EFFECTS OF LIPID EMULSIONS USED IN TOTAL PARENTERAL NUTRITION, AND BREAST MILK OF LEAN AND OBESE WOMEN, ON ADIPOGENESIS IN 3T3-L1 CELLS

by

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ABSTRACT

The growth of fat tissue in infants depends mainly on the differentiation and hypertrophy of existing adipocytes cells; thus, it is a sensitive period for the development of obesity. The objective of this thesis was to investigate the effects of lipid emulsions (Omegaven, SMOFlipid, and Intralipid) and breast milk (BM) on adipogenesis and lipolysis using 3T3-L1 cells. Intralipid, SMOFlipid, and Omegaven had omega (n)-6:n-3 polyunsaturated fatty acids (PUFA) of 7.8:1, 2.72:1 and 0.2:1, respectively. Upon treatment of 3T3-L1 cells with lipid emulsions, Omegaven treatment increased eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in 3T3-L1 adipocytes, reduced triacylglycerol accumulation and inhibited the mRNA expression of lipogenic and lipolytic genes, compared to control or cells treated with SMOFlipid and Intralipid. BM from obese women (BMO) had higher n-6:n-3 PUFA compared to BM from lean women (BML). Upon treatment of 3T3-L1 cells, BML with high n-6:n-3 PUFA increased the mRNA expression of lipogenic genes; however, there was no effect of BMO lipogenesis. There was no effect of BML and BMO on the mRNA expression of lipolytic genes. Our findings suggest that exposure of preadipocytes to Omegaven prevents lipid accumulation and may be beneficial in the prevention of obesity. BMO had no effect suggesting other factors in BM may interfere with adipogenesis, and/or cause adipocyte dysfunction.

CO-AUTHORSHIP STATEMENT

For the work presented in Chapter-3, which was written in manuscript format and in preparation to be submitted for publication in *Pediatric Obesity*, I, Peter Isesele, was involved in the design of the study, conducted experiments, analyzed and interpreted all the data, and prepared the manuscript.

For the work presented in Chapter-4, which was written in manuscript format and in preparation to be submitted for publication in *Maternal and Child Nutrition*, I, Peter Isesele, was involved in the design of the study, measured malondialdehyde levels in breast milk, conducted the cell culture experiments, performed analysis of the data, interpreted data, and prepared the manuscript. Fatty acids analysis was performed by Dr. Raymond Thomas's research group (Department of Boreal Ecosystem and Agricultural Science, Grenfell Campus, Memorial University). Cytokines and leptin analyses were performed by Dr. Sarbattama Sen's research group (Department of Newborn Medicine, Brigham, and Women's Hospital, Boston, Massachusetts).

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ABBREVIATIONS

AA	Arachidonic acid
ACC	Acetyl Coenzyme A carboxylase
ADD1	Adipocyte determination and differentiation-dependent factor 1
ALA	α-Linolenic acid
AMPK	Adenosine monophosphate kinase
ANOVA	Analysis of variance
AP-1	Activating protein-1
aP2	Adipocyte protein 2
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CCAAT enhancer-binding protein
CACT	Carnitine acylcarnitine translocase
cAMP	Cyclic adenosine monophosphate
ChREBP	Carbohydrate response element-binding protein
CPT	Carnitine palmitoyltransferase
DAG	Diacylglycerol
Dex	Dexamethasone
DGAT	Diacylglycerol O-acyltransferase
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
EFA	Essential fatty acid

EFAD	Essential fatty acid deficiency
EGF	Epidermal growth factor
EPA	Eicosapentaenoic acid
FABP	Fatty acid-binding protein
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum-
FFA	Free fatty acids
FID	Flame ionization detector
FXR	Farnesoid X receptor
G3P	Glycerol-3-phosphate
GC	Gas chromatography
GLUT	Glucose transporter
GSK	Glycogen synthase kinase
HSL	Hormone-sensitive lipase
IBMX	Isobutyl-methylxanthine
IFN	Interferon
IR	Insulin receptor
IGF	Insulin-like growth factor
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acids
LPL	Lipoprotein lipase
LXR	Liver X receptor
MCP	Monocyte chemoattractant protein

MCT	Medium-chain triglycerides
MDA	Malondialdehyde
MEK/ERK	Mitogen-activated protein kinase/extracellular signal-regulated kinase
MGL	Monoglyceride lipase
MSD	Meso Scale Discovery
MUFA	Monounsaturated fatty acid
NBCS	Newborn calf serum
NEFA	Non-esterified fatty acids
NF-kB	Nuclear transcription factor kappa-B
NL	Newfoundland and Labrador
PBS	Phosphate buffered saline
PNALD	Parenteral nutrition-associated liver disease
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response elements
Pref	Preadipocyte factor
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RPLPO	Large ribosomal protein
RXR	Retinoid X receptor
SCD1	Stearoyl-Coenzyme A desaturase
SFA	Saturated fatty acid
SOCS	Suppression of cytokine signaling
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
TGF	Transforming growth factor

TNF	Tumor necrosis factor
TPN	Total Parenteral Nutrition
WHO	World Health Organization

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CHAPTER ONE

Introduction and overview

1.0 Introduction

Childhood obesity in Canada is on the rise (Rao et al., 2016) and this has resulted in a wide range of health conditions associated with obesity that are commonly seen in adults, such as type 2 diabetes, high blood pressure and elevated cholesterol (Public Health Agency of Canada, 2012). Proper nutrition is a requisite for growth and development, as well as for maintaining the optimum health of infants. Inadequate nutrition in an infant's first year can result in stunted growth, low intellectual capacity, and high risk of developing a disease in adulthood (Pollitt et al., 1995). Obesity in adolescence is problematic in that it can proceed to obesity in adulthood (Singh et al., 2008). Exclusive breastfeeding is highly encouraged during the infants' first six months of life (Butte et al., 2002), due to the numerous beneficial effects since it contains the ideal nutrient composition required for infant growth (Bernt & Walker, 1999; Butte et al., 2002).

Breast milk (BM) has a unique composition which comprises of carbohydrates, proteins, oligosaccharide, hormones, cytokines, and lipids that are essential for the growth of the newborn (Savino et al., 2013). Among the different sources for infant nutrition, BM is considered as the best option when compared to infant formula feeding (Lessen & Kavanagh, 2015) due to its beneficial effects in mitigating against metabolic disease programming in infants, particularly against obesity and type 2 diabetes (Savino et al., 2013). However, for preterm infants (<37 weeks gestational age), their gastrointestinal tract is not well developed; as such, they cannot be fed enterally. Several complications for preterm infants include respiratory distress syndrome, chronic lung disease, neurological immaturity, and necrotizing enterocolitis (Armanian et al., 2019; Moore et al., 2014; Shulhan et al., 2017). Provision of nutrition intravenously (parenteral nutrition) became a lifesaving therapy for these preterm infants to provide the required nutrients for their survival.

1.1.1 Total parenteral nutrition for preterm infants

The use of total parenteral nutrition (TPN) started in the 1960s to provide required nutrients such as amino acids, electrolytes, minerals, and carbohydrates to infants (Dudrick et al., 1968). The use of TPN has to be reduced as quickly as possible and replaced by enteral nutrition to minimize potential side effects (Koletzko et al., 2009). The early TPN formulation was fat-free, which resulted in infants having an essential fatty acid deficiency (EFAD); this led to the introduction of lipid emulsions in TPN formulation in the 1970s to supply the needed fat requirements to infants sustained on intravenous feeding (Anez-Bustillos et al., 2016). Over the years, several lipid emulsions have emerged with a unique composition to provide necessary nutritional requirements to infants, and to prevent complications experienced with past lipid emulsions. This ranges from the first generation of lipid emulsion, which is soybean-based, to the new generation of lipid emulsion, which contains fish oil. Lipid is an essential component of a diet and is one of the macronutrients that act as an important source of cellular energy (Fell et al., 2015). They play critical roles as membrane components, precursors of modulators of inflammation, and as the second messenger in cellular signaling cascades (Fell et al., 2015).

1.1.2 Lipid emulsions use in total parenteral nutrition formulation

Lipid emulsions are an important component of TPN that provide energy and essential fatty acids (EFA) for the proper growth and development of the newborn. A lipid emulsion is composed of oil suspended in an aqueous dispersion of phospholipid, water, and glycerol (Burrin et al., 2014). Glycerin is added to lipid emulsions to act as an osmotic agent, while sodium oleate is added as a stabilizing agent (Carpentier & Dupont, 2000). Specific oil and/or a mixture are used in a specific lipid emulsion, given each oil a specific fatty acid composition. (Raman et al., 2017).

1.2 Generations of lipid emulsions

1.2.1 Intralipid

The use of lipid emulsions in TPN formulation started in 1961, with the first available formulation being Intralipid (Elia, 2006). Intralipid is a 20 mg/ml oil (pure soybean oil) lipid emulsion. Intralipid was the most commonly used lipid emulsion in TPN formulation (Waitzberg et al., 2006); it contains 50 % linoleic acid (LA), 25 % oleic acid and 10 % α-linolenic acid (ALA), and the remaining 15 % fatty acids are palmitate and stearate (Vanek et al., 2012). Intralipid contains an adequate amount of EFA to prevent EFAD in infants. Intralipid has very high levels of omega (n)-6 polyunsaturated fatty acid (PUFA), low n-3 PUFA, and high n-6:n-3 PUFA. High levels of n-6 to n-3 PUFA and low levels of antioxidants in Intralipid are associated with increased lipid peroxidation, thereby increasing its inflammatory and hepatotoxic potential (Waitzberg et al., 2006). The ratio of n-6 to n-3 PUFA in Intralipid is estimated to be 7:1 (Fell et al., 2015); the high dose of n-6 PUFA in Intralipid is linked to its immunosuppressive effects (Wanten & Calder, 2007).

The cholestatic effect of Intralipid is partly linked to high levels of phytosterols, which downregulate the farnesoid X receptor (FXR), a dominant sensor involved in bile acid homeostasis (Carter et al., 2007). The cholestatic effect is also linked to high levels of n-6:n-3 PUFA in Intralipid (Anez-Bustillos et al., 2016). In preterm infants sustained on long term TPN, cholestatic liver disease is one of the most common problems and can progress to liver cirrhosis. In infants with intestinal failure, the use of Intralipid has resulted in the development of PN-associated liver disease (PNALD), which leads to the development of hepatic inflammation and cirrhosis. High n-6:n-3 PUFA in Intralipid could also be playing a critical role in the development of PNALD in preterm infants. The second-generation lipid emulsion was introduced in Europe in 1984, which contains a 50:50 (w/w) combination of soybean oil and medium-chain triglycerides (MCT), derived from

coconut oil and other tropical nuts (Raman et al., 2017). This aimed at reducing the high levels of n-6 PUFA that had been suspected of inducing several complications related to Intralipid (Anez-Bustillos et al., 2016). MCT is easily metabolized, less pro-inflammatory, and less prone to lipid peroxidation.

ClinOleic was the third-generation lipid emulsion that was introduced in Europe in the 1990s. Its composition was 80:20 (% w/w) olive oil and soybean oil (Vanek et al., 2012). The fat composition of this generation of lipid emulsion was 20 % PUFA, 65 % monounsaturated fatty acids (MUFA), and 15 % saturated fatty acids (SFA) (Raman et al., 2017). MUFA are less inflammatory compared to the soybean oil used in Intralipid and are also less prone to lipid peroxidation compared to PUFA (Raman et al., 2017). Olive oil has 5 % LA, thus reducing the levels of n-6 PUFA by 75 % of the initial soybean oil-based lipid emulsion (Vanek et al., 2012). The fourth-generation lipid emulsions contain fish oil, either used alone (Omegaven) or used in combination with the oils previously used in other lipid emulsions (SMOFlipid) (Vanek et al., 2012). Lipid emulsions containing fish oil (rich in n-3 PUFA) are the only lipid emulsion that provides a large amount of eicosapentaenoic (EPA) and docosahexaenoic (DHA), thus allowing for a lower n-6:n-3 PUFA (Raman et al., 2017). This leads to a shift in the high n-6:n-3 PUFA experienced in older generations of lipid emulsion to a lower ratio, which has an anti-inflammatory effect (Vanek et al., 2012).

1.2.2 SMOFlipid

SMOFlipid (Fresenius Kabi, Germany), is a mixed lipid emulsion with a concentration of 20 g/100 ml oil and contains 30 % soybean oil, 30 % MCT, 25 % olive oil and 15 % fish oil (Fell et al., 2015). SMOFlipid has high levels of α-tocopherols (200 mg/ml) and low levels of

phytosterols (50 mg/L) (Fell et al., 2015). In clinical trials, the use of SMOFlipid was well tolerated by infants placed on TPN for a short term (7-14 days) (Rayyan et al., 2012). In a double-blinded study, supplementation of SMOFlipid did not affect triacylglycerol (TAG), phospholipid, and total cholesterol levels in serum (Mertes et al., 2006). In a small retrospective cohort study of children who developed PNALD, a transition from Intralipid to SMOFlipid resulted in the resolution of cholestasis in 5 of 8 children (Muhammed et al., 2012). Lee et al. reported the development of biochemical PNALD in 2 infants who were placed on TPN and administered SMOFlipid; however, this was reversed when switched to fish oil monotherapy (Lee et al., 2016).

1.2.3 Omegaven

Omegaven (Fresenius Kabi, Germany) is made from fish oil and contains ~30 % of EPA and DHA (Fell et al., 2015). It has minimal phytosterols and abundant levels of α-tocopherol, containing 150-300 mg/L (Meisel et al., 2011). Omegaven is not approved in Canada to be used routinely as a lipid emulsion in TPN formulation, but it can be approved for restricted compassionate use at a dosage of not more than $1 g.kg^{-1}d^{-1}$ for infants with cholestasis (Ng et al., 2016). The use of Omegaven was shown to revert PNALD in infants who were placed on TPN for their survival (de Meijer et al., 2009; Gura et al., 2006; Lam et al., 2014; Park et al., 2014; Premkumar et al., 2013, 2014; Puder et al., 2009). The low levels of n-6 PUFA in Omegaven raised the concern about the possibility of causing EFAD when used as a monotherapy (de Meijer et al., 2010), but studies have shown that the use of Omegaven as monotherapy did not cause EFA (de Meijer et al., 2010; Le et al., 2010).

1.3 Metabolism of essential fatty acids

Fatty acids have varying degrees of saturation with long carboxylic and aliphatic chains (McNaught & McNaught, 1997). The fatty acids that cannot be endogenously synthesized by the body and must be obtained from the diet are referred to as EFA (Shils & Shike, 2006). The two EFA are LA and ALA. EFA are classified as n-3 PUFA, having first double bond at carbon number 3 from the methyl end of the fatty acid chain and carbon number 6 classified as the n-6 PUFA (Engler et al., 2005). Humans lack the Δ-12 and Δ-15 desaturase; thus, they cannot synthesize the EFA. Once obtained from diet, the EFA are metabolized to form longer chain unsaturated PUFAs through the process of elongation and desaturation to form arachidonic acid (AA; C20:4n-6) from LA, and EPA (C20:5n-3) and DHA (C22:6n-3) from ALA through elongation and desaturation (Sprecher, 1981) (Fig 1.1). The conversion rate of ALA to DHA is less than 1 % (Gillingham et al., 2013). A deficiency in LA and ALA can result in an insufficient supply of EFA leading to EFAD, which is characterized by growth impairment, dermatitis, delay in development, renal and pulmonary abnormalities (Fell et al., 2015).

1.3.1 The role of omega-3 and omega-6 PUFA in metabolic function and PNALD

The fish oil used in Omegaven and SMOFlipid is a significant source of n-3 enriched with long-chain (LC) PUFA, DHA, and EPA. Studies have shown that n-3 PUFA increases insulin sensitivity, reduces serum lipids and hepatosteatosis (Chacińska et al., 2019; Ip et al., 2004; Svegliati-Baroni et al., 2006; Thota et al., 2019). Hepatosteatosis can result from the increased levels of circulating free fatty acids (FFA) released from the adipose tissue (Listenberger et al., 2003). The n-3 LCPUFA, particularly DHA and EPA, play a crucial role in regulating metabolic function by modulating the protein and/or gene expression of several transcription factors that are involved in glucose and fat metabolism (Horn et al., 2019; Huang et al., 2017).

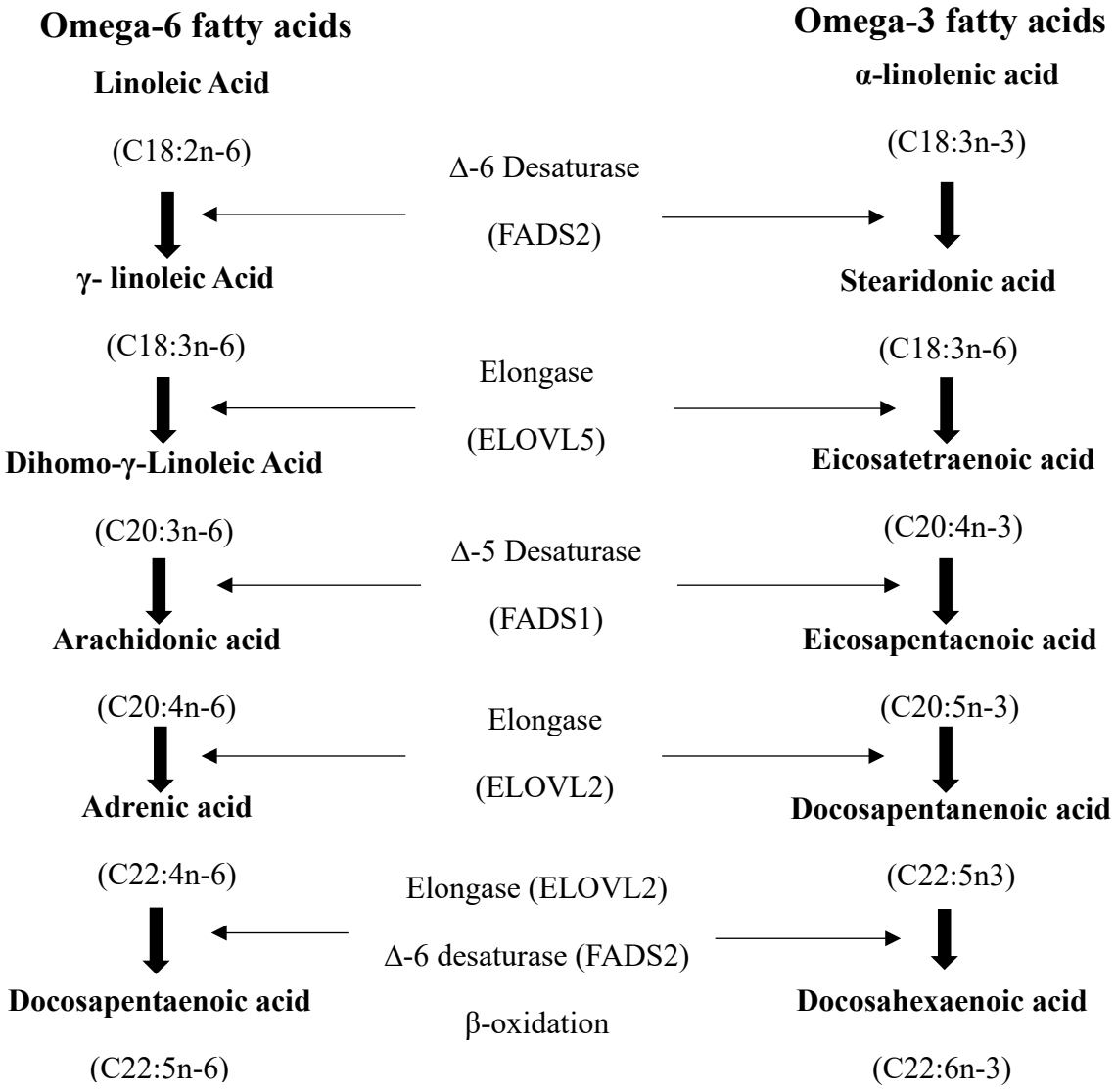

Figure 1.1 Pathway for the elongation and desaturation of the omega-6 and omega-3 polyunsaturated fatty acids

Linoleic acid (LA) and α-linolenic acid are the principal omega-6 and omega-3 fatty acids, respectively. Once consumed, these fatty acids that are converted into other longer chains fatty acids by desaturases (FADS1 and FADS2) and elongases (ELOVL5 and ELOVL2). DHA can be retro converted back to EPA in the peroxisome; however, this conversion occurs at a low basal rate and following DHA supplementation in human (Brossard et al., 1996)

1.4.1 The composition of breast milk

Human BM contains 88.1 % water, 3.8 % fat, 7 % lactose and 0.9 % protein (weight per volume) (Fig 1.2). Lipids account for 45-55 % of total energy provided by human BM and is the main source of energy in infants. The composition of human BM changes over time to adapt to the need of the growing infant (Martin et al., 2016).

1.4.1.1 Bioactive components in breast milk

The bioactive compounds in BM include hormones, cytokines, and immunoglobulins, that are important to meet the needs of newborn infants (Hawkes et al., 2004; Savino et al., 2010). Leptin, adiponectin, insulin, ghrelin, and resistin are the hormones in BM, and they play key roles in energy intake and regulation of body composition (Karatas et al., 2011). Despite being an adipokine, leptin is also produced by the mammary gland and is secreted by epithelial cells in milk fat globules (Smith-Kirwin et al., 1998). Using radioimmunoassay, leptin concentration was found to be higher in whole than in skimmed human BM samples (Houseknecht et al., 1997). Houseknecht *et al.* reported a positive correlation between leptin in BM and maternal plasma leptin concentration (Houseknecht et al., 1997). These authors also observed a positive correlation between breast milk leptin concentration and maternal body mass index (BMI), suggesting that infants of obese women might be exposed to higher levels of leptin than infants of lean women (Houseknecht et al., 1997). A positive correlation between BM leptin levels and infant plasma leptin has been reported by others as well (Ucar et al., 2000).

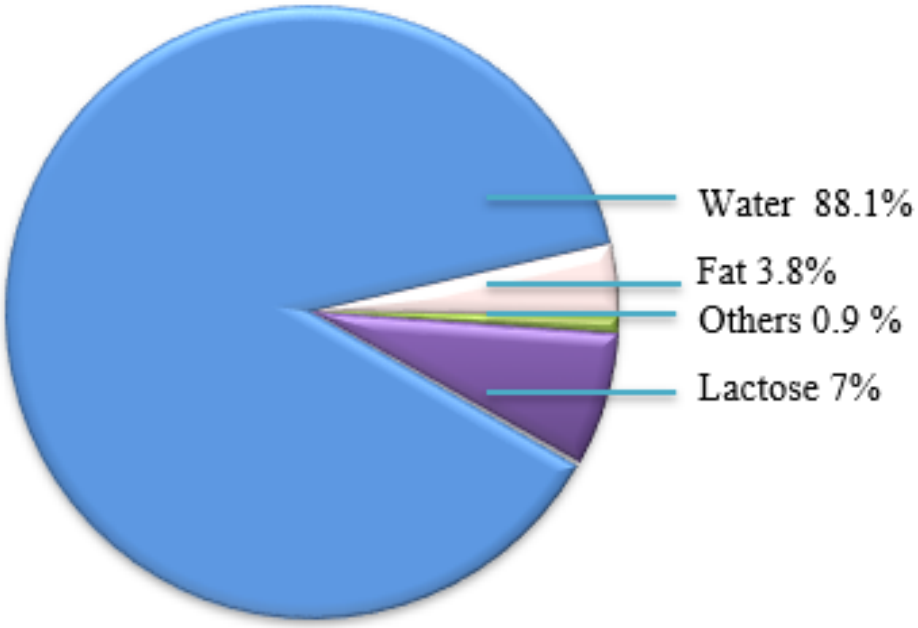

Figure 1.2 The composition of human breast milk

Human breast milk contains water, fat, lactose, and other components such as minerals, protein, and hormones, in different proportions.

Cytokines, such as interleukins (IL)-1, -3, -4, -5, -6, -8, -10, and -12, gamma-interferon (IFN), tumor necrosis factor (TNF)-alpha, and transforming growth factor (TGF)-beta-1 play roles in the normal growth and development of the immune system of the newborn (Garofalo & Goldman, 1998). BM composition is dynamic and varies across lactation stages (Geddes & Perrella, 2019), from colostrum (produced in the first few days postpartum) to mature milk (4 to 6 weeks postpartum) (Ballard & Morrow, 2013). Studies have shown elevated levels of pro-inflammatory cytokines in BM at one week postpartum, which consequently decreased at one month postpartum, suggesting that the cytokine expression is dynamic during lactation (Chollet-Hinton et al., 2014). Similarly, pro-inflammatory cytokine release from adipose is positively correlated with obesity, insulin resistance, and type-2 diabetes in adults (Bastard et al., 2002).

1.4.1.2 Lipid composition of breast milk

The fat in BM is present in the form of milk fat globules that are formed in the mammary alveolar cells (Koletzko et al., 2001). Lipids are the most important component of BM, supplying energy and specific fatty acids that help in the development of the central nervous system (Martin et al., 2016). The major proportion of milk is formed from the circulating lipids derived from the maternal diet, and maternal body stores; thus, maternal diet plays a key role in modulating the fatty acid composition of the BM (Koletzko et al., 2001). Lipids in BM are involved in the regulation of cell function, and inter- and intra-cellular communication in the breastfed infants (Innis, 2014). Triacylglycerol (TAG) is the main fat fraction, and it represents 98-99% of total fat in human BM (Koletzko et al., 2001). The remaining classes of lipids in BM consist predominately of diacylglycerol, monoacylglycerol, free fatty acids, phospholipids, sphingolipids, and cholesterol (Lopez & Ménard, 2011).

1.4.1.3 PUFA in breast milk

LCPUFA is usually transferred through the placenta from mother to fetus in the third trimester of pregnancy; after birth, BM from women act mainly as the source of LCPUFA to infants (Martin et al., 2016). BM contains the two EFA LA and ALA, which serve as precursors for the synthesis of other LCPUFA, such as n-6 (AA) and n-3 PUFA (EPA and DHA) (Guo, 2014). AA, EPA, and DHA are important fatty acids that play a key role in vision, immune function, inflammatory response, growth regulation, and brain development in the newborn (Martin et al., 2016). The balance of the supply of LCPUFA is also important for the optimum maturation of the infant's immune system. The conversion of ALA to DHA is about 1 % in infants and substantially lower in adults; this evidence is from isotopic tracer studies (Brenna et al., 2009). The transport of LCPUFA through the placenta from mother to fetus is limited in preterm infants who were born before the end of the last trimester of pregnancy; thus, they have a limited amount of AA and DHA (Innis, 2014). Lower levels of AA and DHA have been associated with death and premature infants (Innis, 2014).

1.4.2 Maternal obesity and breast milk composition

According to the World Health Organization (WHO), individuals are classified as lean (normal), overweight or obese if their BMI is 20-24.9kg/m^2, 25-29.9kg/$m^{2,}$ and 30-35kg/m^2 or greater, respectively (World Health Organization, 1995). An abnormal maternal environment of obese women is characterized by dysregulated inflammation and oxidative stress; infants born to obese women are twice as likely to become obese adults themselves (Alcala et al., 2018; Ramsay et al., 2002; Ward et al., 2017). This abnormal metabolic environment that occurs during early obesity can influence the risk of obesity through the life span (Simmons, 2008). The dietary EPA

and DHA and the n-6:n-3 fatty acids during late pregnancy influences the DHA and EPA content in mature BM (Nishimura et al., 2014). BM from obese women had lower DHA and higher inflammatory fatty acids profile (Panagos et al., 2016). Neonates of obese women had lower n-3 PUFA and higher n-6:n-3 fatty acids (Lindholm et al., 2013). This implies that maternal obesity has an impact on BM composition. The n-6:n-3 PUFA has increased to 15-20:1 due to the high consumption of n-6 fatty acids as opposed to the recommended ratio of 4:1 (Candela et al., 2011). The fatty acid composition of BM is critical in maintaining metabolic functions, including adipose tissue function.

1.5 Adipose tissue development and metabolism

At infancy and early childhood, fat tissue growth largely depends on differentiation and hypertrophy of existing adipocytes; thus, it is also a sensitive period for the development of obesity (Stettler et al., 2002). Fat cell progenitors are set during the prenatal period. Adipocyte number increases after birth and during adolescence (Rodríguez et al., 2015), which represents critical periods for subsequent obesity development (Spalding et al., 2008). High BMI in early infancy is more strongly associated with early childhood obesity (Roy et al., 2016). Fetal adiposity is associated with elevated protein expression and increased expression of enzymes mediating fatty acid biosynthesis in adipose depots (Long et al., 2012). The adipose tissue is essential in maintaining energy balance by storing and mobilizing TAG (Ali et al., 2013), and it also acts as an endocrine organ (Coelho et al., 2013). Physiologically, adipose tissue mediates numerous processes by secreting factors that control appetite, lipid metabolism, immune function, insulin-stimulated glucose uptake, and inflammatory response (Lefterova & Lazar, 2009; Sarjeant & Stephens, 2012). Storage of TAG in adipocytes results in the expansion of the adipose (Lafontan & Langin, 2009; Tan & Vidal-Puig, 2008). In the period of energy expenditure requirement, the TAG in adipose

undergoes lipolysis to release glycerol and FFA. The FFA and glycerol are transported in the blood and subsequently infiltrate into the muscle, liver, and other organs to modulate whole-body energy balance (Frayn, 2002). Adipose is key to regulate excess caloric intake relative to expenditure, and it produces a metabolic state that regulates adipocyte hyperplasia (increase in number) and hypertrophy (increase in size) (Shepherd et al., 1993). Hypertrophic adipocytes exhibit increased expression of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8 and macrophage inhibitory factor (MIF).

