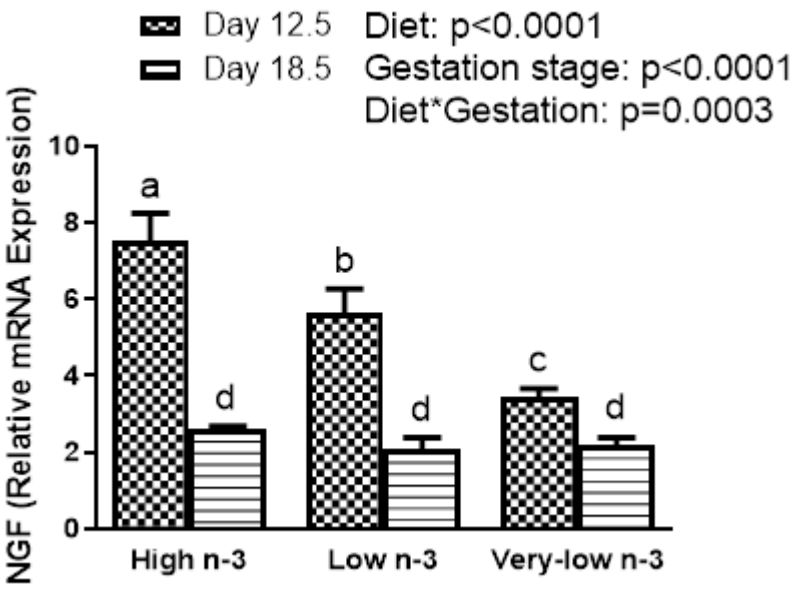
Effect of maternal diets varying in the amount of n-3 PUFA on the placental mRNA expression of fatty acid translocase (FAT/CD36) was measured at gestation day 12.5 and 18.5 as explained in the material and method section. The mRNA expressions were normalized to β-actin (ActB) as the reference gene. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage. $p<0.05$ was considered significant. Data are presented as mean (n = 8 at each gestation stage) ± SD; n-3 PUFA: omega-3 polyunsaturated fatty acids (Chapter 5).

Appendix VIII



Effect of maternal diets varying in the amount of n-3 PUFA on plasma HDLc was measured in non-pregnant and pregnant mice at gestation day 6.5, 12.5 and 18.5. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage. $p<0.05$ was considered significant. Data are presented as mean (n = 8 at each gestation stage) ± SD; n-3 PUFA: omega-3 polyunsaturated fatty acids

Appendix IX



Effects of maternal diets varying in the amount n-3 PUFA on the mRNA expression of nerge growth factor normalized to β-actin (ActB) as the reference gene. Data were analysed using two-way ANOVA to determine the main effects and the interactions between diet and gestation stage; pairwise comparison using Bonferroni's correction was used to determine differences between groups. $p<0.05$ was considered significant. Data are presented as mean (n=8 at each gestation stage) ± SD; n-3 PUFA: omega-3 polyunsaturated fatty acids.