1.5.1 Regulation of adipogenesis

The formation of adipocytes from preadipocytes involves a complex and highly orchestrated program of gene expressions (Lowe et al., 2011). Adipogenesis involves the process whereby committed fibroblast-like preadipocytes differentiate to become lipid-laden adipocytes (Ali et al., 2013; Lefterova & Lazar, 2009). Adipocyte differentiation is characterized by changes in the expression of genes that determine the specific adipocyte phenotype of the cell (Niemelä et al., 2008), and it involves a comprehensive network including transcription factors responsible for the expression of key proteins (Farmer, 2006).

Upon confluency, the growth of preadipocytes is arrested at the G1 phase of the cell cycle. (Niemelä et al., 2008). Following hormonal induction, the growth-arrested preadipocytes re-enter the cell cycle and undergo two rounds of division, referred to as mitotic clonal expansion (Fajas, 2003). The cell then exits the cell cycle, stops proliferating, loses its fibroblast morphology, and the induction of specific genes that determine adipocyte phenotype occurs (Tang & Lane, 2012); these changes are transcriptionally regulated (Niemelä et al., 2008).

1.5.1.1 Mechanisms of hormonal regulation of adipogenesis using *in vitro* cell culture models

Differentiation of preadipocytes is initiated with a cocktail of inducers that include insulin, dexamethasone (Dex), and isobutylmethylxanthine (IBMX) in a fetal bovine serum-containing medium (Kawai et al., 2007). Insulin activates the insulin-like growth factor (IGF)-1 signaling pathway by recruiting and phosphorylating insulin receptor substrate proteins. This leads to the activation of the phosphatidylinositol-3-kinase pathway (Hemati et al., 1997). IBMX is a cyclic nucleotide phosphodiesterase inhibitor that dramatically increases intracellular cAMP levels (Essayan, 2001). The cAMP signal then activates CCAAT enhancer-binding protein (C/EBPβ) gene expression that is required for peroxisome proliferator-activated receptor (PPARγ) and C/EBPα activation via the cAMP response element-binding protein (CREB) transcription factor (Tang & Lane, 2012). Glucocorticoids induce differentiation of preadipocytes; dex, a synthetic glucocorticoid activates glucocorticoid receptor, induces C/EBP-δ, and reduces the expression of preadipocyte factor 1 (Pref-1), a negative regulator of adipogenesis (Smas et al., 1999).

1.5.1.2 The role of PPARγ and CEBPα in adipogenesis

PPARγ, a member of the nuclear receptor superfamily, is a regulator of adipogenesis (Rosen et al., 2002). C/EBPs are induced during adipogenesis and coordinate with PPARγ to regulate adipocyte differentiation (El-Jack et al., 1999; Zuo et al., 2006). PPARγ and C/EBP-alpha then induce each other's expression in a positive feedback loop, thereby maintaining the differentiated cell state (Rosen et al., 2002). PPARs usually form a heterodimer with the retinoid X receptor (RXR) to bind peroxisome proliferator response elements (PPRE) in target genes (Tontonoz & Spiegelman, 2008). PPARγ and C/EBPα induce the expression of genes that are involved in lipogenesis and lipolysis, insulin sensitivity, including those encoding glucose transporter

(GLUT4), fatty acid-binding protein (FABP4) also known as adipocyte protein 2 (aP2), lipoprotein lipase (LPL), perilipin, and the secreted factors such as adiponectin and leptin (Lowe et al., 2011).

1.5.1.3 The role of preadipocyte factor-1 in adipogenesis

Pref-1 is a transmembrane protein highly expressed in preadipocytes, and it contains six epidermal growth factor (EGF)-like repeats at the extracellular domain (Baisong et al., 2002; Smas et al., 1997). Pref-1 participates in maintaining a pre-adipose phenotype, and it rapidly acts by inducing Sox9 expression through activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) pathway (Wang & Sul, 2009). Overexpression of Pref-1 in 3T3-L1 preadipocytes drastically lowers the degree of adipocyte differentiation, while knocking down Pref-1 significantly increases differentiation (Hudak & Sul, 2013). Downregulation of Pref-1 during differentiation coincides with increased C/EBPβ and C/EBPδ, which occurs before C/EBPα and PPARγ induction (Hudak & Sul, 2013), leading to an increase in adipogenesis and lipid accumulation (Wang & Sul, 2009). Dex suppresses Pref-1 expression in preadipocytes, eventually allowing for the induction of PPARγ expression and differentiation of preadipocytes into adipocytes (Hudak & Sul, 2013).

1.5.2 Lipogenesis in the adipose tissue

Lipogenesis in the adipose occurs either as a consequence of re-esterification of FFA with glycerol, or *de novo* synthesis of TAG (Gathercole et al., 2011). *De novo* lipogenesis is the metabolic pathway for the synthesis of fatty acids from excess carbohydrates; fatty acids are incorporated into TAG for energy storage (Ameer et al., 2014). On the other hand, very-low-density lipoproteins (VLDL) secreted from the liver travel to adipose tissue; TAG in VLDL is hydrolyzed into non-esterified fatty acids (NEFA) by the insulin-stimulated action of LPL within the vascular

endothelium in adipose tissues (Merkel et al., 2002). The released NEFAs enter adipocytes, probably through fatty acid transporters such as CD36 and fatty acid transport protein-1 (FATP1) (Endemann et al., 1993; Wu et al., 2006).

1.5.2.1 Transcriptional regulation of lipogenesis

Many of the enzymes involved in *de novo* lipogenesis are regulated at the transcriptional level by sterol regulatory element-binding protein-1 (SREBP1c), carbohydrate response element-binding protein (ChREBP), and liver-X receptor (LXR) (Wang et al., 2015). SREBP-1 is also known as adipocyte determination and differentiation-dependent factor 1 (ADD1) and is a basic helix-loop-helix leucine transcription factor that is associated with adipocyte differentiation (Yokoyama et al., 1993). SREBP1c is proteolytically cleaved when it is activated, and the soluble form becomes translocated to the nucleus where it binds sterol response elements (Horton, Goldstein, & Brown, 2002), and regulates a variety of genes linked to fatty acids and TAG metabolism that include acetyl-CoA carboxylase (ACC1), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1) and, diacylglycerol acyltransferase (DGAT2) (Rosen & Spiegelman, 2000). Over-expression of SREBP1c in adipose leads to insulin-resistant, hypertriglyceridemia, high triglyceride levels, and lipodystrophy (Ali et al., 2013).

Insulin activates SREBP1c and mediates its export from the endoplasmic reticulum to the nucleus, where it binds to the sterol regulatory element present in the promoter of target genes (Shao & Espenshade, 2012). Liver X receptor (LXR) are members of the nuclear receptor superfamily that heterodimerize with retinoid X receptor (RXR) (Chawla et al., 2001). Of the two LXR isomers, LXRα is abundantly expressed in lipogenic tissues and plays an important role in transcriptional regulation of lipogenic genes by activating SREBP-1c (Wong & Sul, 2010).

ACC is a biotin-dependent enzyme, and the two isoforms (ACC1 and ACC2) are encoded by different genes (Brownsey et al., 2006). ACC1 is located in the cytosol and produces malonyl-CoA for *de novo* synthesis of palmitate (Abu-Elheiga et al., 2000; Mao et al., 2006). FAS is a multi-subunit protein that synthesizes palmitate from malonyl-CoA and acetyl-CoA (Menendez et al., 2009). FAS gene expression in adipose tissue is linked to fat accumulation, and inhibition of its activity leads to a reduction in adipocyte size and number (Liu et al., 2004). Leptin has a suppressive effect on FAS gene expression (Bai et al., 1996). Moreover, inflammatory cytokines such as TNF-α reduces the expression of lipogenic genes in adipocytes (Doerrler et al., 1994). SCD1 is the rate-limiting enzyme for the synthesis of MUFA from SFA (Peck & Schulze, 2016). High levels of SCD1 is associated with insulin resistance, cellular inflammation, and stress (Liu et al., 2011). SCD1 deficiency upregulates insulin-sensitizing components and affects glycogen metabolism (Dobrzyn et al., 2010). Loss of SCD1 leads to a reduction of adipocyte inflammation (Liu et al., 2010) and protects mice against adiposity (Ntambi et al., 2002). Macrophage inflammation was regulated by oleate, and not palmitoleate, derived from the adipose tissue(Liu et al., 2010). Reduced levels of SCD1 accentuate palmitate-induced stress in insulin-producing β-cells (Thörn et al., 2010). Both SCD1 and DGAT2 are co-localized in the endoplasmic reticulum and have a similar pattern of expression (Man et al., 2006). DGAT2 is the key enzyme that catalyzes the final step in TAG synthesis for fat storage in the adipocyte. Overexpression of DGAT2 promotes lipid deposition and affects insulin signaling (Zhang et al., 2014).

1.5.3 Lipolysis in the adipose tissue

During fasting and physical exercise, adipocytes mobilize their fat stores to fulfill energy-demanding processes (Morigny et al., 2016). Lipolysis involves the hydrolysis of TAG into fatty acids and glycerol through the action of lipases (Langin, 2006). Perilipin is the major protein

covering large lipid droplets in adipocytes (Sztalryd & Brasaemle, 2017), and functions as a protective barrier that restricts access of TAG lipases to neutral lipid substrates to prevent basal lipolysis (Brasaemle et al., 2000). Activation of perilipin-A by PKA-mediated phosphorylation increases lipolysis by increasing the surface area of neutral lipid droplets accessible for attack by lipases (Marcinkiewicz et al., 2006; Moore et al., 2005). Three lipases act in sequence with the concomitant release of one FFA in each step. First, adipose triglyceride lipase (ATGL), which is the rate-limiting enzyme in the lipolytic pathway, hydrolyzes TAG into diacylglycerol (DAG) (Zimmermann et al., 2004). DAG is then hydrolyzed by hormone-sensitive lipase (HSL) into monoacylglycerol (MAG) (Haemmerle et al., 2002), which is cleaved by monoglyceride lipase (MGL) into glycerol and FFA (Fredrikson et al., 1986). The FFA released are delivered to the peripheral tissues where they serve as a substrate for β-oxidation and adenosine triphosphate (ATP) production, while glycerol may be used as a substrate for gluconeogenesis in the liver (van der Spek et al., 2012). Dysregulation of lipolysis may result in the elevated circulation of FFAs, which is thought to restrict glucose utilization and ultimately induce insulin resistance (Arner, 2002; Jensen, 2007).

ATGL is a 54 kDa lipase that belongs to a family of proteins containing a patatin-like domain (Kienesberger et al., 2009). It exhibits a 10-fold higher substrate specificity for TAG than DAG, indicating its role in the first step of TAG hydrolysis (Zimmermann et al., 2004). ATGL is upregulated by fasting and suppressed by feeding. ATGL is upregulated by glucocorticoids (e.g., dexamethasone) in a concentration- and dose-dependent manner in 3T3-L1 preadipocytes (Villena et al., 2004). Inhibition of ATGL in adipose tissue improves hepatic insulin sensitivity through reduced FFA fluxes and ectopic deposits in other tissues (Morigny et al., 2016).

HSL is an 84 kDa cytoplasmic protein, and it has a relative fatty acid hydrolase activity that is 10-fold greater against DAG than TAG (Racelot et al., 1997). Unlike ATGL, HSL is phosphorylated by PKA, leading to its activation by translocating the enzyme from the cytosol to the lipid droplet; thus, insulin and catecholamine can control the activity of HSL by modulation of cyclic AMP (cAMP) levels (Lafontan & Langin, 2009).

The final step of the lipolytic process is the efflux of fatty acids and glycerol from the adipocytes as a result of the association between FABP4 and HSL. Expression of FABP4 is highly induced during adipocyte differentiation and transcriptionally controlled by PPARγ agonists, fatty acids, and insulin (Furuhashi, 2019; Trojnar et al., 2019). FABP4 plays a key role in the trafficking of NEFA from lipid droplets to the plasma membrane; thus, the loss of FABP4 activity may reduce NEFA release (Lafontan & Langin, 2009). Elevated levels of FABP is associated with insulin resistance and obesity. FABP4-deficient mice showed an increase in body weight but reduced insulin resistance in both high-fat diet-induced and genetic obesity mouse models (Furuhashi, 2019; Nakamura et al., 2017; Wu et al., 2014)

1.5.4 β-oxidation of fatty acids

β-oxidation is the catabolic pathway in the mitochondria that produce energy from TAG hydrolysis. In the cytosol, FFA is converted to acyl-CoA, and the protein carnitine palmitoyltransferase-1 (CPT1) catalyzes the transfer of the acyl group of a long-chain fatty acyl-CoA to carnitine to form acylcarnitine (mainly palmitoylcarnitine); carnitine acyltransferase (CACT) transfers acylcarnitine across the outer mitochondrial membrane (Sharma & Black, 2009). Elevated glucose metabolism inhibits β-oxidation due to increased production of pyruvate that is transformed into malonyl CoA, reducing the fatty acid catabolic pathway (Visiedo et al., 2013)

(Sidossis & Wolfe, 1996). Excessive levels of FFA can impair mitochondrial function leading to abnormal fatty acid oxidation (Li et al., 2008)

1.5.6 Adipokines in adipose tissue

Adipocytes release adipokines such as leptin, which trigger certain intracellular signaling pathways (Pessin & Kwon, 2013). Impaired biosynthesis, assembly, secretion, and signaling transduction of these adipokines are associated with the development of obesity and its related disorders (Deng & Scherer, 2010). Leptin is a satiety hormone of 16 kDa peptide encoded in the obesity (ob) gene that regulates energy balance by inhibiting food intake (Caro et al., 1996; Halaas et al., 1995; Zhang et al., 1994). Leptin inhibits the accumulation of lipids in adipocytes by increasing the turnover of TAG, inhibiting basal and insulin-stimulated *de novo* lipogenesis but stimulates oxidation of glucose and FFA (Harris, 2014). Leptin inhibits insulin-stimulated phosphorylation of insulin receptor (IR) and glycogen synthase kinase-3 (GSK3), and binding of insulin to its receptors in adipocytes (Pérez et al., 2004; Walder et al., 1997). Obese individuals have high levels of circulating leptin, which leads to leptin resistance. This is a result of reduced transport of leptin to the brain and reduced expression of leptin receptors or an increased concentration of suppression of cytokine signaling (SOCS-3), which suppresses leptin signaling by inhibiting leptin-induced STAT activation (Myers et al., 2008).

1.5.7 Adipose tissue dysfunction and metabolic disorder

Obesity is associated with an increase in adipose tissue mass and size (Spalding et al., 2008). In the adipose tissue, immune cells secrete numerous pro- and anti-inflammatory cytokines (Chawla et al., 2011; Huh et al., 2014; Makki et al., 201-3). The pro-inflammatory cytokines, as in the case of obesity, lead to insulin resistance, while the anti-inflammatory cytokines play a role in

maintaining insulin sensitivity. Adipose tissue dysfunction occurs when there is an imbalance between the pro- and anti-inflammatory adipokines, which results in insulin resistance, hypertriglyceridemia, and low-grade systemic inflammation (Faber et al., 2009; Kip et al., 2004; Voulgari et al., 2011)

Adipose tissue inflammation elevates adipose tissue lipolysis and results in a high concentration of FFA in circulation, thereby leading to dyslipidemia (Julius, 2003). Leptin and adiponectin also play an important role in regulating adipocyte function. During the enlargement of the adipose tissue, the levels of leptin and resistin increase to induce insulin resistance, while adiponectin is downregulated (Rezaee & Dashty, 2013). Adipose tissue from lean individuals preferentially secretes anti-inflammatory adipokines, while obese adipose tissue releases pro-inflammatory cytokines (Ouchi et al., 2011).

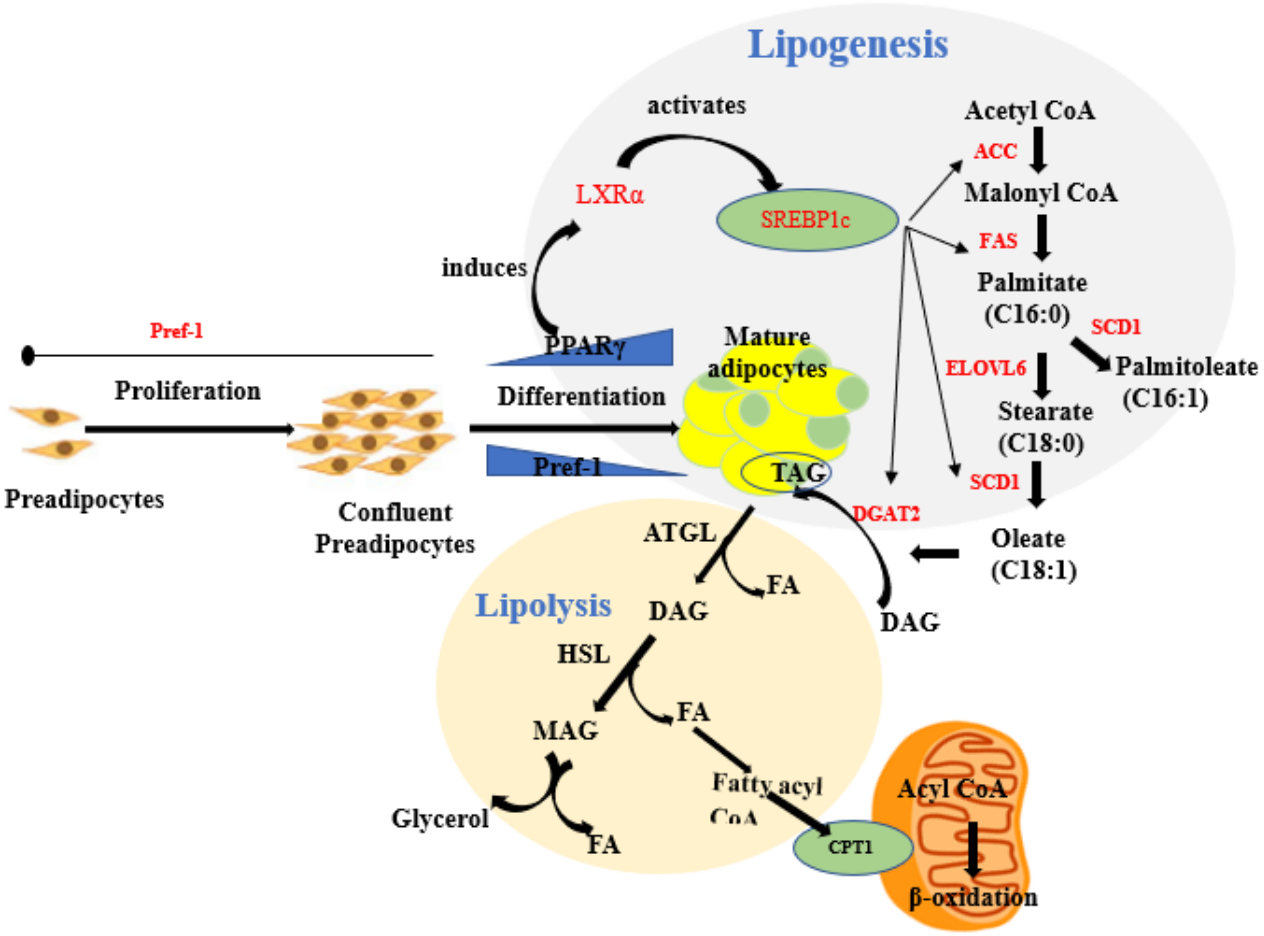

Figure 1.3 The pathway involved in the regulation of adipogenesis, lipogenesis, lipolysis, and β-oxidation in adipocytes

Confluent preadipocytes are differentiated into mature adipocytes upon induction with a differentiation cocktail. Lipolysis involves the hydrolysis of TAG into fatty acids and glycerol through the action of lipases; the β-oxidation catabolic pathway produces energy from TAG. ACC, acetyl-CoA carboxylase; ATGL, Adipose triglyceride lipase; CPT1, carnitine palmitoyltransferase I; DAG, diacylglycerol; DGAT2, Diacylglycerolacyl transferase; FA, fatty acid; FAS, fatty acid synthase; HSL, Hormone-sensitive lipase; LXRα, liver X receptor alpha; PPARγ, peroxisome proliferator-activated receptor-gamma; Pref-1, preadipocyte factor 1; SCD1, stearoyl-CoA desaturase; SREBP1c, sterol regulatory element-binding protein 1; TAG, triacylglycerol

1.5.8 Role of n-3 and n-6 PUFA in adipose tissue metabolism

Both n-6 and n-3 PUFA can act as ligands for the transcription factors involved in adipogenesis. AA, an n-6 PUFA, and its metabolites serve as ligands for PPARγ to induce fat cell differentiation. Adipose tissue inflammation is a key characteristic of obesity, and n-3 PUFA inhibits nuclear transcription factor B (NF-kB), a key transcription factor involved in cytokine gene expression and inflammation (Allam-Ndoul et al., 2016). N-3 PUFA also reduces the expression of FABP4 in 3T3-L1 adipocytes and reduces serum FABP4 concentrations in humans (Furuhashi et al., 2016). The translocation of ChREBP and SREBP1c is inhibited by n-3 PUFA, leading to a reduced expression of genes involved in lipogenesis such as FAS, SCD1, and ACC (Dentin et al., 2005; Kaur et al., 2011; Sekiya et al., 2003; Yahagi et al., 1999). N-3 also promotes the β-oxidation of fatty acids (Ukropec et al., 2003). On the other hand, n-6 PUFA increases lipid accumulation in adipocytes (Madsen et al., 2005), both LA and AA promote the differentiation of preadipocytes to mature adipocytes (Azain, 2004). N-6 and n-3 PUFA play a crucial role in regulating lipid metabolism, inflammatory pathways, and adipogenesis (Schmitz & Ecker, 2008). Downstream metabolites of n-3 and n-6 PUFA are anti- and proinflammatory, respectively (DiNicolantonio & O'Keefe, 2018; Stupin et al., 2019). Thus, a balance of n-3 and n-6 PUFA is important in regulating lipid metabolism in adipocytes.

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CHAPTER TWO

Rational, Objectives and Hypotheses

2.1 Murine 3T3-L1 cells as an *in vitro* model to study adipogenesis

The murine 3T3-L1 cell is an established cell line that represents an appropriate model of adipocyte differentiation and has been used extensively to investigate the mechanisms involved in adipocyte differentiation (Iyer et al., 1998). The 3T3-L1 cells were derived from the Swiss 3T3 mouse embryo, based on their ability to accumulate lipids (Green & Meuth, 1974). Murine 3T3-L1 preadipocytes have a fibroblast morphology; under appropriate hormonal induction with insulin, IBMX, and dex, they can undergo differentiation into a lipid-laden and insulin-sensitive adipocyte-like phenotype (Lefterova & Lazar, 2009).

BM and lipid emulsions whole content are not exposed directly to the infant's adipocytes, rather the chylomicron and triacylglycerol that are digested and absorbed interact with the adipose tissue. However, lipid emulsions are prepared structurally similar to chylomicrons using phospholipids emulsifiers coating a TG core (Carpentier & Dupont, 2000; Raman et al., 2017). Most of the studies that have investigated the mechanistic effects of lipid emulsions on inflammation, apoptosis and reactive oxygen species production have used cell culture model (direct exposure) (Yan et al., 2017; Yan et al., 2016; Yan et al., 2018). Similarly, studies that have investigated the effects of BM on adipogenic differentiation have used a cell culture model (Fujisawa et al., 2013; Lyle et al., 1998; Vaidya & Cheema, 2018). In this study, we used a cell culture model to investigate the mechanistic effects of lipid emulsions and BM on adipogenesis. We have used 3T3-L1 adipocytes in this study because the mechanisms of adipogenesis are very well characterized in this cell line (Li et al., 2017). However, results from this research will be interpreted with caution regarding physiological application to the human context.

2.2 Rationale and hypotheses

The rate of obesity in the province of Newfoundland and Labrador is 35.2 %, which is higher than any other province in Canada (Navaneelan & Janz, 2014). Due to the nutritional value of BM, it is highly encouraged to breastfeed infants during their first few months of life. However, for a preterm infant that depends on TPN, the lipid emulsion is an important component to provide EFA. Intralipid, SMOFlipid, and Omegaven are the lipid emulsions for TPN and contains n-6:n-3 PUFA of 7.8:1, 2.7:1, and 0.2:1, respectively. Adipose stem cells differentiate into adipose lineage during prenatal and early postnatal life (Fu et al., 2014; Panina et al., 2018), and the adipocytes formed during this period remain through life (Spalding et al., 2008). During this period, nutritional factors such as hormones, fatty acids, and cytokines can influence adipose tissue development and fat storage by inducing the differentiation of preadipocytes to lipid-laden adipocytes. The amount and type of food provided to an infant has a critical role in its growth, development, and metabolic regulation. Thus, lipid emulsions and BM containing specific fatty acids and other bioactive compounds may play a role in inducing preadipocytes to differentiate, thereby programming towards an increased risk of obesity in later life. It is therefore important to investigate the effects of lipid emulsions and BM of lean and obese women on the transcriptional regulation of adipogenic/lipogenic and lipolytic genes.

2.2 Overall Aim and specific objectives: -

The overall aim of the work presented in this thesis was to investigate the effects of lipid emulsions (Study 1), and BM from lean and obese women (Study 2) on adipogenesis and lipolysis in 3T3-L1 cells. The specific objectives and hypotheses of the study were:

Study 1: The objectives of the first study using lipid emulsions were:

i. To investigate whether treatment of 3T3-L1 preadipocytes with lipid emulsions, before differentiation, would affect adipogenesis when cells are differentiated without lipid emulsions.

ii. To investigate the effects of lipid emulsions on lipolysis and β-oxidation in mature (day 8) 3T3-L1 adipocytes differentiated in the absence of lipid emulsions and then treated with lipid emulsions.

Hypotheses:

i. Treatment of 3T3-L1 preadipocytes with Omegaven, a fish oil-based lipid emulsion, will inhibit adipogenesis, compared to SMOFlipid and Intralipid.

ii. Treatment of 3T3-L1 preadipocytes with Omegaven will increase β-oxidation and reduce lipolysis in adipocytes, compared to SMOFlipid and Intralipid.

Study 2: The objectives of the second study using BM from lean and obese women were:

i. To determine the levels of malondialdehyde and the n-6:n-3 PUFA of BM from obese and lean women.

ii. To investigate the effects of BM from lean and obese women on adipogenesis in 3T3-L1 adipocytes.

iii. To investigate the effects of BM from lean and obese women on lipolysis in 3T3-L1 adipocytes.

Hypotheses:

i. BM from obese women will have higher n-6:n-3 PUFA, compared to BM from lean women.

ii. BM with higher n-6:n-3 PUFA will have a greater effect on adipogenesis and increase lipolysis.

CHAPTER THREE

Effects of lipid emulsions used in total parenteral nutrition on adipogenesis and lipolysis in 3T3-L1 cells

A version of this chapter will be submitted for publication in Pediatric Obesity

3.0 Abstract

Intralipid, SMOFlipid, and Omegaven are the lipid emulsions available for TPN formulation and contains n-6:n-3 PUFA of 7.8:1, 2.5:1, and 0.2:1, respectively. In this study, the effects of lipid emulsions on adipogenesis, lipogenesis, lipolysis, and β-oxidation were investigated using 3T3-L1 cells. Treatment of preadipocytes with Omegaven for only 48 hours, followed by differentiation (without treatment) for eight days, showed an increase in the levels of DHA and EPA, and a decrease in TAG accumulation. Furthermore, Omegaven decreased the mRNA expression of lipogenic genes compared to control cells; however, Intralipid and SMOFlipid had no effects. Next, the effects of lipid emulsions were investigated on lipolysis and β-oxidation by differentiating preadipocytes for eight days without lipid emulsions, followed by treatment with lipid emulsions for 48 hours. Omegaven-treated cells showed higher levels of DHA and EPA, compared to SMOFlipid and Intralipid. Both SMOFlipid and Omegaven increased the mRNA expression of *Cpt1*, while Omegaven significantly decreased the mRNA expression of *Atgl* and *Hsl* compared to control cells. These findings demonstrate that Omegaven decreases lipid accumulation and increases β-oxidation in 3T3-L1 cells.

3.1 Introduction

Inadequate nutrition in an infant's first year can result in stunted growth, low intellectual capacity, and high risk of developing metabolic disorders in adulthood (Beal et al., 2018; Vohr et al., 2017). Exclusive breastfeeding is highly encouraged during the infant's first six months of life, due to the numerous beneficial effects since it contains the ideal nutrient composition required for infant growth (Bernt & Walker, 1999; Pérez-Escamilla et al., 2019). However, for preterm infants (<37 weeks gestational age), the gastrointestinal tract is not well developed; as such, these infants cannot be fed enterally (Henderickx et al., 2019). Several complications for preterm infants include developmental delay, neurological immaturity, and the threat of developing necrotizing enterocolitis (Baranowski & Claud, 2019; Jin et al., 2019). TPN became a lifesaving therapy for these preterm infants to provide required nutrients for their survival (Moyses et al., 2013; Nandivada et al., 2013; Nandivadae et al., 2013). The early TPN formulation was fat-free, which resulted in infants being deficient in EFA, this led to the introduction of lipid emulsions in TPN formulation to supply fat requirements for infants sustained on intravenous feeding (Anez-Bustillos et al., 2016). Lipid emulsions provide the necessary EFA for infants to avoid EFAD. LA and ALA are the two EFA because these cannot be synthesized *de novo;* once consumed, these fatty acids serve as precursors for the synthesis of other longer chain PUFAs of the n-6 (AA) and n-3 (EPA and DHA) series, respectively (Guo et al., 2014), through elongation and desaturation process. AA, EPA, and DHA are important fatty acids as they play a key role in vision, immune function, inflammatory response, growth regulation, and cognitive development in the newborn (Martin et al., 2016; Parolini, 2019; Tallima & El Ridi, 2018). Therefore, the fatty acid composition of lipid emulsions in TPN is crucial. The oils used in lipid emulsion are major sources of TAG, and each oil has a unique fatty acid composition (Raman et al., 2017). Intralipid (20 mg/ml soybean oil)

contains 50 % of LA, 25 % oleic acid, and 10 % of ALA, thus providing a sufficient amount of EFA to avoid EFAD (Vanek et al., 2012). However, Intralipid has very high levels of n-6 PUFA, low n-3 PUFA, and high n-6:n-3 PUFA of 7.8:1. SMOFlipid (20 g/100 ml) contains 30 % soybean oil, 30 % medium-chain triglycerides, 25 % olive oil, and 15 % fish oil (Fell et al., 2015). SMOFlipid provides EFA from soybean oil, long-chain n-3 PUFA (EPA and DHA) from fish oil, and MUFA from olive oil, with an n-6:n-3 PUFA of 2.5:1 (Donoghue et al., 2018). Omegaven, on the other hand, is a pure fish oil-based lipid emulsion, containing high levels of n-3 PUFA (EPA and DHA), with an n-6:n-3 of 0.2:1 (Fell et al., 2015).

A high n-6:n-3 PUFA has been shown to increase adipogenesis in BM (Vaidya & Cheema, 2018), and is associated with obesity (Simopoulos, 2016), while lower n-6:n-3 PUFA decreases adipogenesis (Rudolph et al., 2018). Both n-6 and n-3 PUFA can act as ligands for transcription factors that are involved in adipogenesis (Riera-Heredia et al., 2019). PPARγ, a member of the nuclear-receptor superfamily, has been shown to act as the master regulator of adipogenesis (Shao et al., 2016). EPA downregulates the mRNA expression of PPARγ (Lee et al., 2008), while DHA inhibits adipocyte differentiation (Kim et al., 2006). LXR and PPAR form heterodimers with RXR to regulate lipid metabolism by activating SREBP1c expression (Xu et al., 2018; Yoshikawa et al., 2001). PUFA also suppress SREBP1c gene expression by inhibiting LXR binding to LXR response element (Yoshikawa et al., 2002). N-3 PUFA decreased SREBP1c protein expression in 3T3-L1 adipocytes (Wójcik et al., 2014) and mRNA expression in mice (Arai et al., 2009), while n-6 PUFA has an opposite effect of regulating SREBP1c gene expression (Muhlhausler et al., 2010). SREBP1c and LXRα are the transcription factors that regulate the key lipogenic genes that express ACC1, FAS, SCD1, and DGAT2 (Dentin et al., 2004). EPA (n-3) downregulates the mRNA expression of *Acc1* (Lee et al., 2008), while AA (n-6) upregulated the mRNA expression of *Acc1*

in 3T3-L1 adipocytes (Vaidya & Cheema, 2015). N-3 PUFA also downregulates FAS (Wójcik et al., 2014) and SCD1 mRNA expression (Barber et al., 2013).

In obesity, basal lipolysis from adipocytes is elevated and is closely associated with insulin resistance (Morigny et al., 2016). Thus, inhibition of adipocyte lipolysis is a promising therapy to reduce the levels of circulating FFA, thereby decreasing ectopic fat deposition (Samuel & Shulman, 2012). The anti-obesity effect of n-3 PUFA is linked to its ability to ameliorate insulin resistance and increase β-oxidation. Studies have reported that the lipid-lowering effect of n-3 PUFA in adipocytes is due to increased lipolysis and β-oxidation (Kim et al., 2006; Lee et al., 2008). In contrast, others have reported no effect of EPA and DHA on lipolysis (Prostek et al., 2014). ATGL is the rate-limiting enzyme in the lipolytic pathway (Zimmermann et al., 2004); it hydrolyzes TAG into DAG, which is then hydrolyzed by HSL into MAG (Haemmerle et al., 2002). Previous studies have reported that n-3 PUFA increased the mRNA expression of ATGL (Prostek et al., 2016), while others have reported that EPA decreased the mRNA expression of *Hsl* in 3T3-L1 adipocytes (Manickam et al., 2010)

Previously, our laboratory has shown that BM of women from Newfoundland and Labrador with higher n-6:n-3 PUFA induced greater differentiation of preadipocytes to mature adipocytes, leading to increased fat accumulation in 3T3-L1 cells (Vaidya & Cheema, 2018). Intralipid, SMOFlipid, and Omegaven contain n-6 to n-3 PUFA ratios of 7.8:1, 2.7:1, and 0.2:1, respectively. However, it is not known whether treatment with these lipid emulsions affects adipogenesis and lipogenesis in 3T3-L1 cells. I investigated the effects of lipid emulsions with low and high n-6:n-3 PUFA on adipogenesis, lipogenesis, and lipolysis in 3T3-L1 cells. I hypothesized that preadipocytes treated with Omegaven, prior to differentiation, will inhibit adipogenesis, compared

to SMOFlipid and Intralipid, by downregulating the mRNA expression of genes involved in adipogenesis and lipogenesis. Furthermore, I hypothesized that Omegaven would inhibit lipolysis and increase β-oxidation in 3T3-L1 mature adipocytes, compared to SMOFlipid and Intralipid.

3.2 Materials and Methods

3.2.1 Lipid emulsions

Intralipid 20 % (w/v), SMOFlipid 20 % (w/v), and Omegaven^{TM} 10% (w/v) were obtained from our collaborator, Renu Gill, Manager, Janeway Children's Hospital Pharmacy. The lipid emulsions obtained were stored under sterile conditions at 4^{o}C until used.

3.2.2 Fatty acid analysis of lipid emulsions

Total lipids were extracted from the lipid emulsions using the method of Folch *et al.* (Folch et al., 1957). Chloroform:methanol (2:1 v/v) and 300 µl of 50 mM NaCl was added to 500 µl of Intralipid, SMOFlipid, and Omegaven. Samples were vortexed and stored overnight. Samples were then centrifuged at 2,000 x g for 4 minutes, and the lower layer was transferred to a transmethylation vial (5 mL V-Vial CLR). Samples were then evaporated under nitrogen gas, and 10 µl of 10 mg/ml heptadecanoate (C17:0) in methanol was added to each sample as an internal standard. Lipids were transmethylated by adding 2 ml of transmethylation reagent (94 % methanol and 6 % of 98 % concentrated sulfuric acid), and approximately 20 mg of hydroquinone was added to prevent oxidation; samples were incubated at 65^{o}C for 2 hours (Arvidson & Olivecrona, 1962). The organic fatty acid methyl esters (FAME) were extracted twice using 1.5 ml of hexane; the extract was washed with 1.5 ml of water, and samples were placed at -20^{o}C overnight. The hexane layer was carefully decanted into a new tube, dried under nitrogen gas (N_2), and re-suspended in 50 µl of 99 % pure carbon disulfide. The fatty acid composition was analyzed using gas chromatography (GC)

(Chechi et al., 2010). Samples were run on an Omegawax X 320 (30 m X 0.32 mm) column from Supelco (Sigma-Aldrich, Canada) for 45 minutes using a flame ionization detector. The parameters of the GC were set as oven temperature, 200ºC; injector, 240ºC; and detector 260ºC. PUFA-2 and 3 (Sigma-Aldrich, Canada) were used as standards for the identification of fatty acids by retention time, and the concentration of each fatty acid was calculated using the internal standard.

3.2.3 3T3-L1 cell culture

3.2.3.1 Materials

3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC, # CL-173, USA). Dulbecco's Modified Eagles Medium (DMEM), sodium pyruvate (100 mM), newborn calf serum (NBCS) and fetal bovine serum (FBS) were obtained from Gibco Life Technologies (Ontario, Canada). Insulin solution (10 mg/ml in 25 mM HEPES, pH 8.2), IBMX and Dex were obtained from Sigma-Aldrich (Ontario, Canada).

3.2.3.2 Cell metabolic activity (MTT assay)

A dose-response experiment was conducted to determine the dose of lipid emulsions to be used to treat 3T3-L1 preadipocytes. Preadipocytes (3T3-L1) were seeded into 96 well microplates at a density of 1 x 10^4 cells per well and incubated for 24 hours. The media were replaced with 200 µL of DMEM + 10 % NBCS with different concentrations of Intralipid, SMOFlipid, and Omegaven (0.2 %, 0.4 %, 1 % and 2 % v/v), and cells were incubated for 48 hours. Control cells received media only. The cell metabolic activity was measured after 48 hours of treatment using the MTT colorimetric assay (Fu et al., 2009). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) was dissolved in 1X phosphate-buffered saline (PBS) at a concentration of 5 mg/ml and 10 % MTT solution in fresh culture media (DMEM + 10 % NBCS)

was prepared. MTT media (200µl) was added to each well, and the plates were incubated for 4 hours in a 37ºC, 5 % CO_2 incubator. Afterward, the media were aspirated, and the formazan crystals were dissolved in 200 µl of dimethyl sulfoxide (DMSO) for 30 minutes at 37ºC. The absorbance of each well was read at 570 nm using a spectrophotometer. The cell metabolic activity was expressed as percentage fold change from control as stated below:

$$\text{Cell metabolic activity (\%)} = [A_{570}\,(\text{sample})/A_{570}\,(\text{control})] \times 100\ \%$$

There was no effect of any of the tested concentrations of all lipid emulsions on cell metabolic activity (data shown in the Results section 3.3.1; Figure 3.3). A concentration of 1% (v/v) was selected based on the MTT results, and the previous studies (Yan et al., 2018; Yan et al., 2017; J. Yan et al., 2018).

3.2.3.3 Treatment of 3T3-L1 preadipocytes with lipid emulsions

The first question was to investigate whether the treatment of 3T3-L1 preadipocytes with lipid emulsions (prior to the induction of adipogenesis) will have an effect on adipogenesis. The 3T3-L1 preadipocytes were cultured in DMEM containing 10 % NBCS in a 5 % CO_2, humidified environment at 37ºC. At 70-80 % confluence, media were changed to DMEM + 1 % NBCS, and cells were serum-starved for 12 hours (Prostek et al., 2014). The media were replaced with DMEM containing 1 % NBCS with or without 1 % (v/v) Intralipid, SMOFlipid, and Omegaven, and then incubated for either 24 or 48 hours. Cells were harvested at 24 hours, and at 48 hours to extract lipids for fatty acid analysis, and total RNA was extracted to measure gene expression.

In an independent set of experiments, after 48 hours of treatment with lipid emulsions, the lipid emulsions were removed, and cells were continued to differentiate for 8 days in the absence

of lipid emulsions. This experimental design allowed us to investigate whether the treatment of preadipocytes with specific lipid emulsions will influence the accumulation of fat during differentiation into lipid-laden adipocytes. After treatment of preadipocytes for 48 hours with lipid emulsions, the cells were washed three times with PBS, and fresh media containing DMEM + 10 % FBS, insulin (10 µg/mL), IBMX (0.5 mM) and dex (1 µM) was added (Day 0) without lipid emulsions. After 48 hours (day 2), the media were changed to DMEM containing 10% FBS and 10 µl/mL insulin (no lipid emulsions). Media were replaced with DMEM + 10% FBS on day 4 and day 6 without lipid emulsions until day 8 when the cells were fully differentiated. Cells were harvested on day 8, lipids were extracted for TAG assay and fatty acid analysis; total RNA was extracted to measure gene expression.

3.2.3.4 Treatment of 3T3-L1 mature adipocytes with lipid emulsions to investigate the effect on lipolysis

To investigate whether lipid emulsions will induce lipolysis in mature adipocytes, 3T3-L1 preadipocytes were cultured in DMEM containing 10 % NBCS in a 5 % CO_2, humidified environment at 37ºC. Two days post confluency, cells were induced to differentiate (day 0), as described in section 3.2.3.2, without lipid emulsions for 8 days. Cells were then serum-starved for 12 hours by changing media to DMEM + 1 % FBS. Cells were treated with media containing 1 % Intralipid, SMOFlipid and Omegaven, and incubated for 48 hours. Control cells received no treatment. Cells were washed with 1X PBS and harvested to extract lipids for fatty acid analysis and total RNA for gene expression.

3.2.3.5 Oil Red O staining

Lipid accumulation in 3T3-L1 adipocytes was measured using an Oil Red O solution in 0.5 % isopropanol (Millipore, Ontario, Canada). After day 8 of differentiation, cells were washed thrice with 1X PBS, and 1 ml of Oil Red O dye was added and incubated for 15 min at room temperature. The dye was then removed, and the cells were washed with 2 ml of distilled water to remove the non-binding dye. Oil Red O-stained adipocytes were viewed using a Leica DMIL LED Tissue Culture Microscope, and images were taken using Infinity Camera Analyze Software at 400x magnification. Oil Red O-stained lipids were eluted by adding 500 µl of 100 % isopropanol to each well, and the plate was set on a shaker for 30 min. The absorbance of extracted dye was measured using a spectrophotometer at 520 nm, and isopropanol was used as a blank.

3.2.4 Fatty acid analysis and triacylglycerol assay

To measure the fatty acid composition and TAG accumulation, 3T3-L1 adipocytes at day 8 of differentiation were washed twice in 1X PBS and then scrapped in 3 ml of 1X PBS. Lipids were extracted using the Folch extraction method (Folch et al., 1957). The lipid fraction was dried under liquid nitrogen and was transmethylated for fatty acid analysis using GC, or reconstituted in 100 µl of isopropanol, and the levels of TAG were measured using a triglyceride assay kit #236-60 (Sekisui Diagnostic chemical Ltd., USA).

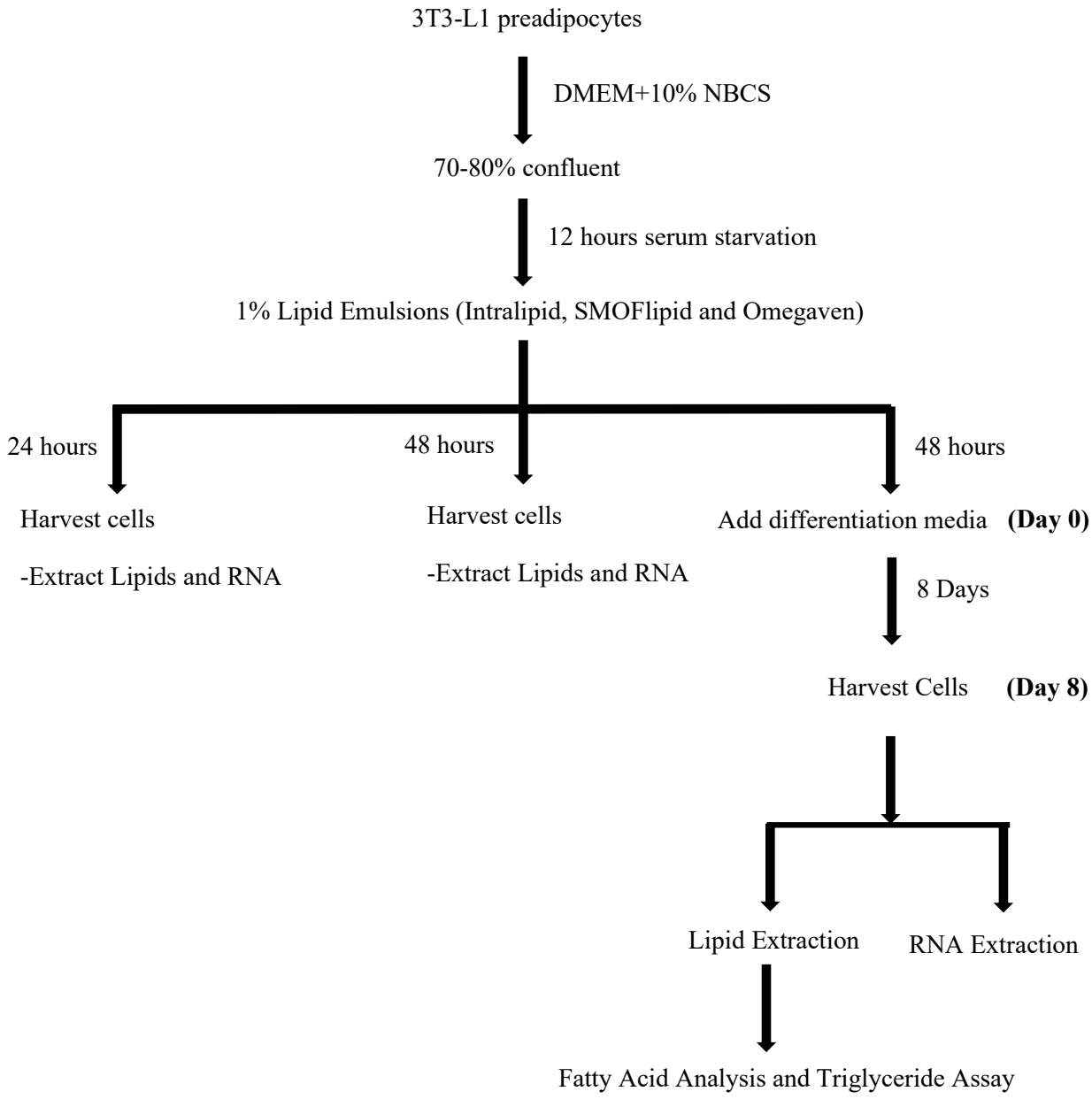

Figure 3.1 Experimental design to investigate the effects of lipid emulsions on adipogenesis in 3T3-L1 adipocytes

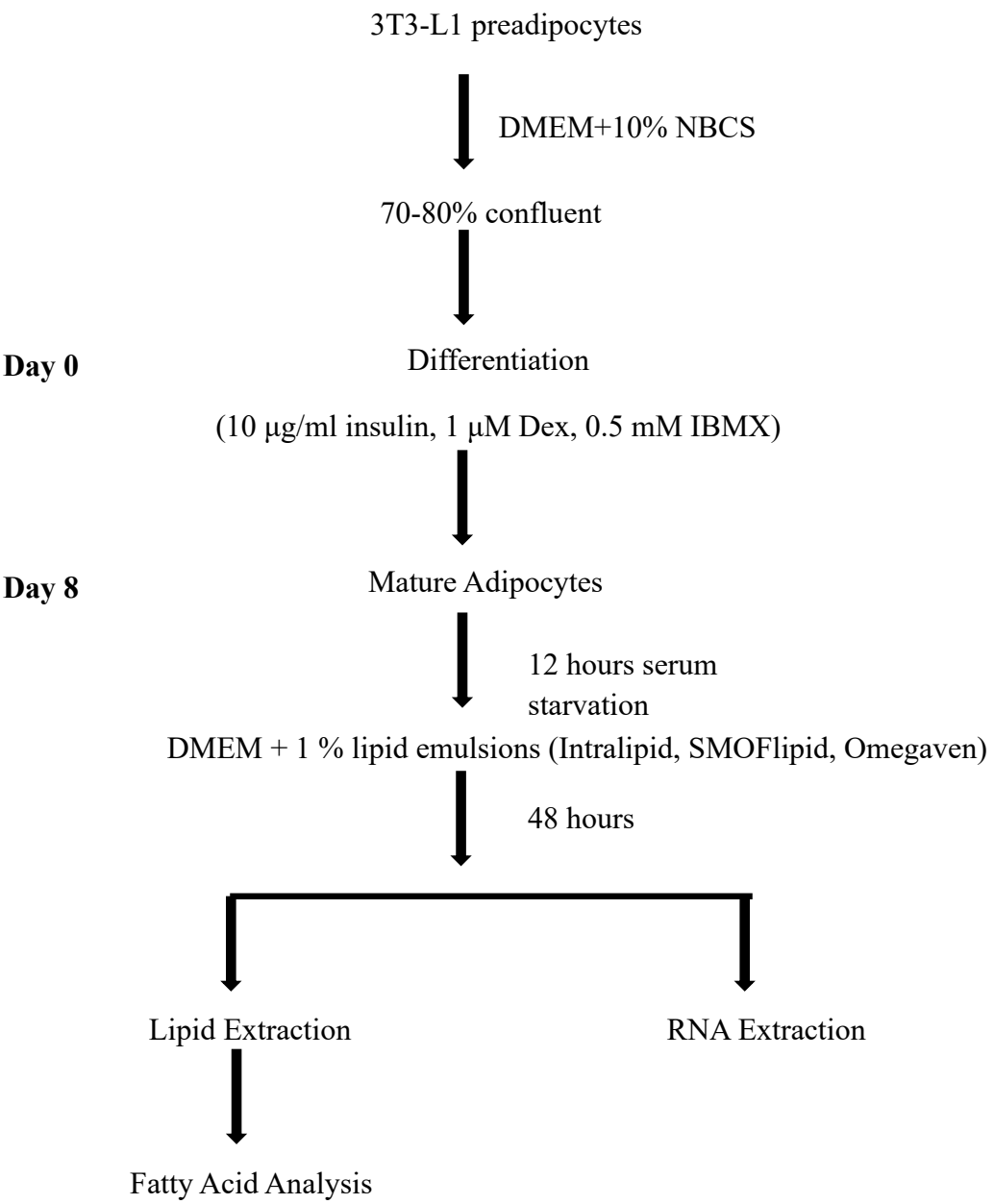

Figure 3.2 Experimental design to investigate the effects of lipid emulsions on lipolysis in 3T3-L1 mature adipocytes.

3.2.5 Total RNA isolation

Total RNA was extracted from 3T3-L1 cells using TRIzol (Invitrogen, Carlsbad, CA, USA) (Chomczynski & Sacchi, 1987) and treated with DNase (Promega, Wisconsin, USA) to remove genomic DNA contamination. The concentration and purity (260/280) of the RNA was measured using a Nanodrop 2000 (Thermo Scientific, USA), and RNA integrity was checked using 1.2 % agarose gel. Synthesis of cDNA from 1 µg of total RNA was carried out using a reverse transcription kit (#A3500, Promega, Wisconsin, USA). Master Mix was prepared by mixing appropriate volumes of 25 mM $MgCl_2$, 10X reverse transcription buffer, 10 mM dNTP mixture, 0.5 µl Recombinant RNasin ribonuclease inhibitor, 15 u AMV reverse transcriptase and random primers (0.5 µg/ml), added to the DNase free RNA and incubated at room temperature for 10 min. Samples were placed in Eppendorf PCR machine with appropriate conditions set for cDNA synthesis (45^0C for 15 minutes. 95^0C for 5 minutes and 5^0C), and cDNA was then stored at -20ºC.

3.2.6 Real-time quantitative polymerase chain reaction

Primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and obtained from IDT Technologies (Coralville, IA, USA); primer sequences are given in Table 3.1. Amplification was performed using iQ SYBR Green Supermix (#1708880, Bio-Rad, USA) with a reaction volume of 10 µl and 50 ng cDNA per reaction. Samples were run for 40 cycles (denaturation at 95ºC for 15mins, annealing at 58-60ºC for 15 seconds and extension at 72ºC for 15 seconds) using the CFX96™ Real-Time System. Data were analyzed using the CFX Manager ™ Software Version 3.0. The delta Ct values for each gene of interest were recorded, and the related expression was normalized against RPLPO (a large ribosomal protein) as the housekeeping gene. The expression levels were measured using the ΔΔCt method (Livak & Schmittgen, 2001). The efficiency of all primers was within the acceptable range of 90-110%.

3.2.7 Statistical Analysis.

Data were analyzed using GraphPad Prism Software (version 6). Gene expression data and fatty acids analysis at 24 and 48 hours were carried out using two-way ANOVA to determine the effect of treatment and time followed by Tukey posthoc test if a significant interaction was observed. Gene expression and fatty acid analysis at day 8 of differentiation were carried out using one-way ANOVA and Tukey post hoc. Results were presented as mean ± standard deviation (SD), n=3, and each experiment was repeated twice. $P<0.05$ was considered significant.

Table 3.1 Primer sequence for qPCR

Gene	Sequence		Ascension No
Acc1	5'-GGCCAGTGCTATGCTGAGAT-3'	Forward	XM_006531957.3
	5'-AGGGTCAAGTGCTGCTCCA-3'	Reverse	
Atgl	5'-GGTTCAGTAGGCCATTCCTC-3'	Forward	NR_028142.1
	5-GGTTCAGTAGGCCATTCCTC-3'	Reverse	
Cpt1	5'-CCAGGCTACAGTGGGACATT-3'	Forward	XM_006531658.3
	5'-GAACTTGCCCATGTCCTTGT-3'	Reverse	
Dgat2	5'-CTGCTGTTGGCTGGTTTCAC-3'	Forward	NM_026384.3
	5'-CAGGAGGATATGCGCCAGAG-3'	Reverse	
Fasn	5'-CTGCGGAAACTTCAGGAAATG-3'	Forward	NM_007988.3
	5'-GGTTCGGAATGCTATCCAGG-3'	Reverse	
Fabp4	5'-CATAACCCTAGATGGCGGGG-3'	Forward	NM_024406.2
	5'-CCAGCTTGTCACCATCTCGT-3'	Reverse	
Hsl	5'-AGACACCAGCCAACGGAT-3'	Forward	XM_006539572.3
	5'-GGGCATAGTAGGCCATAGCA-3'	Reverse	
LXRα	5'-GCTCTGCTCATTGCCATCAG-3'	Forward	XM_006499168.3
	5'-TGTTGCAGCCTCTCTACTTGGA-3'	Reverse	
Pparg	5'-GAGCTGACCCAATGGTTGCTG-3'	Forward	XM_017321456.1
	5'-GCTTCAATCGGATGGTTCTTC-3'	Reverse	
Pref-1	5'-TTCGGGCTTGCACCTCAA-3'	Forward	XM_006515457.3
	5'-GGAGCATTCGTACTGGCCTTT-3'	Reverse	
Rplp0	5'-AATTTCAATGGTGCCTCTGG-3'	Forward	NM_007475.5
	5'-TCACTGTGCCAGCTCAGAAC-3'	Reverse	
Scd1	5'-CACCTGCCTCTTCGGGATTT-3'	Forward	NM_009127.4
	5'-CTTGACAGCCGGGTGTTTG-3'	Reverse	
Srebp1c	5'-CGGCTCTGGAACAGACACTG-3'	Forward	NM_001313979.1
	5'-TGAGCTGGAGCATGTCTTCG-3'	Reverse	

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

Acc1, acetyl-CoA carboxylase; Atgl, Adipose triglyceride lipase; Cpt1, carnitine palmitoyltransferase I; Dgat2, Diacylglycerolacyl transferase; Fabp4, fatty acid-binding protein; Fasn, fatty acid synthase; Hsl, Hormone-sensitive lipase; *LXRα, liver X receptor alpha; Pparg, peroxisome proliferator-activated receptor-gamma; Pref-1, preadipocyte factor 1; Scd1, stearoyl-CoA desaturase; Srebp1c, sterol regulatory element-binding protein 1; Rplp0, ribosomal protein large.*

Table 3.2 Fatty acid composition of lipid emulsions

FA (% w/w)	Intralipid	SMOFlipid	Omegaven
C14:0	0.11 ± 0.01^{c}	1.65 ± 0.04^{b}	5.06 ± 0.17^{a}
C16:0	14.65 ± 0.47^{b}	16.08 ± 4.04^{a}	15.75 ± 0.08^{ab}
C16:1n7	0.43 ± 0.02^{c}	2.57 ± 0.04^{b}	8.95 ± 0.20^{a}
C18:0	5.42 ± 0.35	5.58 ± 3.04	5.38 ± 0.17
C18:1	22.61 ± 0.06^{b}	36.77 ± 0.04^{a}	16.92 ± 0.02^{c}
C18:2n6	49.12 ± 1.00^{a}	25.63 ± 0.04^{b}	5.72 ± 0.18^{c}
C18:3n3	5.47 ± 0.14^{a}	2.49 ± 1.04^{b}	0.87 ± 0.01^{c}
C18:4n3	0.00 ± 0.00	0.54 ± 0.04^{b}	3.38 ± 0.04^{b}
C20:1n9	0.22 ± 0.01^{c}	0.38 ± 0.04^{b}	0.69 ± 0.04^{a}
C20:4n6	1.04 ± 0.15^{b}	1.29 ± 1.04^{b}	2.57 ± 0.08^{a}
C20:4n3	ND	0.07 ± 0.04	ND
C20:5n3	ND	3.55 ± 0.04^{b}	18.74 ± 0.26^{a}
C22:4n6	ND	0.10 ± 0.04^{b}	0.18 ± 0.01^{a}
C22:5n3	0.07 ± 0.01^{c}	0.43 ± 0.04^{b}	1.47 ± 0.02^{a}
C22:6n3	0.86 ± 0.12^{c}	2.92 ± 1.04^{b}	14.32 ± 0.29^{a}
∑ SFA	20.18 ± 0.83^{c}	23.31 ± 0.69^{b}	26.16 ± 0.08^{a}
∑ MUFA	23.26 ± 0.07^{c}	39.72 ± 0.39^{a}	26.91 ± 0.25^{b}
∑ n-3 PUFA	6.40 ± 0.01^{c}	9.94 ± 0.9^{b}	38.84 ± 0.06^{a}
∑ n-6 PUFA	50.16 ± 0.85^{a}	27.03 ± 0.26^{b}	8.12 ± 0.26^{c}
n-6/n-3 ratio	7.84 ± 0.12^{a}	2.72 ± 0.2^{b}	0.219 ± 0.01^{c}

*****Total lipids were extracted, and fatty acids were prepared as described in section 3.2.2. Data are expressed as the weight percentage of the total extracted fatty acids, values are expressed as mean ± SD, n = 3. Data were analyzed using one-way ANOVA after arcsine transformation and superscripts within a row (a,b,c) was used to denote a significant difference between the lipid emulsions. FA = fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ND = Not detected, ΣPUFA = sum of polyunsaturated fatty acids, ΣSFA = sum of saturated fatty acids, Σn-6 = sum of omega-6 PUFA, Σn-3 = sum of omega-3 PUFA.*

3.3 Results

3.3.1 Effects of lipid emulsions on cell metabolic activity of 3T3-L1 preadipocytes

The cell metabolic activity was used to determine the dose at which there is no toxic effect on the metabolic activity of the cells after the treatment of 3T3-L1 preadipocytes with lipid emulsions. The cell metabolic activity of 3T3-L1 preadipocytes treated with 0.2 %, 0.4 %, 1 %, and 2 % (v/v) of lipid emulsions for 48 hours was not statistically different from untreated cells (Fig 3.3A, Fig 3.3B, and Fig 3.3C).

3.3.2 Effects of lipid emulsions on fatty acid composition and markers of adipogenesis in 3T3-L1 preadipocyte

3.3.2.1 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 24 and 48 hours, and the effect on the fatty acid composition

Treatment with all lipid emulsions significantly decreased the levels of C14:0, C16:0, and C18:0 at 24 hours ($p<0.001$), and at 48 hours ($p<0.01$), compared to the control cells (Table 3.3). There was a significant effect of treatment ($p<0.001$) and time ($p<0.05$) on the levels of total SFA (Table 3.3). Omegaven-treated cells had lower total SFA, compared to control cells at both 24 and 48 hours of treatment; however, there was no difference in SFA levels between the lipid emulsions at both 24 and 48 hours of treatment. There was a significant effect of treatment ($p<0.001$), and time ($p<0.001$) on C16:1n7, and there was also an interactive effect ($p<0.01$) (Table 3.4). There was no difference in C16:1n7 between all the treatment groups at 24 hours; however, all lipid emulsions decreased C16:1n7 at 48 hours of treatment, compared to control cells (Table 3.4). There was no difference in C18:1 between Omegaven treated cells and control cells at 24 hours; however, Omegaven-treated cells had higher C18:1 at 48 hours, compared to control cells.

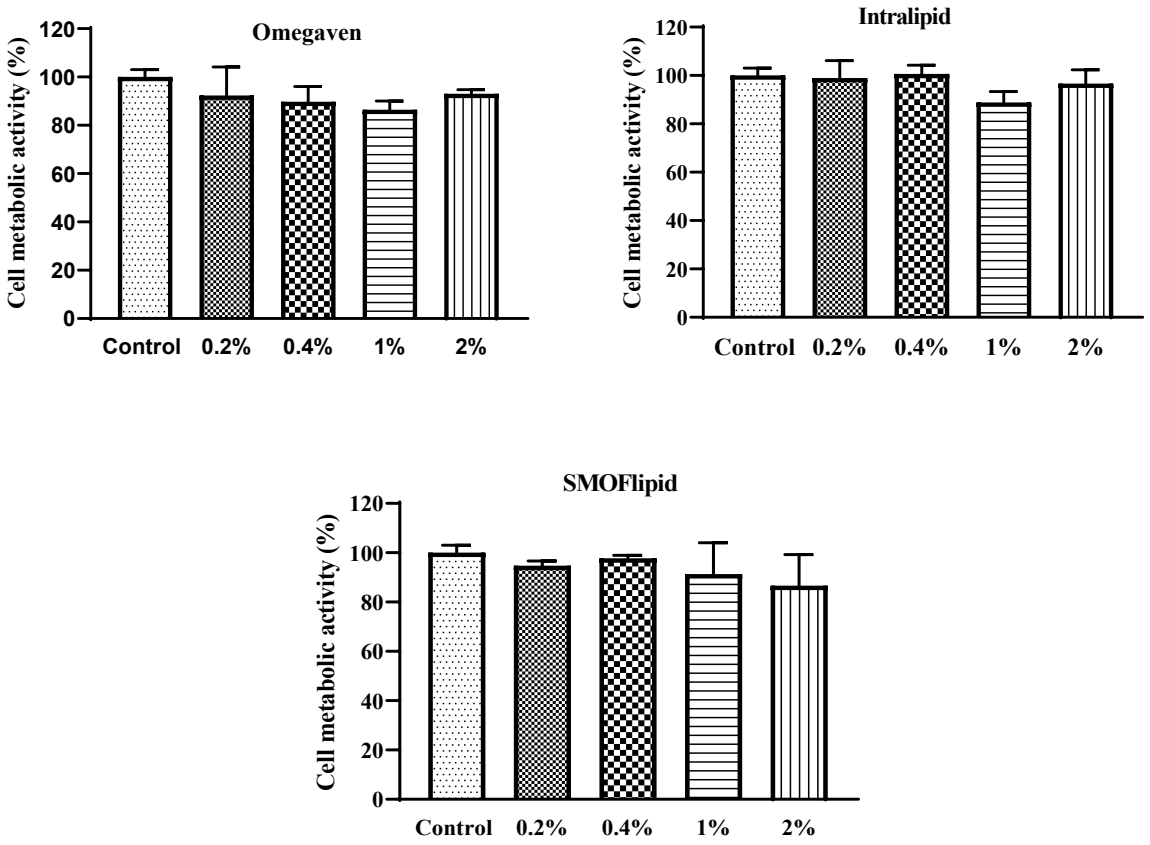

Figure 3.3 Effect of various concentrations of lipid emulsions on cell metabolic activity of 3T3-L1 preadipocytes

The cell metabolic activity of 3T3-L1 preadipocytes was measured using MTT assay after treatment with various concentrations of lipid emulsions, as explained in the methods section 3.2.3.4. (A) Intralipid (B) SMOFlipid (C) Omegaven. Data were analyzed using one-way ANOVA to determine significance ($P<0.05$). Cell metabolic activity was calculated by comparing treatment groups to control cells (No LE). Values are expressed as means ± SD, n=3

SMOFlipid had higher C18:1 at 24 hours of treatment, compared to the control cells, and to other lipid emulsions treatment (Table 3.4). Similarly, SMOFlipid-treated cells had higher levels of C18:1, compared to control cells and Intralipid, but did not difference change compared to Omegaven (Table 3.4). There was a significant effect of treatment (Table 3.4; $p<0.05$) on C20:1n9 levels. The levels of C20:1n9 did not differ between cells treated with Omegaven and control cells at both 24 and 48 hours of treatment. There was no difference change between the levels of C20:19 between the cells treated with Omegaven compared to SMOFlipid and Intralipid treatment at both 24 and 48 hours of treatment (Table 3.4). There was a significant effect of treatment ($p<0.0001$) on the total MUFA levels. Omegaven-treated cells did not affect total MUFA levels compared to control cells at both 24 and 48 hours of treatment. SMOFlipid treatment significantly ($p<0.0001$) increased total MUFA levels at 24 hours of treatment compared to control cells and Intralipid cells, but the levels were not significantly different compared to Omegaven at both 24 and 48 hours (Table 3.4).

Treatment ($p<0.0001$) and time ($p<0.05$) had a significant effect on the levels of LA (C18:2n6) (Table 3.5). Omegaven-treated cells had higher LA levels compared to control cells at both 24 and 48 hours of treatment; however, the levels were lower compared to SMOFlipid- and Intralipid-treated cells at both 24 and 48 hours (Table 3.5). ALA (C18:3n3) was only detected in cells treated with Intralipid at both 24 and 48 hours and was not detected in Omegaven, control, and SMOFlipid treated cells. There was a significant effect of treatment ($p<0.0001$) and time ($p<0.01$) on the levels of AA (C20:4n6) (Table 3.5). Treatment with all lipid emulsions showed an increase in AA levels, compared to the control cells at both 24 and 48 hours (Table 3.5). Omegaven-treated cells had a higher total n-6 PUFA compared to control cells at both 24 and 48 hours of treatment; however, the total n-6 PUFA of Omegaven-treated cells was lower compared to

SMOFlipid and Intralipid at both 24 and 48 hours of treatment (Table 3.5). The delta-6 desaturase (D6D) index, which is the ratio of LA to AA (Mohammadzadeh et al., 2014) was calculated at both 24 and 48 hours of treatment. There was a significant effect of treatment ($p<0.0001$) on D6D activity. Omegaven treated cells had higher D6D compared to control cells at 24 hours; however, there was no difference at 48 hours of treatment (Figure 3.4A). The activity index of Omegaven-treated cells was lower compared to SMOFlipid and Intralipid at both 24 and 48 hours (Fig 3.4A); this indicates lower conversion of LA to AA in Omegaven-treated cells compared to other lipid emulsions.

There was a significant effect of treatment ($p<0.0001$) and time ($p<0.05$) on EPA and DHA levels. Omegaven treated cells had higher EPA and DHA incorporation compared to control cells at both 24 and 48 hours of treatment (Table 3.5). Omegaven-treated cells had similar EPA levels compared to SMOFlipid-treated cells, but the levels were higher compared to Intralipid at 24 hours. However, the levels of EPA were higher in Omegaven-treated cells at 48 hours compared to both SMOFlipid and Intralipid (Table 3.5). Omegaven-treated cells also had higher DHA levels compared to control cells at both 24 and 48 hours of treatment (Table 3.5). Similarly, the levels of DHA in Omegaven-treated cells were higher than SMOFlipid- and Intralipid-treated cells at both 24 and 48 hours of treatment. Omegaven also had a higher total n-3 PUFA at 24 and 48 hours of treatment compared to control cells. Omegaven had higher n-3 compared to other lipid emulsions at 48 hours (Table 3.5). There was no difference in n-6:n-3 PUFA between Omegaven-treated cells and control cells at 24 and 48 hours of treatment (Table 3.5); however, at 48 hours, the n-6:n-3 ratio of Omegaven-treated cells was 1.03; while the ratios for control, SMOFlipid, and Intralipid were 1.20, 2.92, and 4.48 respectively (Table 2.5).

Table 3.3 Saturated fatty acid composition of 3T3-L1 preadipocytes treated with 1 % lipid emulsions for 24 and 48 hours

	FA (%w/w)	C14:0	C16:0	C18:0	∑SFA
24 hours	**No LE**	1.89 ± 0.27^{a}	28.0 ± 0.19^{a}	37.0 ± 1.25^{a}	67.0 ± 1.32^{a}
	Intralipid	0.99 ± 0.14^{b}	22.8 ± 1.64^{b}	25.6 ± 4.42^{b}	49.4 ± 6.09^{b}
	SMOFlipid	1.47 ± 0.57^{b}	20.3 ± 1.22^{b}	20.5 ± 1.09^{bc}	42.4 ± 2.36^{b}
	Omegaven	1.46 ± 0.68^{b}	23.0 ± 9.68^{b}	25.1 ± 8.68^{b}	49.6 ± 8.68^{b}
48 hours	**No LE**	3.02 ± 0.81^{a}	26.7 ± 0.05^{a}	27.8 ± 0.31^{b}	57.6 ± 1.07^{a}
	Intralipid	1.10 ± 0.15^{b}	22.4 ± 2.26^{b}	22.1 ± 3.10^{bc}	45.7 ± 5.50^{b}
	SMOFlipid	1.33 ± 0.28^{b}	22.0 ± 0.58^{b}	21.7 ± 0.20^{bc}	45.1 ± 0.50^{b}
	Omegaven	1.83 ± 0.49^{ab}	20.8 ± 0.86^{b}	18.2 ± 1.84^{c}	40.9 ± 2.21^{b}
Main Effect	**Trt**	$p < 0.001$	$p < 0.0001$	$p < 0.001$	$p < 0.0001$
	Time	$p < 0.05$	NS	$p < 0.01$	$p < 0.05$
	Trt x Time	NS	NS	$p < 0.05$	NS

** 3T3-L1 Preadipocytes were treated for 24 and 48 hours with 1 % lipid emulsions. Total lipids were extracted, and fatty acids were prepared as described in section 3.2.2. Data are expressed as the weight percentage of the total extracted fatty acids, values are expressed as mean ± SD, n = 3. Data were analyzed using Two-way ANOVA after arcsine transformation followed by Turkey Posthoc to determine differences when there was a significant interaction. Superscripts within a column (a,b,c) were used to denote a significant difference between the lipid emulsions. FA = fatty acid, LE = lipid emulsion, ND= Not detected, NS = Not significant, Trt = treatment, ΣSFA sum of saturated fatty acids.*

Table 3.4 Monounsaturated fatty acid composition of 3T3-L1 preadipocytes treated with 1% lipid emulsions for 24 and 48 hours

	FA (%w/w)	C16:1n7	C18:1	C20:1n9	∑ MUFA
24 hours	**No LE**	1.55 ± 0.52^{b}	22.69 ± 2.70^{bc}	0.41 ± 0.28^{ab}	24.5 ± 1.89^{bc}
	Intralipid	1.34 ± 0.06^{b}	18.94 ± 1.99^{c}	0.29 ± 0.08^{b}	20.5 ± 2.12^{c}
	SMOFlipid	2.15 ± 0.24^{b}	30.98 ± 0.45^{a}	0.45 ± 0.01^{ab}	33.5 ± 0.58^{a}
	Omegaven	1.55 ± 8.68^{b}	25.44 ± 4.90^{b}	0.46 ± 3.68^{ab}	27.4 ± 2.68^{ab}
48 hours	**No LE**	3.51 ± 0.93^{a}	23.44 ± 0.75^{b}	0.64 ± 0.05^{a}	27.5 ± 0.19^{b}
	Intralipid	1.67 ± 0.15^{b}	17.9 ± 0.89^{c}	0.30 ± 0.18^{b}	19.8 ± 1.21^{c}
	SMOFlipid	2.12 ± 0.18^{b}	28.55 ± 0.57^{a}	0.36 ± 0.22^{ab}	31.0 ± 0.31^{ab}
	Omegaven	2.07 ± 0.01^{b}	29.06 ± 0.41^{a}	0.49 ± 0.27^{ab}	31.3 ± 0.17^{ab}
Main Effect	**Trt**	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.0001$
	Time	$p < 0.001$	$p < 0.01$	NS	NS
	Trt x Time	$p < 0.01$	$p < 0.01$	NS	$p < 0.05$

Preadipocytes were treated for 24 and 48 hours with 1 % lipid emulsions. Total lipids were extracted, and fatty acids were prepared as described in section 3.2.2. Data are expressed as the weight percentage of the total extracted fatty acids; values are expressed as mean ± SD, n = 3. Data were analyzed using Two-way ANOVA after arcsine transformation followed by Turkey Posthoc to determine differences when there was a significant interaction. Superscripts within a column (a,b,c) were used to denote a significant difference between the lipid emulsions. FA = fatty acids, LE = lipid emulsion, ND = Not detected, NS = Not significant, Trt = treatment, ΣMUFA = sum of monounsaturated fatty acids

Table 3.5 PUFA composition of 3T3-L1 preadipocytes treated with 1 % (v/v) lipid emulsions for 24 and 48 hours

	FA (%w/w)	C18:2n6	C18:3n3	C20:4n6	∑ n-6 PUFA	C20:5n3	C22:5n3	C22:6n3	∑ n-3 PUFA	n-6/n-3 ratio
24 hours	**No LE**	1.59 ± 0.97^{c}	ND	3.68 ± 0.48^{c}	5.27 ± 0.56^{e}	3.42 ± 0.28^{bc}	ND	0.44 ± 0.14^{c}	3.87 ± 0.16^{c}	1.36 ± 0.11^{cd}
	Intralipid	17.1 ± 2.53^{a}	0.72±0.26	6.69 ±0.94^{ab}	21.8 ± 3.18^{b}	2.59 ± 0.40^{c}	0.57 ± 0.10^{c}	2.22 ± 0.39^{b}	6.11 ± 0.93^{b}	3.91 ± 0.27^{a}
	SMOFlipid	9.40 ± 0.55^{b}	ND	6.36 ± 0.14^{b}	15.7 ±0.62^{cd}	3.39 ± 0.14^{bc}	2.00 ± 1.28^{a}	2.82 ± 1.00^{b}	8.21 ± 1.96^{b}	1.99 ± 0.45^{c}
	Omegaven	6.97 ± 9.68^{b}	ND	6.30 ± 9.68^{b}	13.2 ± 8.68^{d}	4.76 ± 4.68^{b}	0.87 ±0.68^{bc}	3.97 ± 4.68^{b}	9.62 ± 9.68^{b}	1.37±3.68^{cd}
48 hours	**No LE**	3.30 ± 0.51^{c}	ND	4.79 ± 0.10^{c}	8.09 ± 0.40^{e}	4.14 ± 0.29^{bc}	1.26 ± 0.02^{b}	1.30 ± 0.75^{b}	6.71 ± 0.47^{b}	1.20±0.02^{cd}
	Intralipid	19.6 ± 2.94^{a}	1.09±0.11	8.36 ± 0.19^{a}	28.0 ± 2.99^{a}	2.29 ± 0.03^{c}	0.38 ± 0.33^{c}	2.98 ± 0.73^{b}	6.39 ± 1.45^{b}	4.48 ± 0.63^{a}
	SMOFlipid	10.8 ± 0.46^{b}	ND	6.92 ±0.40^{ab}	17.7 ± 0.54^{c}	3.10 ± 0.03^{c}	0.56 ±0.49^{bc}	2.41 ± 0.17^{b}	6.09 ±0.35^{bc}	2.92 ± 0.26^{b}
	Omegaven	6.73 ± 0.17^{b}	ND	7.19±0.25^{ab}	13.9 ± 0.40^{d}	7.14 ± 1.45^{a}	0.63±0.56^{bc}	6.04 ± 1.09^{a}	13.8 ± 2.65^{a}	1.03 ± 0.21^{d}
Main Effect	**Trt**	p < 0.0001	NS	p < 0.0001	p < 0.0001	p < 0.0001	NS	p < 0.0001	p < 0.0001	p < 0.0001
	Time	p < 0.05	NS	p < 0.01	p < 0.01	p < 0.05	NS	p < 0.01	p < 0.05	p < 0.05
	Trt x Time	NS	NS	NS	NS	p < 0.01	p < 0.01	NS	p < 0.01	p < 0.05

**Preadipocytes were treated for 24 hours and 48 hours with 1 % lipid emulsions. Total lipids were extracted, and fatty acids were prepared as described in section 3.2.2. Data are expressed as the weight percentage of the total extracted fatty acids. Values are expressed as mean ± SD, n = 3. Data were analyzed using Two-way ANOVA after arcsine transformation followed by Turkey Posthoc to determine differences when there was a significant interaction. Superscripts within a column (a,b,c) denoted a significant difference between the lipid emulsions. FA = fatty acids, LE = lipid emulsion, ND = Not detected, NS = Not significant, Trt = treatment, PUFA = polyunsaturated fatty acids Σn-3 = sum of omega-3 PUFA, Σn-6 = sum of omega-6 PUFA*

3.3.2.2 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 24 and 48 hours altered the mRNA expression of *Pref-1*

Pref-1 is a marker for preadipocytes, which decreases upon induction of differentiation (Hudak & Sul, 2013). There was a significant effect of treatment ($p<0.01$) and time ($p<0.0001$) on *Pref-1* mRNA expression (Fig 3.4B), and there was also an interaction between time and treatment ($p<0.01$) to reveal a higher *Pref-1* mRNA expression at 24 hours of treatment. *Pref-1* mRNA expression was significantly higher in cells treated with Omegaven, SMOFlipid, and Intralipid compared to control cells at 24 hours (Fig 3.4B); however, there was no significant difference between cells treated with SMOFlipid and Omegaven. Interestingly, the mRNA expression of *Pref-1* decreased at 48 hours after treatment with Omegaven compared to the control cells (Fig 3.4B); moreover, *Pref-1* levels of Omegaven-treated cells were lower compared to SMOFlipid and Intralipid-treated cells.

3.3.2.3 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 24 and 48 hours had no effect on the mRNA expression of *Pparg* and *Srebp1c*

I investigated if the treatment of 3T3-L1 preadipocytes with lipid emulsions for 24 or 48 hours will affect the regulation of adipogenic and lipogenic genes. However, we were not able to detect the mRNA expression of *Pparg* and *Srebp1c* at 24 and 48 hours in all treatment groups.

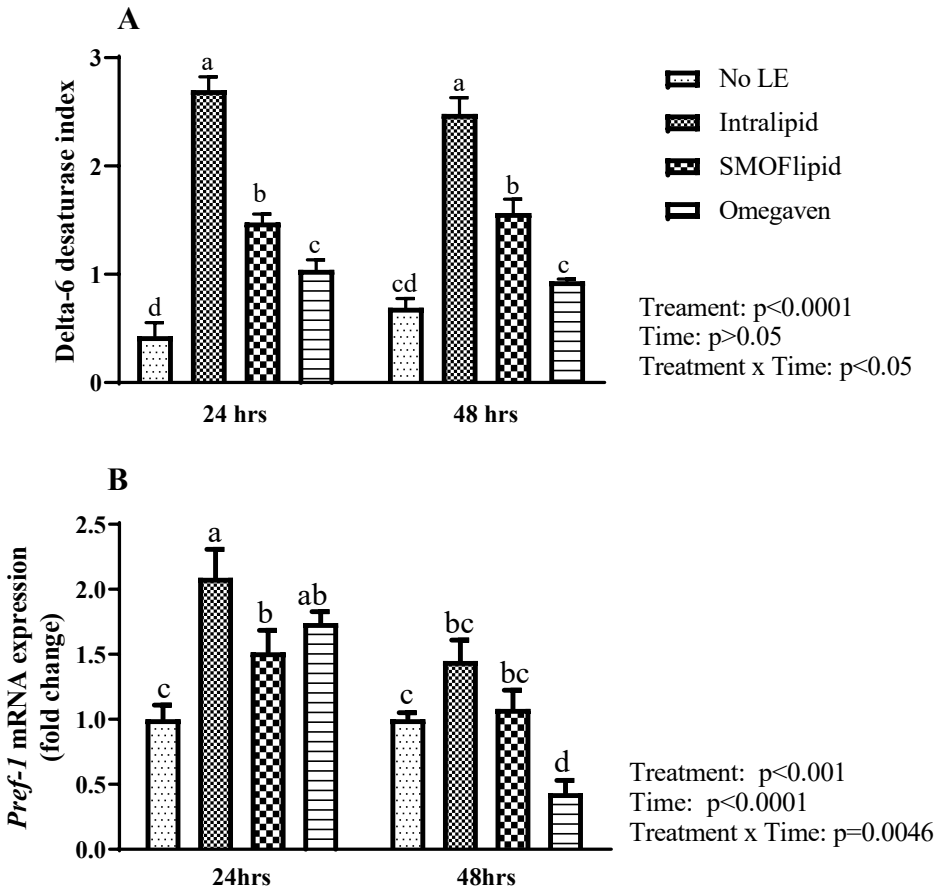

Figure 2.4 Effects of lipid emulsions on the Delta-6 desaturase index and the mRNA expression of Pref-1 in 3T3-L1 preadipocytes treated for 24 and 48 hours

Preadipocytes were treated for 24 and 48 hours with 1 % lipid emulsions, lipids were extracted for fatty acids analysis, and the total RNA was extracted for mRNA expression (A) Delta-6 desaturase index (LA/AA), (B) mRNA expression of preadipocyte factor 1 (Pref-1), normalized with RPLPO as the house-keeping gene. Values are expressed as means ± SD, n = 3. Data were analyzed using two-way ANOVA to determine the main effects and interactions of treatment and time; the pairwise comparison was done using Tukey's test to determine differences when there was an observed significant interaction. LE = lipid emulsion, LA = linoleic acid, AA = Arachidonic acid. letters (a, b, c) represent significant differences between treatment groups, P<0.05 was considered significant.

3.3.3 Effects of lipid emulsions on adipogenesis and lipogenesis in 3T3-L1 mature adipocytes.

3.3.3.1 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 48 hours prior to differentiation affected the levels of triacylglycerol in mature 3T3-L1 adipocytes (8 days)

Preadipocytes were treated for 48 hours with lipid emulsions and differentiated without lipid emulsions for 8 days. The levels of total TAG were measured in 3T3-L1 mature adipocyte at day 8 of differentiation. There was a significant effect of treatment on total TAG levels (Fig 3.5A; $p<0.01$); Omegaven treatment decreased TAG compared to control cells. Similarly, Omegaven treated cells had lower TAG compared to SMOFlipid; however, there was no difference compared to Intralipid treatment.

3.3.3.2 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 48 hours prior to differentiation altered the fatty acid composition of mature 3T3-L1 adipocytes (8 days)

Preadipocytes were treated for 48 hours with lipid emulsions and differentiated without lipid emulsions for 8 days. The fatty acid composition of mature 3T3-L1 adipocytes is given in Table 3.6. The levels of C14:0 did not differ in Omegaven treatment compared to control cells; however, SMOFlipid and Intralipid showed higher C14:0 levels compared to control cells (Table 3.6). Omegaven treatment had lower C14:0 levels compared to SMOFlipid and Intralipid treatment. There was no effect of treatment on the levels of C16:0 levels. There was no difference in C18:0 in Omegaven treated cells compared to control cells. However, cells treated with Omegaven had lower levels of C18:0 compared to SMOFlipid and Intralipid (Table 3.6). There was no difference in total SFA among the treatment groups compared to control cells.

Omegaven treatment showed lower levels of C16:1n7 compared to control cells, SMOFlipid, and Intralipid treatment. However, treatment with all lipid emulsions showed a decrease in C18:1 compared to control cells (Table 3.6). There was no detection of C20:1n9 in Omegaven; Intralipid and SMOFlipid treatment were not different in C20:1n9 levels compared to control. Cells treated with Omegaven had lower total MUFA compared to control cells, and other lipid emulsions. There was no difference in total MUFA in Intralipid and SMOFlipid treatment compared to control cells. All lipid emulsions increased LA (C18:2n6) compared to control cells. However, there was no difference in LA between Omegaven and Intralipid; the levels of LA were lower in SMOFlipid compared to Omegaven (Table 3.6). Treatment had a significant effect ($p<0.0001$) on AA (C20:4n6) levels. Omegaven treatment had a higher AA level compared to control cells and other lipid emulsions. Similarly, Intralipid and SMOFlipid had higher AA levels compared to control, but the levels were lower compared to Omegaven.

There was a significant effect of treatment on EPA (C20:5n3; $p<0.001$), DHA (C22:6n3; $p<0.0001$), DPA (C22:5n3; $p<0.01$) levels (Table 3.6). Treatment with Omegaven increased the levels of EPA, DHA, DPA, compared to control cells, SMOFlipid-treated cells, and Intralipid-treated cells; however, SMOFlipid and Intralipid had no effect on the levels of EPA, DHA and DPA, compared to control cells. Omegaven-treated cells had higher total n-3 PUFA compared to control cells and other lipid emulsions. The n-6:n-3 PUFA was not significantly different between Omegaven and control cells, although the ratio was lower compared to Intralipid and SMOFlipid.

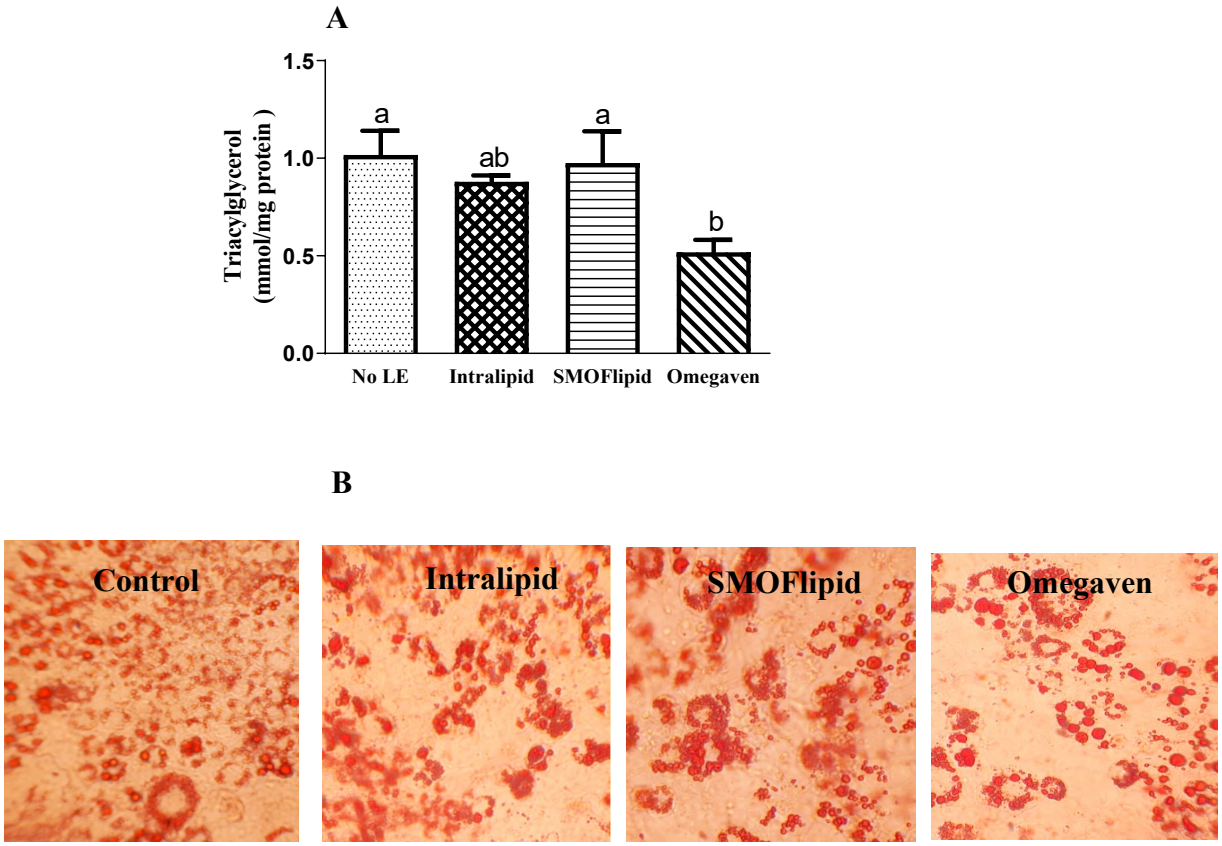

Figure 3.5 Effects of lipid emulsions on lipid accumulation in 3T3-L1 adipocytes

Preadipocytes were treated for 48 hours with 1 % lipid emulsions and differentiated for 8 days without lipid emulsions. Total triacylglycerol concentration was measured at day 8 of differentiation (A) total triacylglycerol levels expressed as millimole per milligram protein (B) Oil Red O picture of Control, Intralipid, SMOFlipid, and Omegaven. Pictures were taken as described in section 3.2.3.5. Values are expressed as means ± SD, n = 3. Data were analyzed using one-way ANOVA to determine the effect of treatment, and superscripts (a,b) were used to denote significant differences between the treatment group. P<0.05 was considered significant. LE = lipid emulsions.

Table 3.6 The fatty acid composition of 3T3-L1 mature adipocytes (day 8) when preadipocytes were treated with 1 % lipid emulsions for 48 hours prior to differentiation

FA (%)	Control	Intralipid	SMOFlipid	Omegaven
C14:0	4.85 ± 0.180^{b}	5.42 ± 0.05^{a}	5.66 ± 0.13^{a}	4.88 ± 0.22^{b}
C16:0	30.46 ± 0.55	29.80 ± 0.68	30.28 ± 1.35	31.75 ± 0.82
C16:1n7	31.71 ± 0.49^{a}	32.92 ± 1.07^{a}	33.13 ± 1.15^{a}	28.66 ± 1.49^{b}
C18:0	6.02 ± 0.39^{ab}	5.08 ± 0.59^{b}	4.71 ± 0.50^{b}	7.23 ± 0.98^{a}
C18:1	17.16 ± 0.59^{a}	15.58 ± 0.10^{b}	15.53 ± 0.30^{b}	15.67 ± 0.14^{b}
--C18:2n6	0.381 ± 0.05^{c}	0.67 ± 0.02^{ab}	0.59 ± 0.04^{b}	0.70 ± 0.04^{a}
C20:1n9	0.09 ± 0.02	0.13 ± 0.05	0.11 ± 0.02	ND
C20:4n6	0.98 ± 0.16^{c}	1.52 ± 0.02^{b}	1.40 ± 0.02^{b}	1.89 ± 0.17^{a}
C20:4n3	ND	0.18 ± 0.03^{b}	0.18 ± 0.07^{b}	0.36 ± 0.09^{a}
C20:5n3	0.28 ± 0.01^{b}	0.22 ± 0.01^{b}	0.22 ± 0.08^{b}	0.55 ± 0.06^{a}
C22:5n3	0.32 ± 0.03^{b}	0.28 ± 0.01^{b}	0.33 ± 0.01^{b}	0.46 ± 0.05^{a}
C22:6n3	0.30 ± 0.01^{b}	0.39 ± 0.01^{b}	0.40 ± 0.02^{b}	0.70 ± 0.08^{a}----
∑ SFA	41.91 ± 0.755	40.3 ± 0.03	40.6 ± 1.930	43.86 ± 1.49
∑ MUFA	48.96 ± 0.78^{a}	48.6 ± 0.08^{a}	48.7 ± 1.469^{a}	44.39 ± 1.42^{b}
∑ PUFA	2.25 ± 0.69^{c}	3.24 ± 0.07^{b}	3.11 ± 0.190^{b}	4.650 ± 0.33^{a}
∑ n-3 PUFA	1.35 ± 0.19^{b}	2.18 ± 0.03^{b}	1.98 ± 0.042^{b}	2.588 ± 0.19^{a}
∑ n-6 PUFA	0.90 ± 0.52^{c}	1.06 ± 0.03^{b}	1.12 ± 0.171^{b}	2.062 ± 0.14^{a}
n-6/n-3	1.507 ± 0.0^{b}	2.05 ± 0.03^{a}	1.76 ± 0.285^{ab}	1.254 ± 0.00^{c}

Preadipocytes were treated for 48 hours with 1 % lipid emulsions (LE) and differentiated for 8 days without lipid emulsions. Total lipids were extracted for fatty acid analysis as mentioned in section 3.2.2. Data were analyzed using One-way ANOVA after arcsine transformation followed by Tukey Posthoc to determine differences among treatment groups when there was a significant effect. Values are expressed as mean ± SD n = 3. FA = fatty acids, ΣSFA = sum of saturated fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ΣPUFA = sum of polyunsaturated fatty acids, Σn-6 = sum of omega-6 PUFA, Σn-3 = sum of omega-3 polyunsaturated fatty acids, ND = Not detected, $P<0.05$ was considered significant.

3.3.3.3 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 48 hours prior to differentiation altered the mRNA expression of *Pparg*, *LXRα*, and *Srebp1c* in mature 3T3-L1 adipocytes (day 8)

Omegaven decreased the mRNA expression of *Pparg* at day 8 of differentiation ($p<0.05$; Fig 3.6A) compared to control; however, SMOFlipid and Intralipid had no effect compared to control cells. There was no difference in the mRNA expression of *Pparg* in Omegaven-treated cells compared to SMOFlipid, but the expression was lower compared to Intralipid treatment. Omegaven significantly decreased the mRNA expression of *LXRα* compared to control cells ($p<0.05$; Fig 3.6B); however, no difference observed in mRNA expression of *LXRα* with SMOFlipid and Intralipid treatment compared to control cells. No difference was observed among the treatment groups in the mRNA expression of *LXRα*. Treatment with Omegaven decreased the mRNA expression of *Srebp1c* compared to control cells, while Intralipid increased the expression compared to control cells ($p<0.01$, Fig 3.6C). There was no difference in the mRNA expression of *Srebp1c* after SMOFlipid treatment compared to control cells. Omegaven treatment had lower mRNA expression of *Srebp1c* compared to other lipid emulsions; SMOFlipid treatment had lower expression compared to Intralipid (Fig 3.6C).

3.3.3.4 Treatment of 3T3-L1 preadipocytes with lipid emulsions for 48 hours prior to differentiation altered the mRNA expression of lipogenic genes in mature 3T3-L1 adipocytes (day 8)

There was a significant effect of treatment on the mRNA expression of *Acc1* ($p<0.05$; Fig 3.7A). Treatment with Omegaven had no effect on the mRNA expression of *Acc1* compared to control cells. Treatment with lipid emulsions had a significant effect on the mRNA expression of

Fasn and *Scd1*. Omegaven treatment significantly decreased the mRNA expression of *Fasn* ($p<0.01$; Fig 3.7B) and *Scd1* ($p<0.01$; Fig 3.7C) compared to control cells. Similarly. Omegaven treatment also had lower mRNA expression of *Fasn* and *Scd1* compared to SMOFlipid and Intralipid treatment. There was a significant effect ($p<0.05$) of treatment on the mRNA expression of *Dgat*2 Omegaven-treated cells significantly decreased ($p<0.05$) the mRNA expression of *Dgat2* compared to control cells (Fig 3.7D). There was no difference in the mRNA expression of *Dgat2* with Omegaven treatment compared to SMOFlipid and Intralipid treatment (Fig 3.7D).

3.3.4 Effects of lipid emulsions on lipolysis and β-oxidation in 3T3-L1 adipocytes

3.3.4.1 Fatty acid composition of mature 3T3-L1 adipocytes differentiated for 8 days without lipid emulsions and then treated for 48 hours with 1 % lipid emulsions

The fatty acid composition of 3T3-L1 mature adipocytes treated for 48 hours post differentiation is given in Table 3.7. Treatment had a significant effect ($p<0.0001$) on the levels of C14:0, where SMOFlipid and Intralipid treated cells had lower levels compared to Omegaven. There was a significant effect ($p<0.0001$) of treatment on the level of C16:0; Omegaven-treated cells had lower levels of C16:0 compared to control cells. Omegaven-, SMOFlipid, and Intralipid-treated cells had lower levels of C18:0 compared to control cells. However, SMOFlipid-treated cells had higher C18:0 levels compared to Omegaven, but the levels were not different compared to Intralipid. Cells treated with Omegaven had lower total SFA compared to control cells. Omegaven treatment had lower total SFA compared to SMOFlipid treatment, but the levels were not different compared to Intralipid.

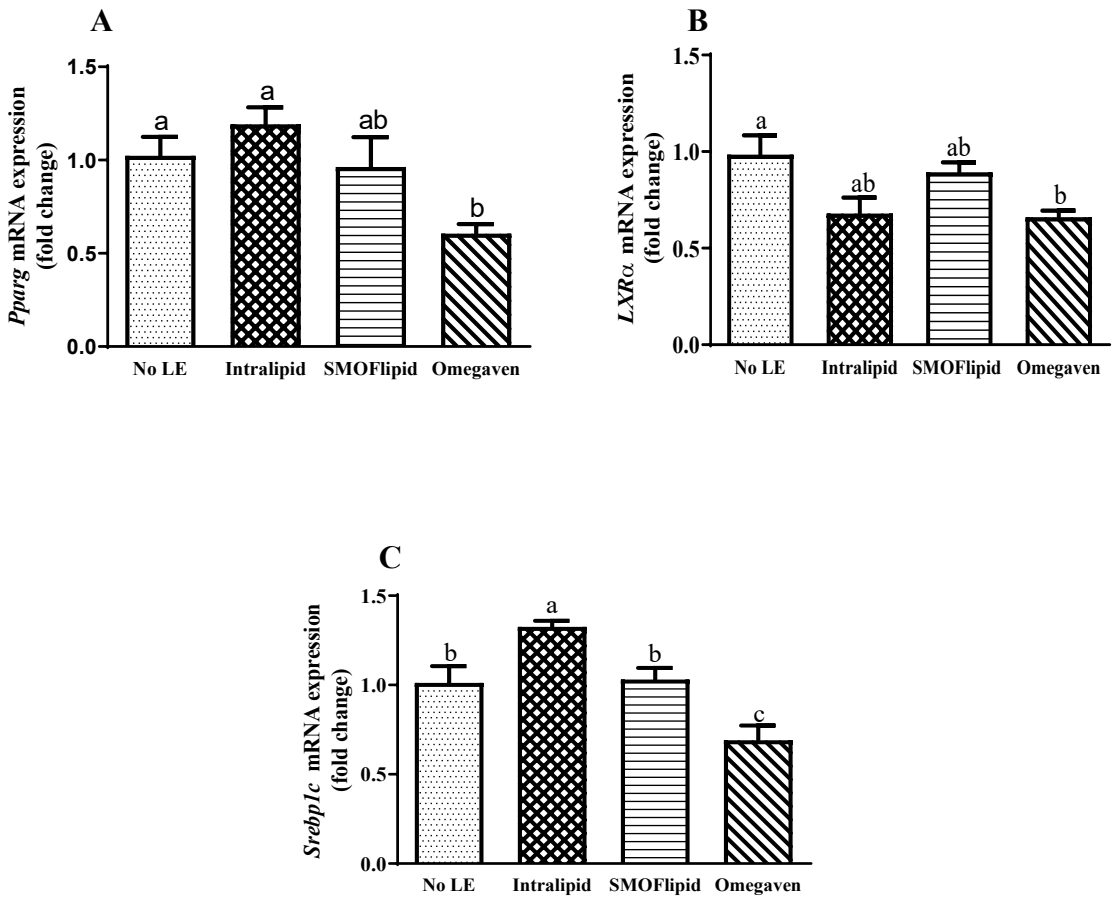

Figure 3.6 Effects of lipid emulsions on the mRNA expression of Pparg, LXRα, and Srebp1c in mature (day 8) 3T3-L1 adipocytes

Preadipocytes were treated for 48 hours with 1% (v/v) lipid emulsions and differentiated for 8 days without lipid emulsions lipids and the mRNA expression was measured on day 8. The data represent mRNA expression of (A) peroxisome proliferator activator receptor protein-gamma (Pparg), (B) liver X receptor (LXRα) (C) sterol regulatory element-binding protein 1 (Srebp1c), normalized with Rplp0 as the house-keeping gene. Values are expressed as means ± SD, n = 3. Data were assessed using one-way ANOVA to determine the effect of treatment and Tukey's test was used to determine differences when there was an observed significant difference. Letters (a, b, c) represent significant differences between treatment groups, P<0.05 was considered significant. LE = lipid emulsions.

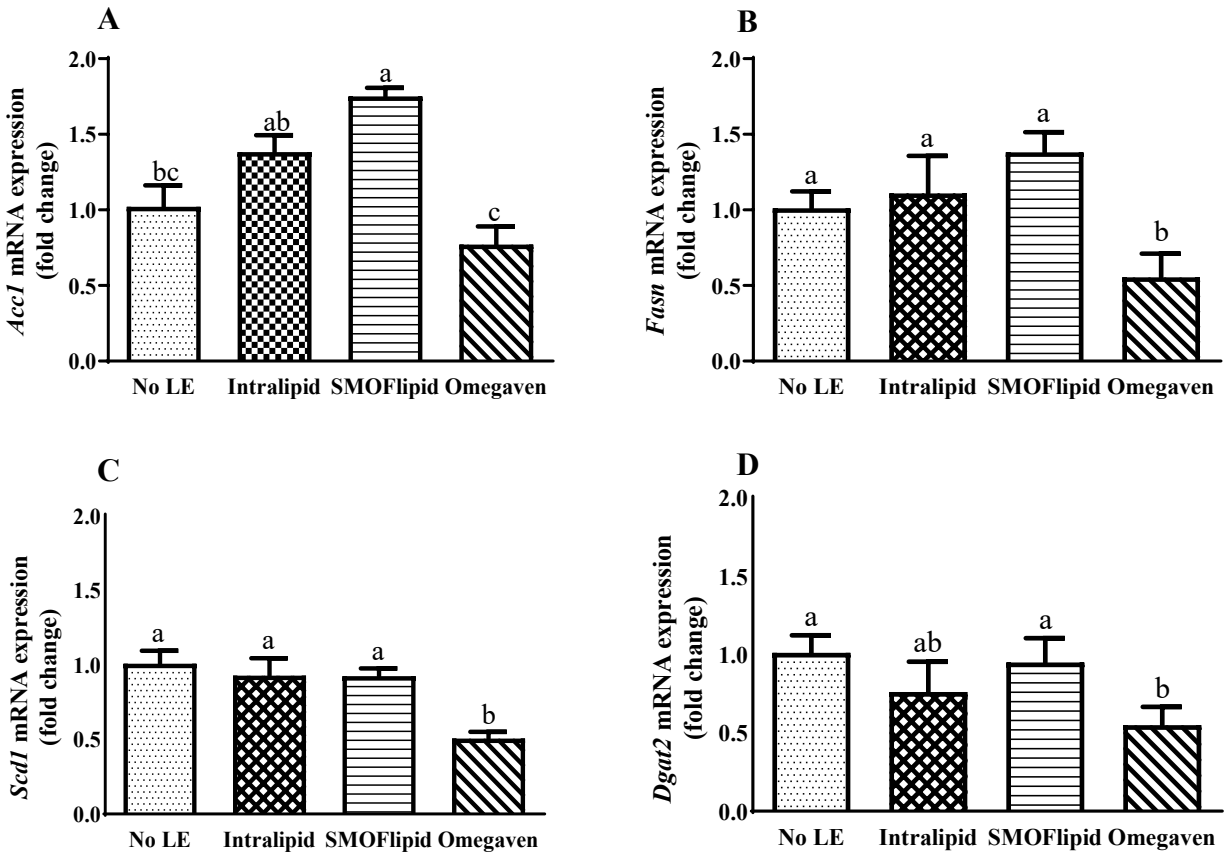

Figure 3.7 Effects of lipid emulsions on the mRNA expression of lipogenic and adipogenic genes in mature 3T3-L1 adipocytes (day 8) differentiated without lipid emulsions

Preadipocytes were treated for 48 hours with 1% (v/v) lipid emulsions (LE) and differentiated for 8 days without lipid emulsions and the mRNA expression was measured on day 8. The data represent mRNA expression of (A) acetyl CoA carboxylase (Acc1) (B) fatty acid synthase (Fasn) (C) stearoyl-CoA desaturase (Scd1) (D) diacylglycerol O-acyltransferase (Dgat2) normalized with Rplp0 as the house-keeping gene. Values are expressed as means ± SD, n = 3. Data were assessed using one-way ANOVA to determine the effect of treatment and Tukey's test was used to determine differences when there was an observed significant difference. Letters (a, b, c) represent significant differences between treatment groups, $P<0.05$ was considered significant. LE = lipid emulsions.

There was a significant effect ($p<0.0001$) of treatment on C16:1n7 levels. Cells treated with Omegaven, SMOFlipid, and Intralipid had lower levels of C16:1n7 compared to control cells (Table 3.7). The levels of C16:1n7 in Omegaven-treated cells were not different compared to SMOFlipid but were higher compared to Intralipid. Omegaven-treated cells had higher C20:1n9 compared to SMOFlipid and Intralipid. Total MUFA in Omegaven-treated cells was lower compared to control cells. The levels of total MUFA in SMOFlipid-treated cells were not different from control; total MUFA was higher in SMOFlipid compared to Omegaven and Intralipid (Table 3.7).

There was a significant effect ($p<0.0001$) of treatment on LA (C18:2n6) levels whereby SMOFlipid and Intralipid treated cells had significantly ($p<0.0001$) higher levels of LA compared to the control cells. Intralipid-treated cells had higher LA levels compared to SMOFlipid- and Omegaven-treated cells. AA levels in Omegaven-treated cells were not different from control cells. SMOFlipid-treated cells had higher AA compared to Intralipid but were not different from cells treated with Omegaven. Omegaven treated cells had higher total n-6 PUFA compared to control cells, but lower compared to other lipid emulsions.

Treatment had a significant effect ($p<0.0001$) on EPA (C20:5n3) and DHA levels (Table 3.7). Treatment with Omegaven had significantly ($p<0.0001$) higher levels of EPA and DHA compared to control cells, and cells treated with SMOFlipid and Intralipid. Total n-3 PUFA was higher in cells treated with Omegaven compared to control cells and compared to other lipid emulsions. Omegaven, SMOFlipid, and Intralipid had n-6:n-3 PUFA of 0.16, 2.05, and 6.63, respectively.

Table 3.7 The fatty acid composition of mature 3T3-L1 adipocytes differentiated without lipid emulsions for 8 days and then differentiated for 48 hours with 1 % lipid emulsions

FA (%)	Control	Intralipid	SMOFlipid	Omegaven
C14:0	4.34 ± 0.19^{a}	1.30 ± 0.24^{c}	2.99 ± 0.16^{b}	4.78 ± 0.22^{a}
C16:0	38.24 ± 0.3^{a}	18.93 ± 2.16^{c}	28.40 ± 2.76^{b}	20.42 ± 1.60^{c}
C16:1n7	22.48 ± 0.24^{a}	5.97 ± 1.35^{c}	11.65 ± 1.66^{b}	12.89 ± 0.51^{b}
C18:0	9.94 ± 1.28^{a}	5.74 ± 1.06^{bc}	7.86 ± 0.29^{ab}	5.40 ± 0.19^{c}
C18:1	18.45 ± 0.61^{c}	22.78 ± 0.37^{b}	29.04 ± 2.18^{a}	18.5 ± 1.00^{c}
C18:2n6	0.80 ± 0.07^{c}	38.14 ± 4.46^{a}	11.40 ± 2.36^{b}	2.90 ± 0.07^{c}
C18:3n3	ND	4.24 ± 0.5^{a}	1.10 ± 0.28^{b}	0.78 ± 0.08^{b}
C20:1n9	ND	0.27 ± 0.01^{c}	0.37 ± 0.02^{b}	0.71 ± 0.05^{a}
C20:4n6	2.00 ± 0.12^{a}	0.93 ± 0.18^{b}	1.76 ± 0.0^{a}	2.05 ± 0.05^{a}
C20:4n3	ND	ND	ND	0.71 ± 0.06
C20:5n3	0.42 ± 0.07^{c}	0.14 ± 0.03^{c}	1.79 ± 0.14^{bc}	15.99 ± 1.78^{a}
C22:5n3	2.30 ± 0.82^{a}	1.02 ± 0.38^{b}	1.69 ± 0.25^{ab}	2.42 ± 0.27^{a}
C22:6n3	0.92 ± 0.08^{b}	0.48 ± 0.10^{b}	1.84 ± 0.09^{b}	12.06 ± 1.17^{a}
∑ SFA	52.52 ± 1.24^{a}	25.98 ± 3.39^{c}	39.24 ± 3.14^{b}	30.61 ± 1.57^{c}
∑ MUFA	41.04 ± 0.58^{a}	29.01 ± 1.06^{c}	41.06 ± 0.53^{a}	32.10 ± 1.49^{b}
∑ PUFA	6.43 ± 0.74^{d}	44.95 ± 4.41^{c}	19.59 ± 2.78^{c}	36.92 ± 3.06^{b}
∑ n-3 PUFA	3.64 ± 0.73^{b}	5.88 ± 0.17^{b}	6.43 ± 0.59^{b}	31.97 ± 3.11^{a}
∑ n-6 PUFA	2.79 ± 0.09^{c}	39.07 ± 4.29^{a}	13.17 ± 2.31^{b}	4.95 ± 0.12^{c}
n-6/n-3 ratio	0.79 ± 0.17^{c}	6.63 ± 0.60^{a}	2.05 ± 0.26^{b}	0.16 ± 0.02^{c}

Cells were differentiated for 8 days without lipid emulsion and then treated for 48 hours with 1 % lipid emulsions. Total lipids were extracted for fatty acid analysis as mentioned in section 3.2.2. Data were analyzed using One-way ANOVA after arcsine transformation followed by Turkey Posthoc to determine differences among treatment groups when there was a significant effect. Values are expressed as mean ± SD, n = 3. Superscripts (a,b,c) within a row were used to denote significant differences between the treatment group. FA = fatty acids, ΣSFA = sum of saturated fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ΣPUFA = sum of polyunsaturated fatty acids, Σn-6 = sum of omega-6 PUFA, Σn-3 = sum of omega-3 polyunsaturated fatty acids, ND = not detected, $P<0.05$ was considered significant

3.3.4.2 The mRNA expression of lipolytic genes in mature 3T3-L1 adipocytes differentiated without lipid emulsions for 8 days and then treated for 48 hours with 1 % lipid emulsion

Treatment of mature 3T3-L1 adipocytes with Omegaven significantly ($p<0.05$) decreased the mRNA expression of *Atgl* (Fig 3.8A), compared to the control and SMOFlipid-treated cells; however, the levels were not different compared to Intralipid. Omegaven treatment also significantly ($p<0.05$) decreased the mRNA expression of *Hsl* (Fig 3.8B), compared to control, SMOFlipid-treated, and Intralipid treated cells. All lipid emulsions significantly ($p<0.01$) decreased the mRNA expression of *Fabp4* (Fig 3.8C), compared to the control cells.

3.3.4.3 The mRNA expression of *Cpt1* in mature 3T3-L1 adipocytes differentiated without lipid emulsions for 8 days and then differentiated for 48 hours with 1 % lipid emulsion

Treatment of mature adipocytes with Omegaven and SMOFlipid for 48 hours post differentiation significantly ($p<0.001$) increased the mRNA expression of *Cpt1* (Fig 3.8D), compared to control cells. There was no difference in *Cpt1* mRNA expression in Intralipid-treated cells compared to control cells; however, the mRNA expression of *Cpt1* was lower in Intralipid-treated cells compared to SMOFlipid and Omegaven.

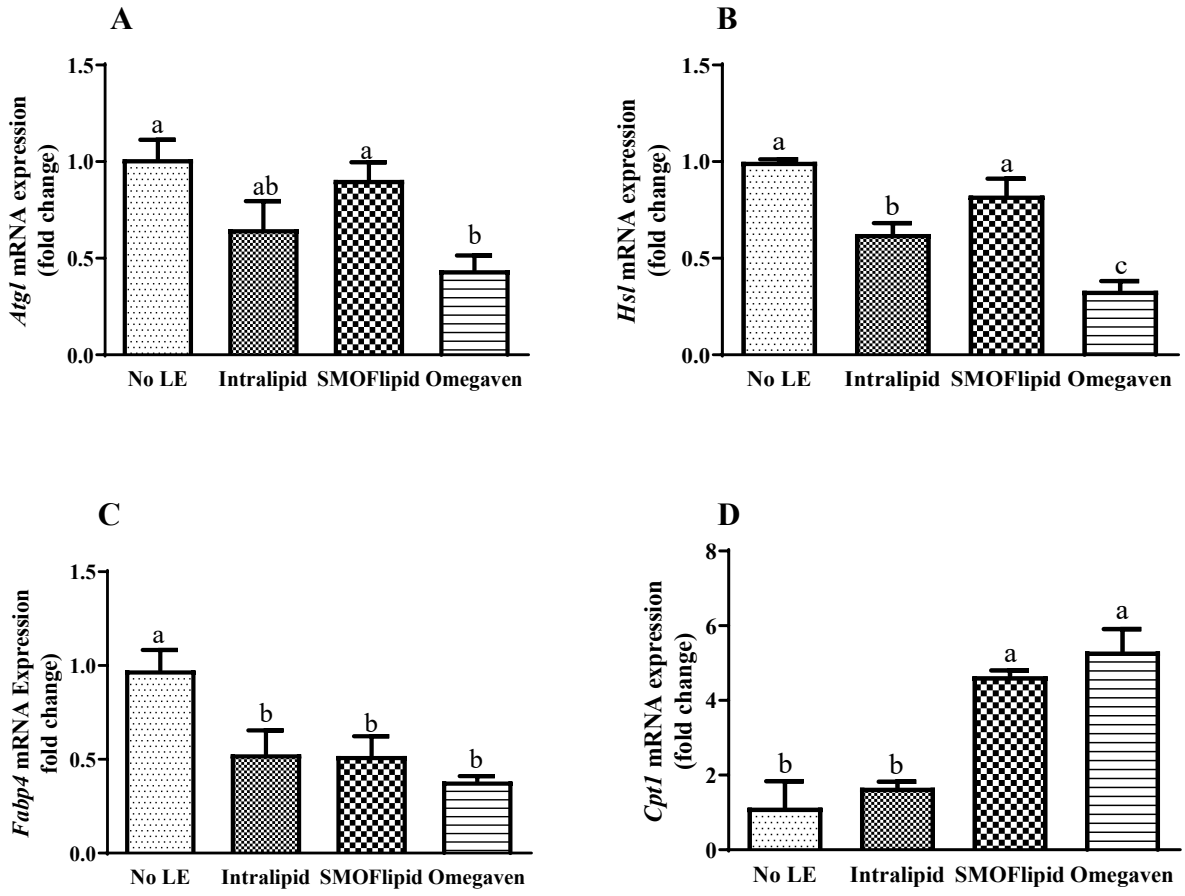

Figure 3.8 Effects of lipid emulsions on the mRNA expression of genes involved in lipolysis and β-oxidation in mature 3T3-L1 adipocytes treated with lipid emulsions for 48 hours post differentiation

Cells were differentiated for 8 days without lipid emulsion and then treated for 48 hours with 1 % lipid emulsion. The mRNA expression was measured at 48 hours of treatment. The data represent mRNA expression of (A) adipose triglyceride lipase (Atgl) (B) hormone-sensitive lipase (Hsl) (C) fatty acid-binding protein (Fabp4) (D) carnitine palmitoyltransferase-1 (Cpt1) normalized with Rplp0 as the house-keeping gene. Values are expressed as means ± SD, n = 3. Data were assessed using one-way ANOVA to determine the effect of treatment, where Tukey's test was used to determine differences when there was an observed significant difference. Letters (a, b, c) represent significant differences between treatment groups, $P<0.05$ was considered significant. LE = lipid emulsion.

3.4 Discussion

At infancy and early childhood, fat tissue growth largely depends on differentiation and hypertrophy of existing adipocytes; thus, it is also a sensitive period for the development of obesity (Taylor & Poston, 2007). Both n-6 and n-3 PUFA act as ligands for the transcription factors that are involved in adipogenesis. Previous studies from our laboratory have shown that BM with high n-6:n-3 PUFA increased the mRNA expression of lipogenic and adipogenic genes in 3T3-L1 adipocytes (Vaidya & Cheema, 2018). However, there are no studies to date to show whether lipid emulsions used in TPN with low and high n-6:n-3 PUFA affect adipogenesis, lipolysis, and β-oxidation in adipocytes. In the current study, the effects of lipid emulsions with low and high n-6:n-3 PUFA on the mRNA expression of genes involved in adipogenesis, lipogenesis, lipolysis, and β-oxidation were examined using 3T3-L1 adipocytes. Our findings reveal that Omegaven reduced lipid accumulation by downregulating adipogenic genes and increasing the mRNA expression of *Cpt1* involved in β-oxidation. We also found that Omegaven decreased lipolytic gene expression, which could have a potential benefit in lower circulating levels of NEFA released from TAG hydrolysis.

I measured the fatty acid composition of 3T3-L1 adipocytes after treatment with lipid emulsions for 24 and 48 hours to examine the incorporation of fatty acids from lipid emulsions. Intralipid treatment had higher AA levels at 48 hours of treatment. LA (C18:2n6) is converted to AA by sequential Δ6 desaturation, elongation, and Δ5 desaturation reactions (Sprecher, 2000). The high AA in Intralipid treatment may be due to a higher conversion of LA to AA because Intralipid has a lower concentration of AA compared to Omegaven. Thus, I calculated the delta- 6 desaturase index (LA/AA); it was interesting to note that Intralipid had a higher delta-6 desaturase index compared to other treatments. Interestingly, ALA (C18:3n3) was only detected in the Intralipid

treatment at both 24 and 48 hours, which corresponds to high levels of ALA in Intralipid compared to other lipid emulsions.

Omegaven-treated cells had higher EPA and DHA at 24 hours of treatment, compared to other lipid emulsions, and the levels increased further at 48 hours. Intralipid treatment showed the highest n-6:n-3 PUFA, followed by SMOFlipid, and Omegaven at both 24 and 48 hours, which is consistent with the n-6:n-3 PUFA of the lipid emulsions, suggesting that treatment of preadipocytes for 24 and 48 hours with lipid emulsions altered the fatty acid composition of the cells. The question arises whether the changes in the fatty acid composition will alter the gene expression of genes involved in adipogenesis. *Pref-1* is highly expressed in preadipocytes to maintain its fibroblast nature; its expression is reduced upon induction of differentiation (Hudak & Sul, 2013). *Pref-1* induces Sox9 expression through the activation of MEK/ERK pathway, leading to increased proliferation of preadipocytes and inhibition of adipocyte differentiation (Kim et al., 2007; Wang & Sul, 2009). Treatment with Intralipid, SMOFlipid, and Omegaven showed higher mRNA expression of *Pref-1* at 24 hours, compared to control cells, indicating inhibition of adipocyte differentiation. However, at 48 hours of treatment, Omegaven treatment decreased Pref-1 mRNA expression, which could likely be due to increased growth arrest of the cells caused by high levels of EPA and DHA in Omegaven. Previous studies have shown that DHA inhibits 3T3-L1 preadipocyte growth (Awad et al., 2000), inhibits adipocyte differentiation and induces apoptosis in 3T3-L1 preadipocytes (Kim et al., 2006). Upon induction of differentiation, adipocytes undergo growth arrest and then mitotic clonal expansion followed by subsequent expression of adipogenic genes that are essential for lipid accumulation (Fajas, 2003; Tang et al., 2003). DHA supplementation inhibits differentiation-associated mitotic clonal expansion in post confluent preadipocytes and subsequently reduced lipid accumulation in adipocytes. Since Omegaven-treated

cells had higher DHA and EPA levels, it could possibly lead to the inhibition of mitotic clonal expansion; hence the cells were already growth-arrested, thereby I observed decreased *Pref-1* mRNA expression.

After 48 hours of treatment of preadipocytes with lipid emulsions, we then differentiated the cells for 8 days without lipid emulsions and measured the fatty acids levels. Omegaven treated cells still had higher levels of DHA and EPA on day 8 of differentiation, suggesting that 48 hours of treatment with Omegaven prior to differentiation maintained higher levels of EPA and DHA in mature adipocytes. EPA and DHA, and their metabolites are natural ligands for PPARγ (Li et al., 2014; Magee et al., 2012). Omegaven treatment decreased the mRNA expression of *Pparg* at day 8 of differentiation, suggesting inhibition of differentiation of preadipocytes to mature adipocytes. This result is similar to the results obtained by Li et al. who also reported that enrichment of EPA and DHA downregulated the mRNA expression of *Pparg* in 3T3-L1 adipocytes (Li et al., 2017). Omegaven treatment also decreased the mRNA expression of *LXRα* in mature adipocytes compared to control cells, which corresponds with the downregulation of *Srebp1c* mRNA expression. LXRα has been shown to activate *Srebp1c*, the transcription factor that regulates the expression of lipogenic genes (Repa et al., 2000). N-3 PUFA suppresses the transcription of *Srebp1c* via the reduced trans-activating capacity of LXRα (Howell III et al., 2009), which explains the effects of Omegaven on *LXRα* and *Srebp1c*.

Fasn is a key lipogenic gene that catalyzes the steps involved in the synthesis of palmitate (C16:0) from acetyl-CoA and malonyl-CoA (Wang et al., 2004). Omegaven treatment decreased the mRNA expression of *Fasn* compared to control cells, suggesting inhibitory effects on lipogenesis. Similarly, Li et al. reported that n-3 PUFA downregulates the mRNA expression of *Fasn* in 3T3-L1 cells (Li et al., 2017). Our results are also consistent with that of Wortman et al.

who reported that EPA decreased the mRNA expression of *Fasn* in 3T3-L1 adipocytes (Wortman et al., 2009). Synthesis of C16:0 by *Fasn* provides substrates for the synthesis of MUFA, specifically oleic acid (C18:1) from stearic acid (C18:0), a reaction catalyzed by *Scd1* (Paton & Ntambi, 2009). MUFA are important for the synthesis of TAG (Ntambi & Miyazaki, 2004). Omegaven treatment decreased the mRNA expression of *Scd1*, compared to control cells. Others have also reported that n-3 PUFA downregulates the mRNA expression of *Scd1* in 3T3-L1 adipocytes (Li et al., 2017; Manickam et al., 2010). The downregulation of *Scd1* is associated with increased insulin sensitivity and resistance to obesity (Paton & Ntambi, 2009). Both *Scd1* and *Dgat2* are colocalized, implying the preference of endogenous MUFA for TAG synthesis (Man et al., 2006). DGAT2 catalyzes the final step of mammalian TAG synthesis (Harris et al., 2011); thus, it is an important lipogenic gene responsible for TAG accumulation in adipocytes. Treatment of preadipocytes with Omegaven decreased the mRNA expression of DGAT2 in mature adipocytes. Suppression of DGAT2 is protective against excessive lipid accumulation, obesity and improved insulin resistance (Choi et al., 2007). TAG accumulation was lower in adipocytes treated with Omegaven, which corresponds with inhibition of *Acc1*, *Fasn* and *Dgat2* mRNA expression. Our laboratory has previously reported that blue mussel and sea cucumber extracts rich in n-3 PUFA decreased TAG levels in 3T3-L1 adipocytes (Vaidya & Cheema, 2014). Other studies have also reported that EPA and DHA reduce TAG accumulation in 3T3-L1 cells (Kim et al., 2006; Manickam et al., 2010).

The stored fat in adipocytes is mobilized by the hydrolysis of TAG into FFA and glycerol (Langin, 2006). The FFA released are delivered to the peripheral tissues where they serve as a substrate for β-oxidation (van der Spek et al., 2012). Elevated lipolysis generates excess FFA, which is implicated in ectopic lipid accumulation and insulin resistance (Kumashiro et al., 2011).

To investigate the effects of lipid emulsions on the regulation of lipolysis and β-oxidation in mature adipocytes, cells were differentiated without lipid emulsions and then treated for 48 hours with lipid emulsions. Omegaven and SMOFlipid treatment for 48 hours showed an increase in the levels of EPA and DHA. Furthermore, Omegaven-treated cells showed a decrease in the mRNA expression of *Atgl* and *Hsl* compared to control cells. *Atgl* is the rate-limiting enzyme in the lipolytic pathway (Zimmermann et al., 2004); it hydrolyzes TAG into DAG, which is then hydrolyzed by HSL into MAG (Haemmerle et al., 2002). The final step in lipolysis is the cleavage of MAG by MGL into glycerol and FFA (Fredrikson et al., 1986). Previous studies have also shown that n-3 PUFA inhibited lipolysis by inhibiting the mRNA expression of *Atgl* (Wang et al., 2017). However, others have reported that both EPA and DHA increased the mRNA expression of *Atgl* in 3T3-L1 adipocytes (Prostek et al., 2016), and EPA increased the mRNA expression of *Hsl* (Manickam et al., 2010). Omegaven contains both EPA and DHA, along with other fatty acids; it is possible that a combination of EPA, DHA and other fatty acids, elicits a different effect on *Atgl* and *Hsl* gene expression, compared to individual fatty acids. One possible beneficial effect of Omegaven inhibiting lipolysis from reducing NEFA trafficking from adipose, thereby preventing ectopic lipid accumulation. *Fabp4* plays a role in the efflux of products of lipolysis (fatty acids and glycerol) from the adipocytes. High levels of *Fabp4* is associated with obesity, inflammation and insulin resistance (Kucharski & Kaczor, 2017). Omegaven and other lipid emulsions downregulated the mRNA expression of *Fabp4* compared to control cells. Similarly, another study also reported that n-3 PUFA decreases *Fabp4* mRNA expression in 3T3-L1 adipocytes (Furuhashi et al., 2016).

Next, I measured the mRNA expression of *Cpt1* in mature adipocytes as *Cpt1* is involved in β-oxidation. Omegaven and SMOFlipid, which contain high levels of n-3 PUFA, increased the mRNA expression of *Cpt1* in mature adipocytes. CPT1 is the rate-limiting enzyme for the β-

oxidation of long-chain fatty acids in the mitochondria by catalyzing the conversion of cytoplasmic long-chain acyl CoA to acylcarnitine, which then enters the mitochondria for β-oxidation (Schreurs et al., 2010). The upregulation of CPT1 enhances insulin-stimulated glucose metabolism and protects against insulin resistance (Perdomo et al., 2004; Sebastián et al., 2007). Our results are similar to others who reported that EPA and DHA increased *Cpt1* mRNA expression in 3T3-L1 adipocytes (Lee et al., 2008; Prostek et al., 2016).

Findings from this study reveal that Omegaven inhibits lipid accumulation by downregulating the mRNA expression of *Pparg*, *LXRα*, and *Srebp1c*. Furthermore, Omegaven decreased the mRNA expression of *Fasn*, *Scd1*, and *Dgat2* mRNA that coincided with reduced fat accumulation. Omegaven and SMOFlipid increased the mRNA expression of *Cpt1* compared to Intralipid. These findings suggest the administration of Omegaven at an early stage may not only be efficacious in treating parenteral nutrition-associated hepatic diseases but may also have a beneficial effect on long term prevention of excess lipid accumulation in the adipocytes. Reduced lipid accumulation in the adipocytes enhances insulin sensitivity. Thus, the early administration of Omegaven could potentially prevent insulin resistance in later life of infants. The lower lipolytic effects seen with Omegaven treatment may be important to reduce the release of FFA from the adipose, thereby preventing ectopic lipid accumulation.

In conclusion, findings from this study reveal that early exposure of Omegaven to preadipocytes increases the incorporation of EPA and DHA and decreases the adipogenesis process, which coincided with reduced-fat accumulation. Inhibition of lipid accumulation in the adipocyte and increased mRNA expression of *Cpt1* may contribute to the prevention of later childhood obesity and enhance insulin sensitivity.

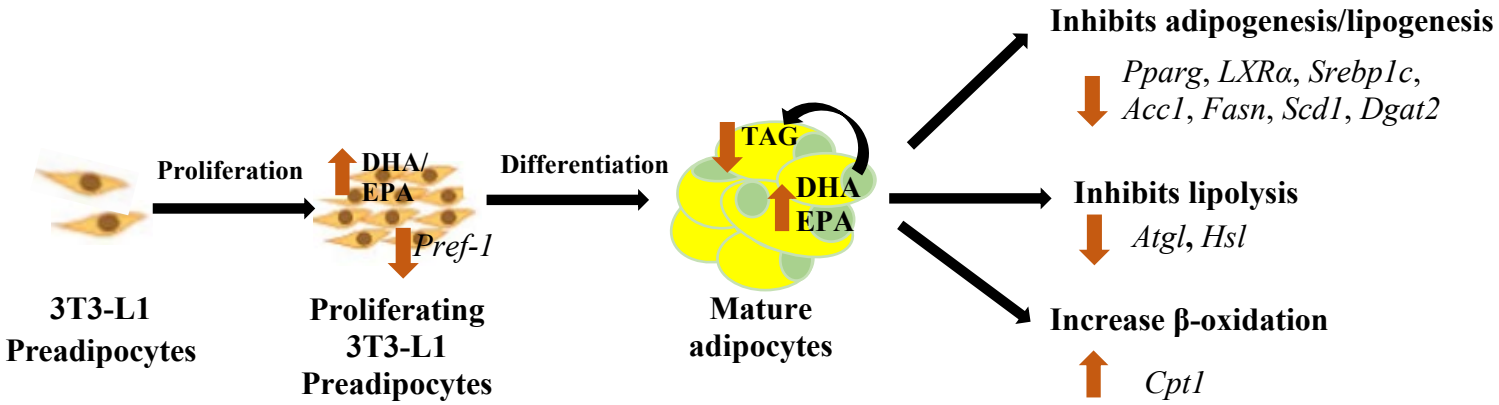

Figure 3.9 Schematic representation of the effect of Omegaven on the mRNA expression of genes involved in lipogenesis, lipolysis, and β-oxidation in 3T3-L1 adipocytes

Acc1, acetyl-CoA carboxylase; Atgl, Adipose triglyceride lipase; Cpt1, carnitine palmitoyltransferase I; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Fasn, fatty acid synthase; Hsl, Hormone-sensitive lipase; *LXRα, liver X receptor alpha; peroxisome proliferator-activated receptor-gamma; Pref-1, preadipocyte factor 1; Scd1, stearoyl-CoA desaturase; ; Srebp1c, sterol regulatory element-binding protein 1; TAG, triacylglycerol*

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CHAPTER FOUR

Maternal obesity modulates the essential fatty acids content in breast milk: potential implications in adipogenesis and lipolysis, using 3T3-LI adipocytes as a model

A version of this chapter will be submitted for publication in Maternal and Child Nutrition

4.0 Abstract

Maternal obesity impacts the fatty acid composition of breast milk (BM). Omega-6 fatty acids increase adipogenesis and are associated with obesity. The objective of this study was to investigate the effects of BM from lean and obese women on adipogenesis and lipolysis in 3T3-L1 cells. BM samples were collected from lean (BML) and obese (BMO) women (n=20 each) at one month postpartum and fatty acid composition measured using GC-MS/FID. BM samples were then grouped into quartiles [(low (Q1) and high (Q4)] for both BML and BMO women based on the levels of n-6:n-3 PUFA levels. BM samples in Q1 and Q4 were pooled separately, each for BML and BMO, to prepared whey to treat 3T3-L1 cells. Cells treated with BMO with high n-6:n-3 PUFA appeared to have larger lipid droplets compared to BMO with low n-6:n-3 PUFA. BML with high n-6:n-3 PUFA significantly increased the mRNA expression of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase compared to BML with low n-6:n-3 PUFA. Interestingly, there was no effect of BMO on lipogenesis. Both BMO and BML had no effect on the mRNA expression of genes involved in lipolysis, compared to control cells. Our findings demonstrate that high n-6:n-3 PUFA in BML is associated with increased lipogenesis, while there was no effect of BMO on lipogenesis. These findings may have implications in maternal lactation programming of transgenerational childhood obesity.

4.1 Introduction

Obesity has become a global epidemic (Blüher, 2019). High rates of pre-pregnancy obesity correspond closely with increased risks of adverse maternal and perinatal outcomes (Singh & DiBari, 2019; Stubert et al., 2018), which has been linked to the abnormal metabolic profile of obese women such as insulin resistance and dyslipidemia. Both obesity and excessive weight gain during pregnancy are risk factors for childhood adiposity and later cardiovascular and respiratory morbidity of the offspring (Gaillard et al., 2014).

Diet plays a key role in the regulation of the infant's growth and development. Exclusive breastfeeding is recommended for infants in the first six months of life because BM is thought to contain the ideal nutrient composition (Bernt & Walker, 1999; Pérez-Escamilla et al., 2019). BM is comprised of carbohydrates (lactose and oligosaccharide), proteins, vitamins, minerals, digestive enzymes, cytokines, hormones, fatty acids, and other bioactive components (Savino et al., 2013). Maternal dietary fat intake and adiposity are both associated with the fatty acids composition of BM (Martin et al., 2016). LCPUFA are critical for the development of the fetal central nervous system (Bernardi et al., 2012). BM contains essential fatty acids such as LA, an n-6 PUFA and ALA, an n-3 PUFA (Koletzko, 2016). LA is converted to AA, while ALA is converted to EPA and DHA. These fatty acids are important for regulating growth, inflammatory responses, immune function, vision, cognitive development, and motor systems in the fetus and newborn (Martin et al., 2016). N-6 PUFA are generally linked to inflammation because the conversion of LA to AA provides a substrate for potent pro-inflammatory mediators including prostaglandins and leukotrienes (Innes & Calder, 2018). On the other hand, n-3 PUFA are often categorized as anti-inflammatory in nature due to: inhibitory effects on the secretion of pro-inflammatory mediators, reduction of

macrophage migration into the adipose tissue and production of anti-inflammatory eicosanoids (Wang & Huang, 2015).

The n-6:n-3 PUFA plays a role in the maintenance of lipid metabolism, oxidative stress, and inflammation (Yang et al., 2016). N-6:n-3 PUFA in human BM was positively associated with early infant adipose deposition during the first 4 months of life, independent of maternal BMI (Rudolph et al., 2017). BM of obese women has been reported to have a pro-inflammatory fatty acid profile at 2 months postpartum (Panagos et al., 2016), indicating that maternal obesity impacts the fatty acids composition of BM, which may, in turn, be associated with childhood obesity. During infancy and early childhood, fat tissue growth largely depends on differentiation and hypertrophy of existing adipocytes; thus it is a sensitive period for the programming and development of obesity (Stettler et al., 2002). Obesity is an enlargement of adipose tissue to store excess energy intake (Jo et al., 2009) Adipose tissue expansion occurs by two distinct mechanisms: hypertrophy (increase in adipocyte size) and hyperplasia (increase in adipocyte number (Jang et al., 2016). Adipose hypertrophy results from increased expression of lipogenic genes are implicated in the onset of adipocyte dysfunction (Chan & Hsieh, 2017; Laforest et al., 2015). Hyperplastic growth appears only at early stages in adipose tissue development (Spalding et al., 2008). Both n-6 and n-3 PUFA act as ligands to regulate the expression of genes involved in lipogenesis and adipogenesis (Riera-Heredia et al., 2019). Low neonatal plasma n-6:n-3 PUFA regulated offspring adipogenic potential, preventing the onset of obesity in mice during later life (Rudolph et al., 2018). ATGL and HSL are the main enzymes that regulate lipolysis in adipocytes (Nielsen et al., 2014), while perilipin-1 is the most abundant protein in adipocytes that coat lipid droplets and inhibit lipolysis in the basal state (Sohn et al., 2018).

Maternal pre-pregnancy weight has been shown to influence the levels of PUFAs, leptin, TNF-α, and IL-6 of BM (4 to 8 weeks postpartum), and also influence infant growth (Nuss et al., 2019). Similarly, obesity in pregnancy is associated with elevated levels of plasma IL-6 (Ramsay et al., 2002). Maternal systemic IL-6 levels are closely associated with increased fetal adiposity (Radaelli et al., 2006). Obese individuals also have higher levels of plasma leptin, which are implicated in the onset of leptin resistance (Myers Jr et al., 2010). Thus, a chronic maternal pro-inflammatory state can cause metabolic abnormalities in the offspring, impacting their health in later life.

We have previously shown that BM of healthy women from Newfoundland and Labrador with higher n-6 PUFA contained a higher concentration of soluble pro-inflammatory cytokines, which induced events similar to insulin resistance (upregulation of SCD1 and DGAT2 mRNA expression) in 3T3-L1 adipocytes (Vaidya & Cheema, 2018). However, the effect of maternal obesity on the fatty acid composition of BM, and its impact on adipogenesis and lipolysis in 3T3-L1 cells is not known. We hypothesized that BM with higher n-6:n-3 PUFA will increase adipogenesis in adipocytes. We further hypothesized that the BM of obese women will increase lipolysis and induce adipocyte dysfunction in 3T3-L1 cells.

4.2 Methods

4.2.1 Collection of breast milk (BM) samples

This study was approved by the Health Research Ethics Board (2017.231) Memorial University of Newfoundland and Labrador, Canada. BM samples were received from Dr. Sarbattama Sen, a neonatologist at the Department of Pediatric Newborn Medicine, Brigham and Women's Hospital, Harvard University, Boston, Massachusetts. Participants were

selected from a randomized controlled trial of maternal vitamin D supplementation in exclusively lactating women living in Charleston, South Carolina, or Rochester, New York, United States (NCT00412074) (November 2005 - August 2012) (Hollis et al., 2015). Participants were included in the parent study if the mothers were breastfeeding exclusively at the time of recruitment. Participants were excluded if they had maternal pre-existing type 1 and type 2 diabetes, hypertension, parathyroid disease, and uncontrolled thyroid disease. In the present study, participants were included if they had BM archived at one month postpartum and BMI available. Participants were categorized based on BMI into two groups; BMI <30kg/m^2 categorized as lean and ≥30kg/m^2 as overweight/obese. A total of 40 women (20 lean and 20 overweight/obese) were selected for the study. BM samples were collected from the participants at one-month postpartum using a hospital-grade electric pump. Participants were asked to collect a complete feed from the opposite breast from which the infants were feeding. BM samples were shipped frozen to Memorial University, and immediately stored at - 80^{o}C until analysis.

4.2.2 Preparation of BM whey

BM whey was separated from the whole BM to prevent the interference of milk fat with immunoassays (Chan et al., 2018). BM whey was separated from whole BM, according to the method described (Yuksel et al., 2015), and modified by (Vaidya & Cheema, 2018). Briefly, whole BM was centrifuged at 500 x *g* for 25 minutes at 4^{o}C to remove cellular components. The supernatants were then centrifuged at 13,700 x *g* for 10 minutes at 4^{o}C to separate the whey from the lipid layer. Aqueous whey (clear phase) was pooled based on the n-6:n-3 PUFA (low and high) separately for the lean and obese women, and divided into

aliquots to avoid repeated freeze-thaw cycles, and stored at -80ºC until needed for further analysis.

4.2.3 Validation of thiobarbituric acid reactive substances (TBARS) assay for measuring malondialdehyde (MDA) levels

A TBARS assay kit (Cat No: KGE013, R & D Systems, Minnesota, USA) was standardized for MDA analysis in human BM.

4.2.3.1 Spike and recovery assay to measure MDA levels in BM

The spike and recovering protocol for validating untested samples were carried out following the protocol outlined in the kit:

Samples required acid treatment prior to assay to clarify the sample by precipitating interfering proteins and other substances. To acidify the BM samples, 1 ml of TBARS acid reagent from the kit (see section 4.2.3) was added to 1 ml of BM whey, mixed gently, and incubated at room temperature for 15 minutes. Samples were then centrifuged at 12,000 x *g* for 4 mins, and the supernatant was collected. From the spiking stock (167 µM of TBARs standard from the kit mentioned in (section 4.2.3), 15 µl was added to the 'control' and 'spike' and vortexed briefly. A 1:2 serial dilution was prepared by adding distilled water to the sample spike, control spike, and unspiked sample. The concentration of the treated samples was calculated from the standard curve and multiplied by a dilution factor of 2. Recoveries for the spiked samples were calculated by measuring the recovery of the spiked diluent control. After the validation, percentage recovery was within the recommended range of 80-120 % (Fig 4.1); hence the kit was validated and used to measure MDA levels in BM.

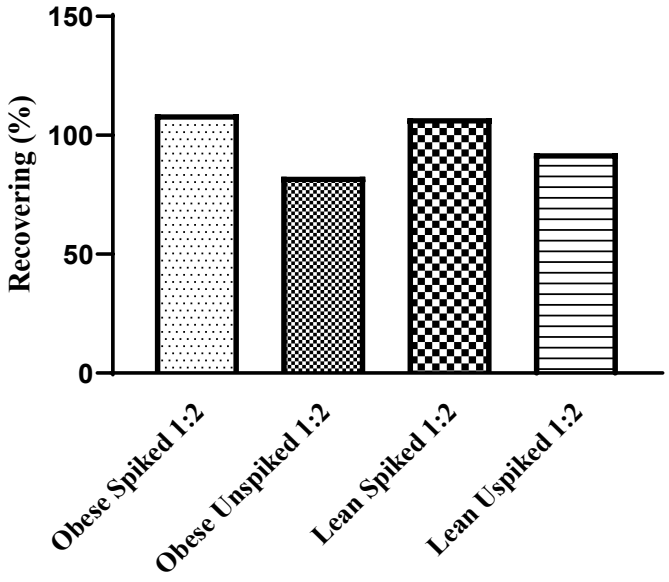

Figure 4.1 *Validation of linearity of MDA assay in breast milk*

4.2.3.2 Measurement of MDA levels in BM

The TBARS assay kit (standardized above) was used to measure MDA levels in BM whey. The standard was converted to MDA by adding 100 µl of TBARs standard to 200 µl of TBARs acid reagent to produce a stock solution of 167 µM. Serial dilution of the standard was prepared; 150 µl of standards and 150 µl of the acidified BM were added to separate wells in a 96 well microplate for standard curve and samples. TBA reagent (75 µl) was added to each well, and the pre-OD of each well was read at 532 nm. The microplate was covered with adhesive strips and incubated for 2 hours at 55ºC before measuring optical density at 532 nm. The pre-OD readings were subtracted from the OD after 2 hours of incubation to get the final concentration and multiplied by the dilution factor to determine the MDA concentration in the samples. Data were expressed as micromole (µM).

4.2.3 Fatty acids analysis of BM samples

Fatty acid analysis of BM samples was performed by Dr. Raymond Thomas's research group (Department of Boreal Ecosystem and Agricultural Science, Grenfell Campus, Memorial University), according to the method of Vidal et al. (Prieto Vidal et al., 2018). Briefly, GC-MS/FID analysis was conducted on a Thermo Scientific Trace 1300 gas chromatography (GC) (Mississauga, ON, Canada) coupled to a Thermo Scientific TSQ 8000 flame ionization detector (FID). Methylated fatty acids were separated with a BPX70 high-resolution column (10 m × 0.1 mm ID × 0.2 μm) (Canadian Life Science, Canada) using helium as the carrier gas at a flow rate of 1 ml/min. One (1 μl) of each sample was applied to the injection system in split mode (15:1) using a Tri-plus auto-sampler (Thermo Scientific, Canada). The oven temperature was programmed as follow: the initial oven temperature of 50°C was held for 0.75 min, then programmed to increase at 40°C/min to 155°C, then increased at 6°C/min to 210°C, and a further increase at 15°C/min to 250°C, where it was held for 2 min, total time: 17 mins. The methylated fatty acids were determined from a comparison of retention times and mass spectra obtained from commercial standards (Supelco 37 component mix, Supelco PUFA No. 3, Supelco FAME mix C8-C24; Sigma Aldrich, Canada) and the NIST database (ThermoScientific, Canada). The amounts of individual fatty acids identified were calculated using standard curves prepared from the standard mixtures and values were presented as % nmol for each BM sample.

4.2.4 Treatment of 3T3-L1 pre-adipocytes with BM

BM samples from both lean and obese women were each grouped into quartiles: low (Q1) and high (Q4), based on the n-6:n-3 PUFA due to variations in the individual n-6:n-3 PUFA composition in both lean and obese BM. Samples in the 1st quartile (low) and the 4th

quartile (high) were pooled separately each for BM of obese and lean women to prepare whey, as explained above in section 4.2.2, to treat 3T3-L1 pre-adipocytes. The n-6:n-3 PUFA in BM has been shown to be directly correlated with proinflammatory cytokines in BM whey (Vaidya & Cheema, 2018). The 3T3-L1 cells were obtained from American Type Culture Collection (ATCC # CL-173, USA). Insulin, IBMX, Dex, and DMEM were obtained from Sigma Aldrich, USA. FBS, antibiotic-antimycotic (100X), and NBCS were obtained from Gibco (USA). Preadipocytes (3T3-L1) were grown to 70 % confluency and were induced to differentiate by adding the differentiation cocktail [insulin (10 µg/ml), 1 µM dex and 0.5 mM IMBX] in DMEM containing 10% FBS along with 1% BM whey as per our previous publication (Vaidya and Cheema, 2017). After 48 hours, the media were changed to DMEM containing 10% FBS and insulin (10 µg/ml) along with 1 % (v/v) BM whey. The media were replaced with DMEM containing 10 % FBS + 1 % BM whey every 48 hours till day 8 when the cells were fully differentiated and then harvested. Control cells received media only (no treatment). The experimental design is shown in Fig 4.2.

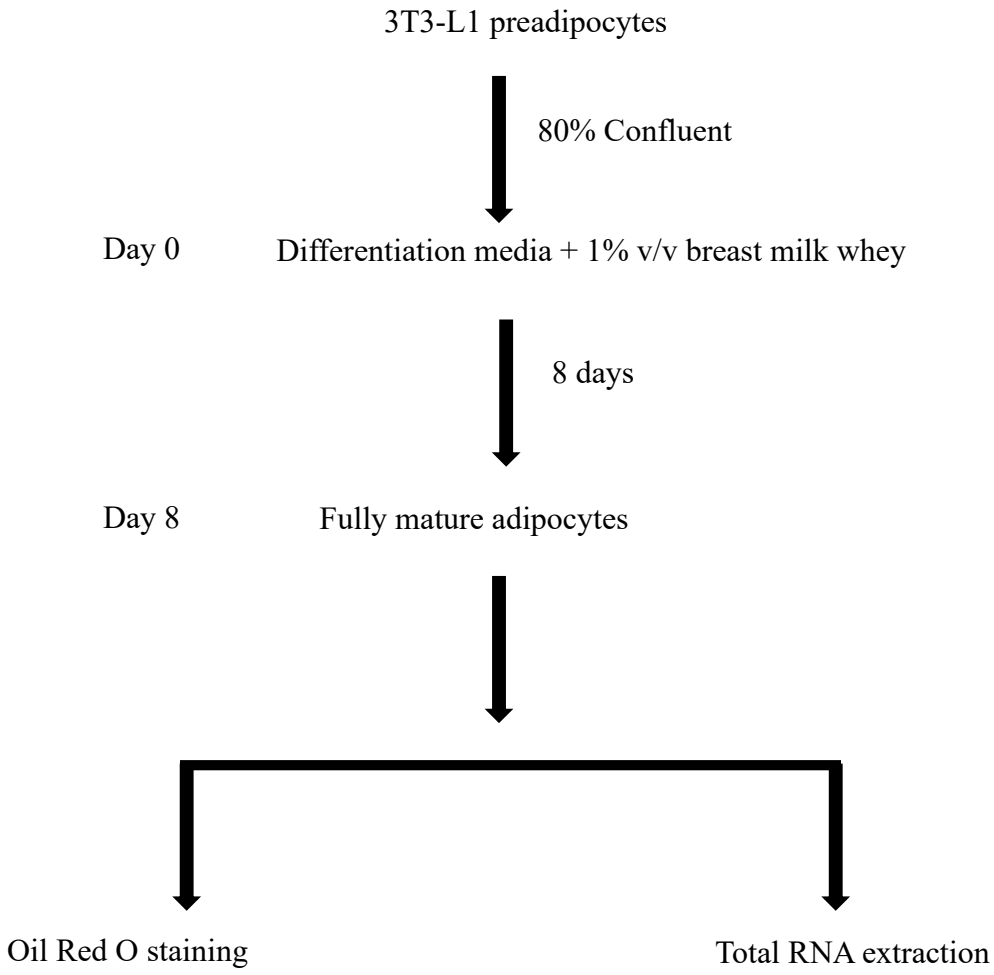

Figure 4.2 Schematic representation of experimental design to investigate the effects of breast milk from lean and obese women with high and low n-6:n-3 PUFA on adipogenesis.

n-3 = omega- 3, n-6 = Omega -6, PUFA = polyunsaturated fatty acids

4.2.5 Oil-Red-O staining

Lipid accumulation in 3T3-L1 mature adipocytes was measured using an Oil-Red-O solution in 0.5 % isopropanol (Millipore, Canada). After day 8 of differentiation, cells were washed thrice with 1X phosphate-buffered saline (PBS), and 1ml of Oil-Red-O dye was added; cells were incubated for 15 min at room temperature. The dye was removed, and cells were washed with 2 ml of distilled water to remove the non-binding dye. Stained adipocytes were viewed using a Leica DMIL LED Tissue Culture Microscope (Leica Microsystems Inc, Canada), and images (3 per well) were taken using Infinity Camera Analyze Software (Lumenera Corporation, Canada) at 400X magnification. Stained lipids were eluted by adding 500 µl of 100 % isopropanol to each well, and the plate was set on a shaker for 30 min. The absorbance of dye extract was measured using a spectrophotometer at 520 nm, and isopropanol was used as a blank.

4.2.6 RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from mature 3T3-L1 cells on day 8 using TRIzol (Chomczynski & Sacchi, 1987). Primers used for qPCR were designed using NCBI primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast/) and obtained from IDT Technologies (Coralville, IA, USA); primer sequences are given in Table 4.1. Amplification was performed using iQ SYBR Green Supermix (#1708880, Bio-Rad, USA) with a reaction volume of 10 µl and 50 ng cDNA per reaction. Samples were run for 40 cycles (denaturation at 95ºC for 15mins, annealing at 58-60ºC for 15 seconds and extension at 72ºC for 15 seconds) using the CFX96^{TM} Real-Time System. Data were analyzed using the CFX Manager^{TM} Software Version 3.0. The delta Ct values for each gene of interest were recorded, and the gene expression was normalized against RPLP0 (large ribosomal protein) as the housekeeping

gene. The expression levels were measured using the ΔΔCt method (Livak & Schmittgen, 2001).

4.2.7 Statistical Analysis.

Data were analyzed using Graph Pad Prism Software (version 6). MDA and fatty acids analysis between BM of lean and obese women was carried out using Student Unpaired T-test. Gene expression data were analyzed using two-way ANOVA followed by Bonferroni post hoc multiple comparisons to compare the main effects of quartiles, maternal status, and interaction between them. For all experiments, results were presented as mean ± standard deviation (SD), n=3, and each experiment was repeated twice. $P < 0.05$ was considered significant.

Table 4.1 Primer sequence for qPCR

Gene	Sequence		Ascension No
Acc1	5'-GGCCAGTGCTATGCTGAGAT-3'	Forward	XM_006531957.3
	5'-AGGGTCAAGTGCTGCTCCA-3'	Reverse	
Atgl	5'-GGTTCAGTAGGCCATTCCTC-3'	Forward	NR_028142.1
	5-GGTTCAGTAGGCCATTCCTC-3'	Reverse	
Fasn	5'-CTGCGGAAACTTCAGGAAATG-3'	Forward	NM_007988.3
	5'-GGTTCGGAATGCTATCCAGG-3'	Reverse	
Hsl	5'-AGACACCAGCCAACGGAT-3'	Forward	XM_006539572.3
	5'-GGGCATAGTAGGCCATAGCA-3'	Reverse	
Mif	5'-TCAAGCGAAGGTGGAACCGTT-3'	Forward	NM_010798.3
	5'-CGGACCGGGTCTACATCAA-3'	Reverse	
Pparg	5'-GAGCTGACCCAATGGTTGCTG-3'	Forward	XM_017321456.1
	5'-GCTTCAATCGGATGGTTCTTC-3'	Reverse	
Plin1	5'-TGCTGGATGGAGACCTC-3'	Forward	XM_011250776.3
	5'-ACCGGCTCCATGCTCCA-3'	Reverse	
Rplp0	5'-AATTTCAATGGTGCCTCTGG-3'	Forward	NM_007475.5
	5'-TCACTGTGCCAGCTCAGAAC-3'	Reverse	
Scd1	5'-CACCTGCCTCTTCGGGATTT-3'	Forward	NM_009127.4
	5'-CTTGACAGCCGGGTGTTTG-3'	Reverse	

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

Acc1, acetyl-CoA carboxylase; Atgl, Adipose triglyceride lipase; Fasn, fatty acid synthase; Hsl, Hormone-sensitive lipase; Mif, macrophage inhibitory factor; Pparg, peroxisome proliferator-activated receptor; Plin1, Perilipin 1; Rplp0, ribosomal protein large. SCD1, stearoyl-CoA desaturase.

4.3 Results

4.3.1 Effects of maternal obesity on fatty acids composition of BM

The fatty acid composition of BM of lean and obese women is shown in Table 4.2. There was no difference in C14:0 and C16:0 between the BM of lean and obese women. However, BM from lean women had higher C18:0 ($p<0.05$), compared to BM from obese women. There was no significant difference in the total SFA. There was no difference in C16:1; however, BM from lean women had higher C18:1 ($p<0.05$), compared to BM from obese women. There was no significant difference in the total MUFA levels in the BM of lean and obese women. There was no difference in AA (C20:4n6) levels between the BM of lean and obese women; however, BM from obese women had significantly ($p<0.01$) higher LA (C18:2n6 and total n-6 PUFA ($p<0.05$), compared to BM from lean women. There was no difference in DHA, EPA, and total n-3 PUFA between lean and obese BM; however, BM from obese women had a higher ($p<0.05$) n-6 to n-3 PUFA ratio (9.15), compared to BM from lean women (7.97).

BM samples from lean and obese women were grouped into quartiles (low and high) based on the n-6 to n-3 PUFA due to variations among the individual samples. The fatty acid composition of BM based on quartile is given in Table 4.3. BM samples of lean women grouped into quartile 1 had n-6:n-3 PUFA of 6.77 (low; designated as Q1), while quartile 4 (high; designated as Q4) had n-6:n-3 PUFA of 9.76; BM of obese women in quartile 1 (low; designated as Q1) had n-6:n-3 PUFA of 7.63, while quartile 4 (high; designated as Q4) had n-6:n-3 PUFA of 11.35.

Table 4.2 The fatty acids composition of breast milk of lean and obese women

Fatty Acids (% nmol)	Lean	Obese
C16:0	22.57 ± 2.45	21.41 ± 2.96
C18:0	6.77 ± 1.08[a]	5.62 ±0.99[b]
C16:1	2.55 ± 0.74	2.49 ± 0.76
C18:1	33.02 ± 3.19[a]	30.46 ± 3.96[b]
C20:1n9	0.35 ± 0.06	0.33 ± 0.06
C18:2n6	22.98 ± 4.55[a]	27.10 ± 4.23[b]
C18:3n6	0.26 ± 0.04	0.28 ± 0.06
C20:3n6	0.72 ± 0.09	0.83 ± 0.12
C20:4n6	0.75 ± 0.11	0.79 ± 0.12
C18:3n3	1.71 ± 0.53	1.81 ± 0.46
C20:3n3	0.19 ± 0.04	0.20 ± 0.04
C20:4n3	0.16 ± 0.02	0.16 ± 0.02
C20:5n3	0.23 ± 0.06	0.22 ± 0.05
C22:5n3	0.34 ± 0.06	0.36 ± 0.08
C22:6n3	0.46 ± 0.11	0.41 ± 0.12
ΣSFA	36.27 ± 3.77	34.56 ± 4.66
ΣMUFA	35.92 ± 3.12	33.28 ± 6.68
ΣPUFA	27.81 ± 5.04[b]	32.16 ± 4.52[a]
Σn6-PUFA	24.70 ± 4.58[b]	29.00 ± 4.24[a]
Σn3-PUFA	3.11 ± 0.59	3.17 ± 0.49
n-6:n-3 PUFA	7.95 ± 1.38[a]	9.15 ± 1.56[b]

**Total lipids were extracted from breast milk samples as described in section 4.2.4. Data were expressed as % nmol of the total extracted fatty acids; values are expressed as mean ± SD, n = 17-20. Data were analyzed by Student T-test, and superscripts (a,b) were used to denote significant differences between the treatment group. FA = fatty acids, ΣSFA = sum of saturated fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ΣPUFA = sum of polyunsaturated fatty acids, Σn-6 = sum of omega-6 PUFA, Σn-3 = sum of omega-3 polyunsaturated fatty acids, P<0.05 was considered significant.*

Table 4.3 The fatty acids composition of breast milk by low and high n-6:n-3 PUFA

	Lean		Obese	
FA (% nmol)	Q1	Q4	Q1	Q4
C14:0	8.00 ± 1.74[a]	5.96 ± 1.03[b]	7.56 ± 0.96[ab]	7.50 ± 3.20[ab]
C16:0	23.90 ± 2.44	20.78 ± 1.12	22.51 ± 1.08	20.49 ± 1.49
C18:0	7.80 ± 1.06	6.17 ± 0.98	6.03 ± 0.83	5.99 ± 1.25
C16:1	2.45 ± 0.16	2.56 ± 0.64	2.63 ± 0.41	2.00 ± 0.79
C18:1	33.35 ± 2.71	33.65 ± 4.97	30.29 ± 2.53	32.05 ± 3.76
C20:1n9	0.36 ± 0.06	0.37 ± 0.09	0.30 ± 0.02	0.36 ± 0.07
C18:2n6	19.35 ± 2.88[a]	25.91 ± 4.41[b]	25.26 ± 5.03[b]	27.21 ± 5.02[b]
C18:3n6	0.26 ± 0.06	0.25 ± 0.02	0.29 ± 0.06	0.29 ± 0.06
C20:3n6	0.68 ± 0.13	0.78 ± 0.04	0.74 ± 0.07	0.78 ± 0.05
C20:4n6	0.74 ± 0.08	0.74 ± 0.13	0.84±0.12	0.77 ± 0.09
C18:3n3	1.56 ± 0.21	1.62 ± 0.64	2.01 ± 0.51	1.30 ± 0.27
C20:3n3	0.20 ± 0.04	0.17 ± 0.03	0.20 ± 0.03	0.20 ± 0.02
C20:4n3	0.18 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.15 ± 0.01
C20:5n3	0.27 ± 0.08	0.19 ± 0.03	0.25 ± 0.05	0.21 ± 0.03
C22:5n3	0.37 ± 0.07	0.31 ± 0.05	0.42 ± 0.07	0.32 ± 0.04
C22:6n3	0.52 ± 0.09	0.42 ± 0.12	0.51 ± 0.16	0.38 ± 0.03
ΣSFA	39.71 ± 3.26[a]	32.91 ± 2.59[b]	36.09 ± 2.42[ab]	33.98 ± 5.23[ab]
ΣMUFA	36.16 ± 2.76	36.58 ± 4.65	33.23 ± 2.28	34.41 ± 4.57
ΣPUFA	24.13 ± 3.33[b]	30.51 ± 5.16[a]	30.68 ± 5.17[a]	31.61 ± 5.41[a]
Σn6-PUFA	21.03 ± 2.88[b]	27.68 ± 4.50[a]	27.13 ± 4.90[a]	29.05 ± 5.02[a]
Σn3-PUFA	3.10 ± 0.44	2.84 ± 0.69	3.55 ± 0.34	2.56 ± 0.39
n-6:n-3 PUFA	6.77 ± 0.07[c]	9.76 ± 1.31[b]	7.63 ± 1.06[c]	11.35 ± 0.50[a]

Data were expressed as % nmol of the total extracted fatty acids, values are expressed as mean ± SD, n = 4-5. Data were analyzed by two-way ANOVA and superscripts (a,b) were used to denote significant differences between the treatment group. Q= quartile, FA = fatty acids, ΣSFA = sum of saturated fatty acids, ΣMUFA= sum of monounsaturated fatty acids, ΣPUFA = sum of polyunsaturated fatty acids, Σn-6 sum of omega-6 PUFA, Σn-3 sum of omega-3 polyunsaturated fatty acids P<0.05 was considered significant

4.3.2 MDA levels in the BM of lean and obese women

BM from obese women had significantly ($p<0.01$) higher levels of MDA, compared to the BM of lean women (Figure 4.3).

4.3.3 Effects of BM on lipid accumulation in 3T3-L1 adipocytes

There was no significant difference in lipid accumulation between the cells treated with the BM of lean and obese women (low and high n-6:n-3 PUFA) compared to control cells, as shown by the Oil Red O staining (Fig 4.4A). However, cells treated with BM from obese women with high n-6:n-3 PUFA (Q4) appeared to have a larger lipid droplet size (Fig 4.4B) compared to the cells treated with BM from lean women (Q1 and 4) and control cells.

4.3.4 Effects of BM on the mRNA expression of lipogenic genes in 3T3-L1 mature adipocytes

There was a significant effect of maternal BMI status on the mRNA expression of *Acc1*, revealing higher expression in the cells treated with BM from lean women, compared to the cells treated with BM from obese women ($p<0.01$; Fig 4.5A). There was a significant interaction between maternal BMI status and quartile (interaction, $p<0.05$) to reveal an increase in the mRNA expression of *Acc1* in cells treated with BM of lean women with high n-6:n-3 PUFA ($p<0.001$, Q4), compared to control cells and low n-6:n-3 PUFA (Q1). Interestingly, BM from obese women (both low and high n-6:n-3 PUFA (Q1 and 4) did not have an effect on the mRNA expression of *Acc1*, compared to the control cells.

There was a significant effect of maternal BMI status on the mRNA expression of *Fasn*; cells treated with BM from lean women had -a higher expression, compared to the cells treated with BM from obese women ($p<0.01$; Fig 4.5B). There was also a significant

interaction between maternal status and quartile (interaction $p<0.05$), with an increase in the mRNA expression of *Fasn* in cells treated with BM of lean women with high n-6:n-3 PUFA ($p<0.01$, Q4), compared to control cells. Interestingly, there was no significant effect of treatment with BM of obese women (both quartile 1 and 4) on the mRNA expression of *Fasn*, compared to the control cells. There was a significant effect of maternal BMI status on the mRNA expression of *Scd1*, revealing higher expression in the cells treated with BM from lean women, compared to the cells treated with BM from obese women ($p<0.01$; Fig 4.5C). There was a significant interaction between maternal BMI status and quartile (interaction, $p<0.01$), indicating an increase in the mRNA expression of *Scd1* in cells treated with BM of lean women with high n-6:n-3 PUFA ($p<0.01$, Q4), compared to control cells. Interestingly, as seen for *Acc1* and *Fasn* mRNA expression, BM of obese women (both quartile 1 and 4) did not have any effect on the mRNA expression of *Scd1*, compared to the control cells. BM from lean and obese women with high and low n-6:n-3 PUFA did not have any effect on the mRNA expression of *Pparg*, compared to control cells (Fig 4.5D).

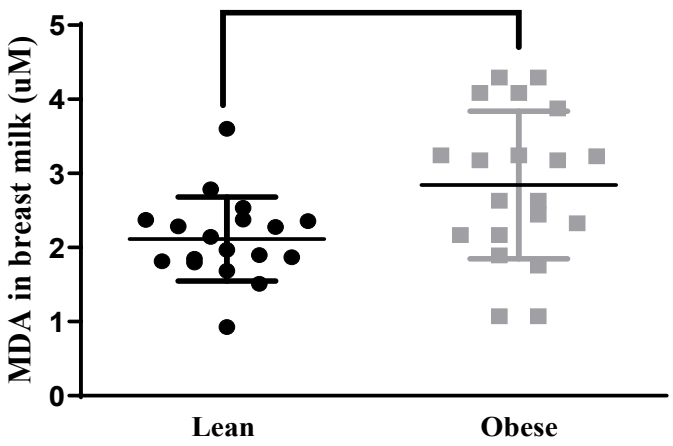

Figure 4.3 The effect of maternal obesity on malondialdehyde levels in breast milk

Malondialdehyde (MDA) levels in the breast milk of lean and obese women at one month postpartum were measured as explained in section 4.2.3.2. Data are expressed as micromoles and values are expressed as mean ± SD, n = 20. Data were analyzed using Student's t-test to determine the difference between the group, P<0.05 was considered significant.

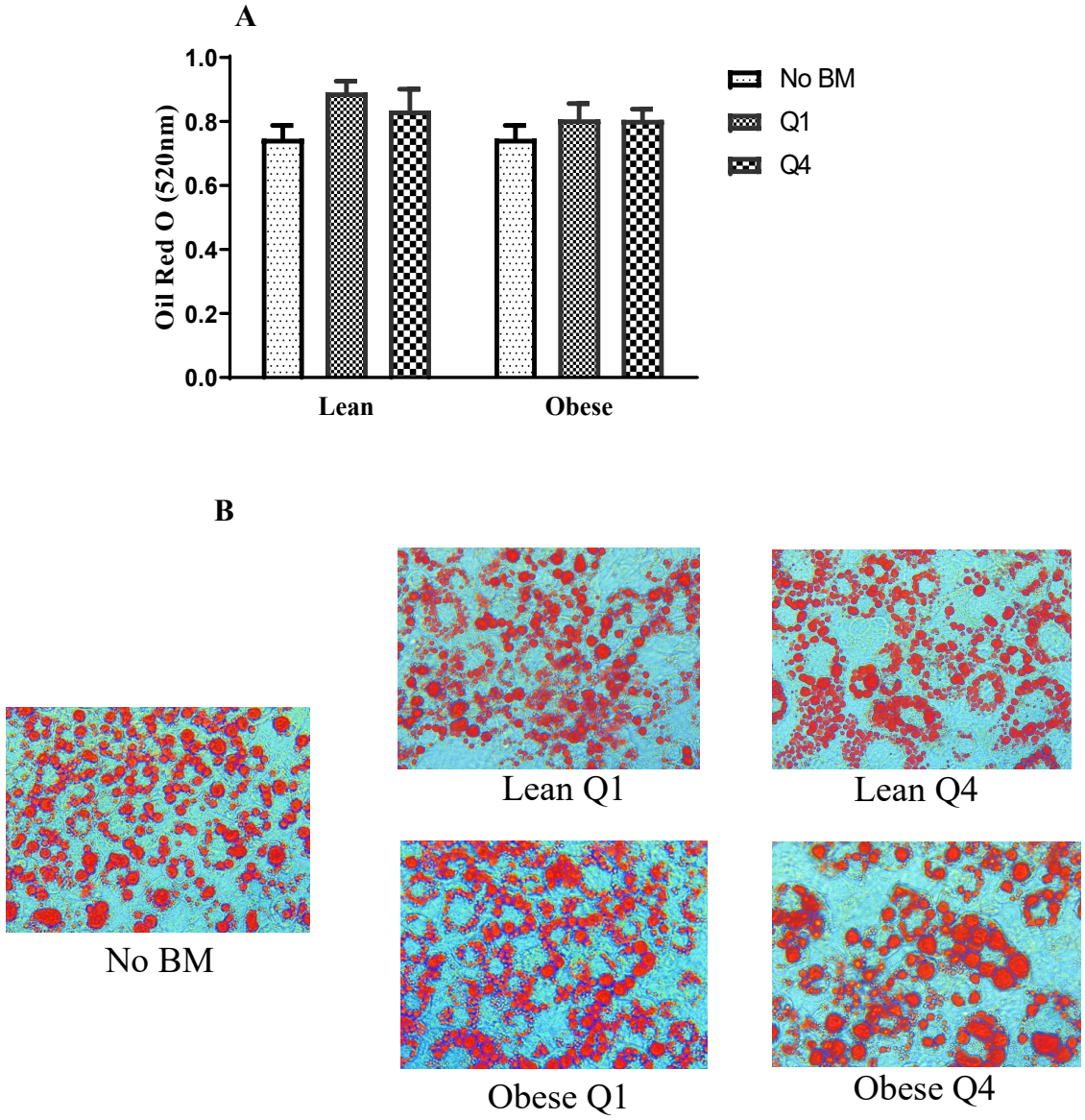

Figure 4.4 Effect of lean and obese breast milk whey with high and low n-6: n-3 PUFA on lipid accumulation in 3T3-L1 adipocytes*:*

Preadipocytes were differentiated for 8 days in the presence or absence of 1 % breast milk whey, and Oil red O staining was performed on day 8. (A) Relative lipid content measured spectrophotometrically as explained in section 4.2.6, and (B) representative images of the cells stained with Oil Red O on day 8 as explained in section 4.2.6 (400x, magnification). Values are expressed as mean ± SD, n = 3. PUFA= polyunsaturated fatty acids, Q = Quartile

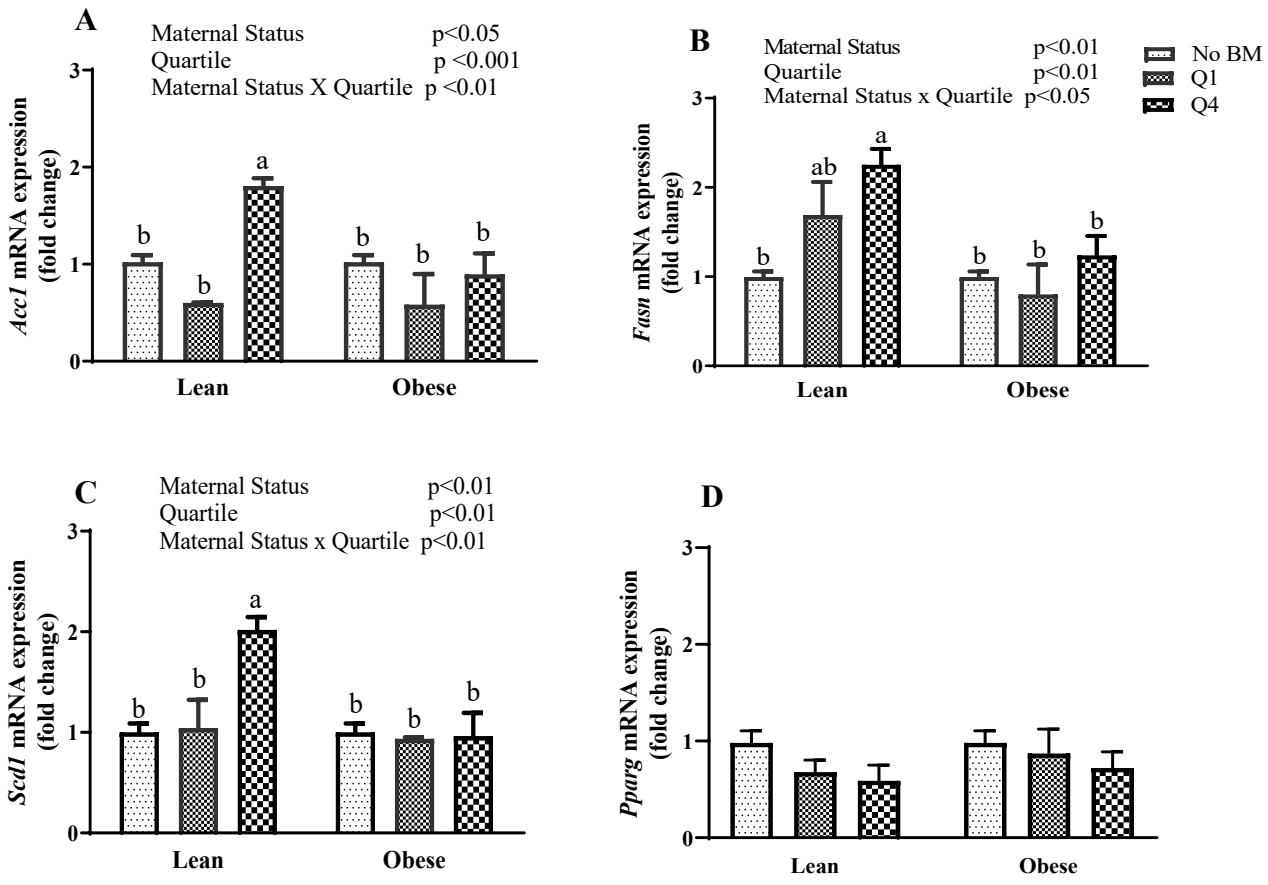

Figure 4.5 Effects of lean and obese breast milk whey with high and low n-6:n-3 PUFA on the mRNA expression of lipogenic and adipogenic genes in 3T3-L1 adipocytes

Preadipocytes were differentiated for 8 days in the presence or absence of 1 % breast milk (BM) whey, and mRNA expression was measured on day 8 as described in section 4.2.6. (A) Acetyl CoA carboxylase (Acc1), (*B) Fatty acid synthase (Fasn), (C) Stearoyl-CoA desaturase-1, and (D) Peroxisome proliferator-activated receptor (Pparg). The mRNA expression was normalized with Rplp0 as the house-keeping gene. Values are expressed as mean ± SD, n = 3. Data were assessed using Two-way ANOVA to determine the effect of treatment and Bonferroni post hoc test was used to determine differences when there was a significant interaction. Letters (a, b) represent significant differences between treatment groups, P<0.05 was considered significant. PUFA = polyunsaturated fatty acids, Q = quartile.*

4.3.5 Effects of lean and obese BM whey with high and low n-6:n-3 PUFA on the mRNA expression of lipolytic genes in 3T3-L1 mature adipocytes

BM whey from lean and obese women with both high (Q4) and low (Q1) n-6:n-3 PUFA downregulated the mRNA expression of *Plin1* ($p<0.01$, Fig 4.6A), compared to control cells. There was no effect of the BM of lean and obese women on the mRNA expression of *Atgl* (Fig 4.6B) and *Hsl* (Fig 4.6C).

4.3.6 Effects of BM of lean and obese women with high and low n-6:n-3 PUFA on the mRNA expression of macrophage inhibitory factor in 3T3-L1 mature adipocytes

There was a significant effect of maternal BMI status ($p<0.05$) on the mRNA expression of macrophage inhibitory factor (*Mif*), revealing higher expression in the cells treated with BM from obese women with high n-6:n-3 PUFA (Q4), compared to cells treated with BM from obese women with low n-6:n-3 (Q1) (Fig 4.7A). Although the 2-way ANOVA shows no statistical difference between the BM of obese women with high n-6:n-3 PUFA treatment and control cells, a Student's t-test revealed that BM from obese women with high n-6:n-3 PUFA had significantly higher mRNA expression of *Mif*, compared to the control cells ($p=0.0031$, Fig 4.7B).

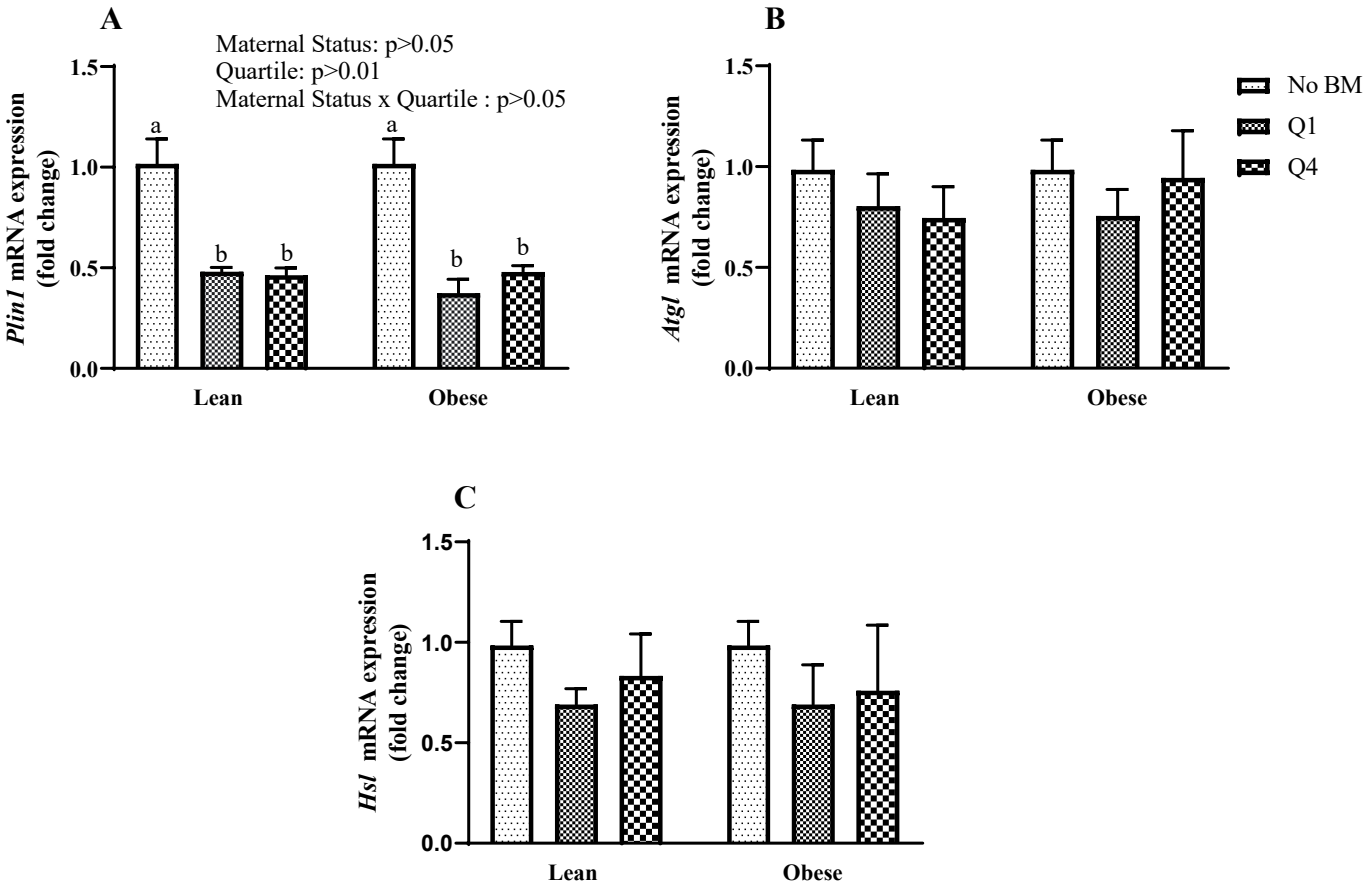

Figure 4.6 Effects of lean and obese breast milk whey with high and low n-6:n-3 PUFA on the mRNA expression of lipolytic genes in 3T3-L1 adipocytes

Preadipocytes were differentiated for 8 days in the presence or absence of 1 % breast milk (BM) whey, and the mRNA expression was measured on day 8 as explained in section 4.2.7 (A) Perilipin (Plin1) (*B) adipose triglyceride lipase (Atgl)* and *(C) hormone-sensitive lipase (Hsl). The gene expression was normalized with RPLPO as the house-keeping gene. Values are expressed as mean ± SD, n = 3. Data were assessed using Two-way ANOVA to determine the effect of treatment, and the Bonferroni post hoc test was used to determine differences when there was a significant interaction. Letters (a, b) represent significant differences between treatment groups, P<0.05 was considered significant. PUFA = polyunsaturated fatty acids, Q= Quartile*

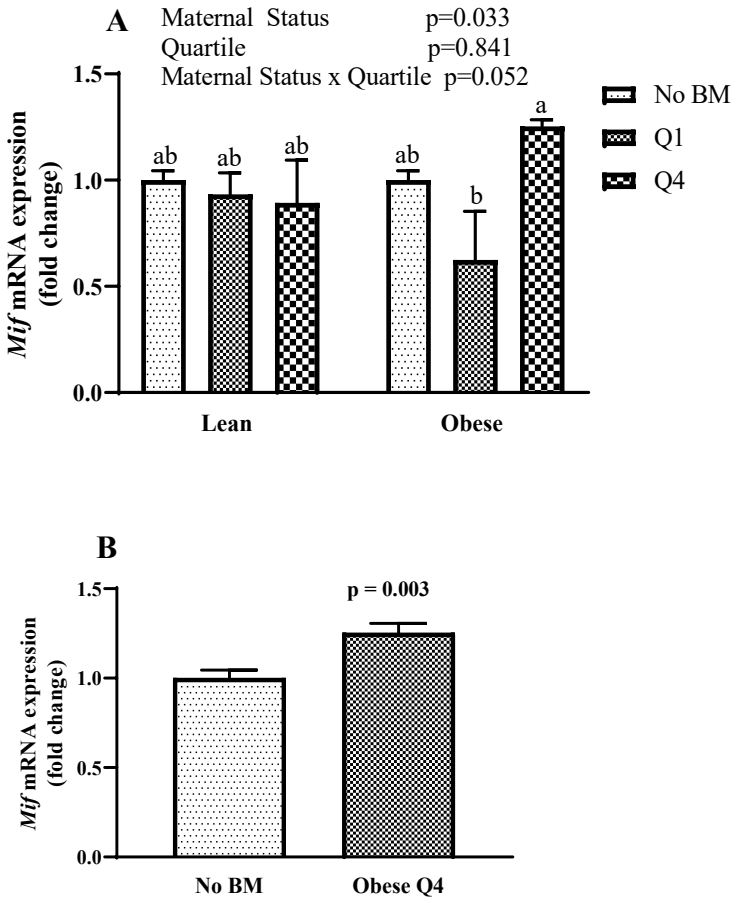

Figure 4.7 Effects of lean and obese breast milk whey with high and low n-6:n-3 PUFA on the mRNA expression of Mif in 3T3-L1 adipocytes

Preadipocytes were differentiated for 8 days in the presence or absence of 1 % breast milk (BM) whey and the mRNA expression was measured on day 8 as explained in section 4.2.7 (A) Macrophage inhibitory factor (Mif, all groups) and (B) Macrophage Inhibitory factor (Mif, control and Obese Q4) normalized with RPLP0 as the house-keeping gene. Values are expressed as mean ± SD, n = 3. Data were assessed using Two-way ANOVA to determine the effect of treatment (A). Student's t-test was used to analyze differences between control cells and obese Q4 (high n-6:n-3 PUFA) (B). Letters (a, b) represent significant differences between treatment groups, P<0.05 was considered significant. PUFA = polyunsaturated fatty acids, Q = quartile.

4.4 Discussion

Our laboratory has previously shown that BM from healthy women from Newfoundland and Labrador had higher n-6:n-3 PUFA, compared to other Canadian populations (Vaidya et al., 2016), and that BM with high n-6:n-3 PUFA induced events similar to insulin resistance (upregulation of SCD1 and DGAT2 mRNA expression) in 3T3-L1 adipocytes (Vaidya & Cheema, 2018). In this study, the effects of BM composition, particularly the n-6:n-3 PUFA, on lipogenic and lipolytic gene expression were investigated using 3T3-L1 adipocytes. Our findings have shown that the BM of obese women had higher MDA and n-6:n-3 PUFA compared to BM from lean women at one month postpartum. Furthermore, BM of lean women with high n-6:n-3 PUFA increased the mRNA expression of lipogenic genes, compared to BM of lean women with low n-6:n-3 PUFA.

The fat component of BM is variable (Saarela et al., 2005), and it reflects changes in the maternal diet (Bravi et al., 2016), particularly in the consumption of LC PUFA. We found that BM of obese women had higher levels of LA (C18:2n6), compared to the BM of lean women. LA is the principal n-6 PUFA consumed in a Western diet (Innes & Calder, 2018), and is the precursor for AA. The conversion of LA to AA occurs by sequential delta Δ-6 desaturation, elongation and Δ-5 desaturase reaction (Sprecher, 2000). Eicosanoids derived from AA play a role in infants' immune response by mediating and regulating inflammation (Hadley et al., 2016). We did not find differences in the AA levels in the BM of lean and obese women, suggesting that maternal obesity has minimal effect on AA levels. Similarly, there was a statistical difference between DHA levels in the BM of obese and lean women. Panagos et al. have reported that BM from obese women had lower DHA, compared to lean women at 2 months postpartum (Panagos et al., 2016). Both AA and DHA are involved in the

development of infant neural and immune systems (Koletzko, 2016). After birth, LCPUFA is transferred to infants from BM (Herrera, 2002). The composition of n-6, n-3 and the n-6:n-3 PUFA in BM correlates with LC PUFA in maternal plasma at 6 weeks postpartum (Much et al., 2013). The n-6 PUFA are generally pro-inflammatory in nature and are implicated in oxidative stress (DiNicolantonio & O'Keefe, 2018; Henniget al., 2001).

The n-6:n-3 PUFA in BM is associated with an increased risk of allergic diseases in infants (Waidyatillake et al., 2018). Generally, an increase in n-6:n-3 PUFA is associated with obesity and inflammation (Simopoulos, 2016). We found that BM from obese women had higher n-6:n-3 PUFA, compared to BM of lean women. This finding is similar to that obtained by Panagos et al., who also reported that BM of obese women has a pro-inflammatory fatty acid profile (Panagos et al., 2016). Thus, maternal obesity appears to impact the fatty acids composition of BM. Early infant adipose deposition is associated with n-6:n-3 PUFA in BM independent of maternal BMI at 2 weeks and 4 months postpartum (Rudolph et al., 2017). It has previously been reported that there is no correlation between n-6:n-3 PUFA in BM of obese women and infant growth at 4-8 weeks postpartum (Nuss et al., 2019). Our collaborator Dr. Sen is following up with the children born to these mothers; thus, we will be able to make an association between the BM composition and obesity in these children in our future studies.

MDA is a major secondary product of lipid peroxidation (Gallardo et al., 2015; Negro et al., 2017; Yuksel et al., 2015). I found that the BM of obese women had elevated levels of MDA (2.76 ± 0.51 μM), compared to BM of lean women (2.11 ± 0.31 μM) at one month postpartum. Our results are consistent with others who reported MDA values of 2.14 ± 0.11 μM in the BM of non-obese women (Yuksel et al., 2015). Normal range of MDA values in

BM at 1 month post-partum is 2.11 - 2.16 μM (Mahdavi et al., 2017; Nikniaz et al., 2013) Ahmed et al. reported that serum of obese and overweight individuals has higher levels of MDA, compared to normal-weight individuals (Ahmed et al., 2016). Furthermore, MDA levels of newborns increased with maternal BMI (Gallardo et al., 2015), suggesting that higher maternal MDA is an indication of the infant's oxidative stress levels. PUFA are prone to peroxidation due to the presence of a double bond in the chain leading to oxidative stress. Lipid peroxides may affect the infant's intestinal homeostasis due to the production of reactive oxygen species (Kwiecien et al., 2014). Regulation of oxidative stress is important in maintaining cell proliferation and differentiation during embryonic development and early infancy (Dennery, 2007). However, high oxidative stress in the newborn is a risk factor for tissue damage in early infancy and later in adolescence (Buonocore et al., 2017). There is a high correlation between leptin and MDA levels in the blood of obese individuals (Ahmed et al., 2016); other studies also link leptin to oxidative stress (Berger & Polotsky, 2018; Fortuño et al., 2010; Morawietz et al., 2006). BM of obese mothers has elevated leptin concentration, compared to normal weight mothers (De Luca et al., 2016). Increased oxidative stress, as demonstrated by high levels of MDA in BM of obese women, may be regulated by hyperleptinemia. (Berger & Polotsky, 2018). Our findings show that the BM of obese women has higher levels of lipid peroxides, which could potentially affect the health of the newborn.

Our laboratory has previously reported that BM with high n-6:n-3 PUFA induces events similar to insulin resistance (upregulation of SCD1 and DGAT2 mRNA expression)in 3T3-L1 adipocytes (Vaidya & Cheema, 2018). Thus, I investigated the effects of low and high n-6:n-3 PUFA in the BM of lean and obese women on adipogenesis in 3T3-L1 cells. Cells treated with BM from obese women with a high n-6:n-3 PUFA appeared to have larger

lipid droplet size, compared to cells treated with lean BM and control (no treatment); however, there was no difference in total lipid accumulation using Oil Red O quantification among the treatment groups. Lipid accumulation in the adipose occurs through the sequential expression of genes involved in TAG synthesis (Hellerstein, 1999; Nagai et al., 2018). *Acc1* is the rate-limiting enzyme in lipogenesis that catalyzes the conversion of acetyl-CoA to malonyl-CoA (Brownsey et al., 2006), which is provided as a substrate for fatty acid synthesis by *Fasn* (Menendez et al., 2009). BM from lean women with high (Q4) n-6:n-3 PUFA increased the mRNA expression of *Acc1* and *Fasn*, compared to control cells and the low n-6:n-3 PUFA. BM from obese women with high n-6:n-3 PUFA did not have any effect on the mRNA expression of *Acc1* and *Fasn*, compared to the control cells and the low n-6:n-3 PUFA. Increased expression of *Acc1* would imply an increase in lipogenesis (Song et al., 2018). SCD1 catalyzes the rate-limiting step in the conversion of SFA (C16:0) into MUFA (palmitoleic acid and oleic acid) (Liu et al., 2011). BM from lean women with high n-6:n-3 PUFA upregulated the mRNA expression of *Scd1*, compared to control cells and low n-6:n-3 PUFA. These results are similar to those previously reported from our laboratory, where BM from healthy women of NL with high n-6:n-3 PUFA upregulated *Acc1* and *Scd1* mRNA expression (Vaidya & Cheema, 2018). High expression of *Scd1* is correlated with insulin resistance (ALJohani et al., 2017; Liu et al., 2011; Popeijus et al., 2008). BM from obese women with high n-6:n-3 PUFA did not have any effect on the mRNA expression *Scd1*, similar to the results obtained for *Fasn* and *Acc1* mRNA expression. Our findings suggest that a higher n-6:n-3 PUFA in the BM of lean women is associated with increased lipogenesis; however, this is not true for BM from obese women. Despite an increase in the mRNA expression of lipogenic genes with BM from lean women with high n-6:n-3 PUFA, there was

no difference in the mRNA expression of *Pparg*. This suggested that the regulation of lipogenic genes is independent of *Pparg* mRNA expression. Another reason for no effect on lipogenic gene expression by BM of obese women with high n-6:n-3 PUFA could be that the large lipid droplets are limiting the expansion of the TAG store. Roberts *et al*. suggested that the large adipocytes may downregulate lipogenic genes in order to limit the expansion of the TAG store (Roberts et al., 2009).

Plin1 is the dominant protein associated with the periphery of lipid droplets in adipocytes (Zhang et al., 2018), which plays a key role in regulating TAG hydrolysis by restricting access of lipolytic genes to lipid droplets, thereby preventing unrestrained basal lipolysis (Kimmel & Sztalryd, 2016). Both BM from lean and obese women with low and high n-6:n-3 PUFA downregulated the mRNA expression of *Plin1* compared to control cells. *Atgl* and *Hsl* are the dominant lipolytic genes that hydrolyze TAG into FFA and glycerol; surprisingly, there was no effect of BM from lean and obese women on the mRNA expression of *Atgl* and *Hsl*. These findings suggest that BM of lean and obese women, irrespective of their n-6:n-3 PUFA ratio have no effect on lipolysis.

As mentioned earlier, although the lipid quantification shows no difference in lipid accumulation, cells treated with BM from obese women with high n-6:n-3 PUFA appeared to have larger lipid droplets. The mRNA expression of *Mif* is associated positively with adipocyte size and negatively with insulin action (Koska et al., 2009). Thus, I measured the mRNA expression of *Mif* in cells treated with BM from lean and obese women with high and low n-6:n-3 PUFA. Cells that visually appeared to have larger lipid droplet size had higher mRNA expression of *Mif* compared to cells treated with BM of obese women with low n-6:n-

3, suggesting that the larger lipid droplets could be a result of increased *Mif* expression. Further studies need to be conducted to confirm if an increased mRNA expression of *Mif* negatively correlates positively with insulin resistance.

Apart from the n-6:n-3 PUFA in the BM of both lean and obese women, leptin, IL-1β, IL-6, and IL-8 has been shown to affect the expression of lipogenic genes. Leptin has been shown to induce fat oxidation and downregulate the expression of genes involved in lipogenesis (Bai et al., 1996; Sari, 2013; Soukas et al., 2000; Stern et al., 2016). Thus, high levels of leptin in the BM of obese women (Appendix I, performed by Dr. Sarbattama Sen's research group) may influence the lipogenic gene regulation, having a negative effect on the mRNA expression of lipogenic genes. IL-1β and IL-6 have been shown to induce insulin resistance in adipocytes (Al-Sulaiti et al., 2019; Henriksbo et al., 2019). Since BM from obese women had higher levels of IL-1β and IL-6 (Appendix I, performed by Dr. Sarbattama Sen's research group), this could have affected insulin sensitivity and the regulation of lipogenic genes in 3T3-L1 cells. There was a positive correlation between pro-inflammatory cytokines (IL-1β and IL-6) and n-6:n-3 PUFA in the BM of obese women (Appendix II). There was no correlation between n-6:n-3 PUFA with leptin and IL-8 (Appendix III)

In conclusion, our findings reveal that maternal obesity is associated with higher 6:n-3 PUFA in BM of obese women, compared to BM of lean women. We also found that higher n-6:n-3 PUFA in the BM of lean women is associated with increased lipogenesis, independent of *Pparg* gene expression (Fig 4.8). However, this effect was not observed with BM from obese women, which suggests that high levels of leptin and pro-inflammatory cytokines in the BM of obese women may induce adipocyte dysfunction The larger lipid droplet size

observed may result in dysfunction of adipocytes leading to metabolic abnormalities, thereby predisposing the infant to a higher risk of diseases in later life. Findings from this study may have implications in maternal lactation programming of transgenerational childhood obesity.

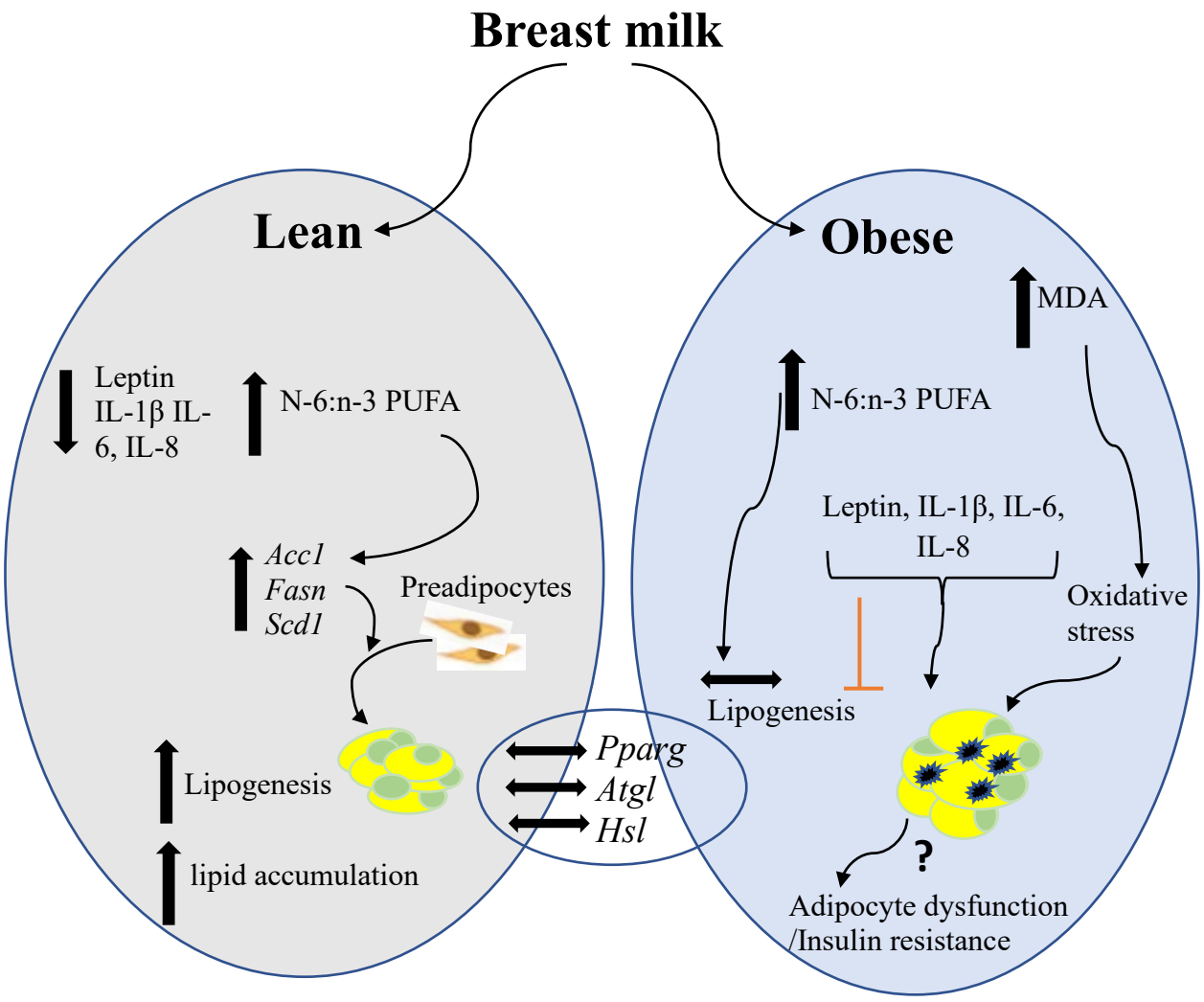

Figure 4.8 Schematic representation of the effect of maternal obesity on breast milk composition and implication in adipogenesis and lipolysis in 3T3-L1 cell

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CHAPTER FIVE

Summary and conclusions

5.1 Summary and conclusion

This thesis focused on exploring the effects of lipid emulsions and BM of lean and obese women on adipogenesis and lipolysis using 3T3-L1 adipocytes. An increase in n-6:n-3 PUFA induces adipogenesis, and it increases the risk for obesity (Simopoulos, 2016). First, I studied the effects of lipid emulsions (Intralipid, SMOFlipid, Omegaven) with n-6:n-3 PUFA of 7.8:1, 2.5:1, and 0.25:1 on the mRNA expression of genes involved in adipogenesis, lipogenesis, lipolysis, and β-oxidation. I hypothesized that lipid emulsion with low n-6:n-3 PUFA (Omegaven) will inhibit adipogenesis and increase β-oxidation, compared to SMOFlipid and Intralipid. Lipid emulsions were exposed to preadipocytes for 48 hours and then differentiated for 8 days without lipid emulsions to investigate the effect of lipid emulsions on the process of adipogenesis. Furthermore, to explore the effects of lipid emulsions on lipolysis and β-oxidation, mature adipocytes (day 8), differentiated without lipid emulsions, were treated with lipid emulsions for 48 hours to study the effects on lipolysis. Secondly, I investigated the effects of maternal obesity on BM fatty acid composition, and the regulation of the mRNA expression of lipogenic and lipolytic genes in 3T3-L1 adipocytes. I hypothesized that BM from obese women will have higher n-6:n-3 PUFA, compared to BM from lean women and that BM with higher n-6:n-3 PUFA will have a greater effect on adipogenesis and increase lipolysis.

5.1.2 Key observations

A) Lipid emulsion study

i. Treatment of 3T3-L1 preadipocytes with Omegaven increased EPA and DHA at 24 and 48 hours of treatment compared to other lipid emulsions. Treatment with Intralipid, SMOFlipid, and Omegaven increased the *Pref-1* mRNA expression at

24 hours of treatment of preadipocytes, indicating inhibition of differentiation. However, at 48 hours of treatment, Omegaven treatment decreased *Pref-1* mRNA expression, which could likely be due to increased growth arrest of the cells caused by high levels of EPA and DHA in Omegaven.

ii. Preadipocytes treated for 48 hours with lipid emulsions and then differentiated without lipid emulsions for 8 days, showed higher levels of DHA and EPA after treatment with Omegaven. Omegaven treatment also revealed a decrease in triacylglycerol levels at day 8 of differentiation. This indicates that Omegaven inhibits lipid accumulation in 3T3-L1 adipocytes.

iii. Preadipocytes treated for 48 hours with lipid emulsions and then differentiated without lipid emulsions for 8 days, showed that Omegaven treated cells downregulated the mRNA expression of *Pparg*, *LXRα*, *Srebp1c*, *Fasn*, *Scd1*, *Dgat2*. This could possibly be due to high levels of EPA and DHA in Omegaven that act as ligands for the transcription factors, thus preventing activation of the lipogenic and adipogenic genes.

iv. In mature adipocytes differentiated without lipid emulsions and then treated with lipid emulsions for 48 hours, Omegaven and SMOFlipid increased the mRNA expression of *Cpt1*, but only Omegaven treatment downregulated the mRNA expression of lipolytic genes (*Atgl* and *Hsl*).

These results support our hypotheses and show that Omegaven with low n-6:n-3 PUFA inhibits lipid accumulation and increases the mRNA expression of *Cpt1* involved in β-oxidation.

B). BM Study

i. BM from obese women had elevated levels of MDA and n-6:n-3 PUFA compared to BM of lean women. High n-6:n-3 PUFA in the BM of lean women increased the mRNA expression of lipogenic genes independent of *Pparg*. However, BM from obese women with high n-6:n-3 PUFA did not have any effect of the mRNA expression of lipogenic genes, which could likely be due to high levels of leptin and pro-inflammatory cytokines known to induce insulin resistance. However, these results are interpreted with caution, as I did not measure the independent effect of BM leptin and cytokines on lipogenic gene expression.

ii. The null effect of the BM of obese women on lipogenic gene expression is not due to increased lipolytic gene expression.

Our first hypothesis was supported by the results, with BM of obese women having higher n-6:n-3 PUFA. However, our second hypothesis on adipogenesis was only supported by the BM of lean women that had high n-6:n-3 PUFA.

5.1.3 Conclusion

The use of Omegaven for a preterm infant has been shown to improve hepatic function. Little was known on its role in adipocyte function. This study provides useful mechanistic insight into the regulation of long-term lipogenesis by early exposure to Omegaven. This study suggests that preterm infants given Omegaven will have reduced lipid accumulation, and this could potentially prevent the incidence of childhood obesity. Findings from the BM study may have implications in maternal lactation programming of transgenerational childhood obesity. The results from the lipid emulsion

and BM study suggest that the type of fatty acids that the infants are exposed to in early life could potentially disrupt adipocytes function thereby predisposing them to a higher risk of metabolic disorders in later life.

5.2 Limitations and future direction

In both studies, I only measured the mRNA expression of the lipogenic, adipogenic, and lipolytic genes. These genes may also be regulated at the post-transcriptional levels (Brunmeir & Xu, 2018; Song et al., 2018; Strable & Ntambi, 2010; Xu et al., 2013). Thus, it would be important to look at the protein expression of the genes that are affected by treatment. We observed that cells treated with the BM from obese women with high n-6:n-3 PUFA appeared to have larger lipid droplets. Thus, it will be important to measure the lipid droplet size in the future. Even though we did not find any difference in the mRNA expression of the lipolytic genes, it will be important the measure glycerol and FFA release in the media to clearly explain whether lipolysis occurred. In addition, we did not have any information on the food records of both the lean and obese women, as diet could impact BM composition. We are proposing that the BM of obese women with high levels n-6:n-3 PUFA, MDA, and pro-inflammatory cytokines might be inducing events similar to inflammation and insulin resistance in 3T3-L1 adipocytes. Further studies need to be conducted to explore glucose uptake, and the mechanisms of insulin resistance after treatment with BM of obese and lean women.

5.3 References

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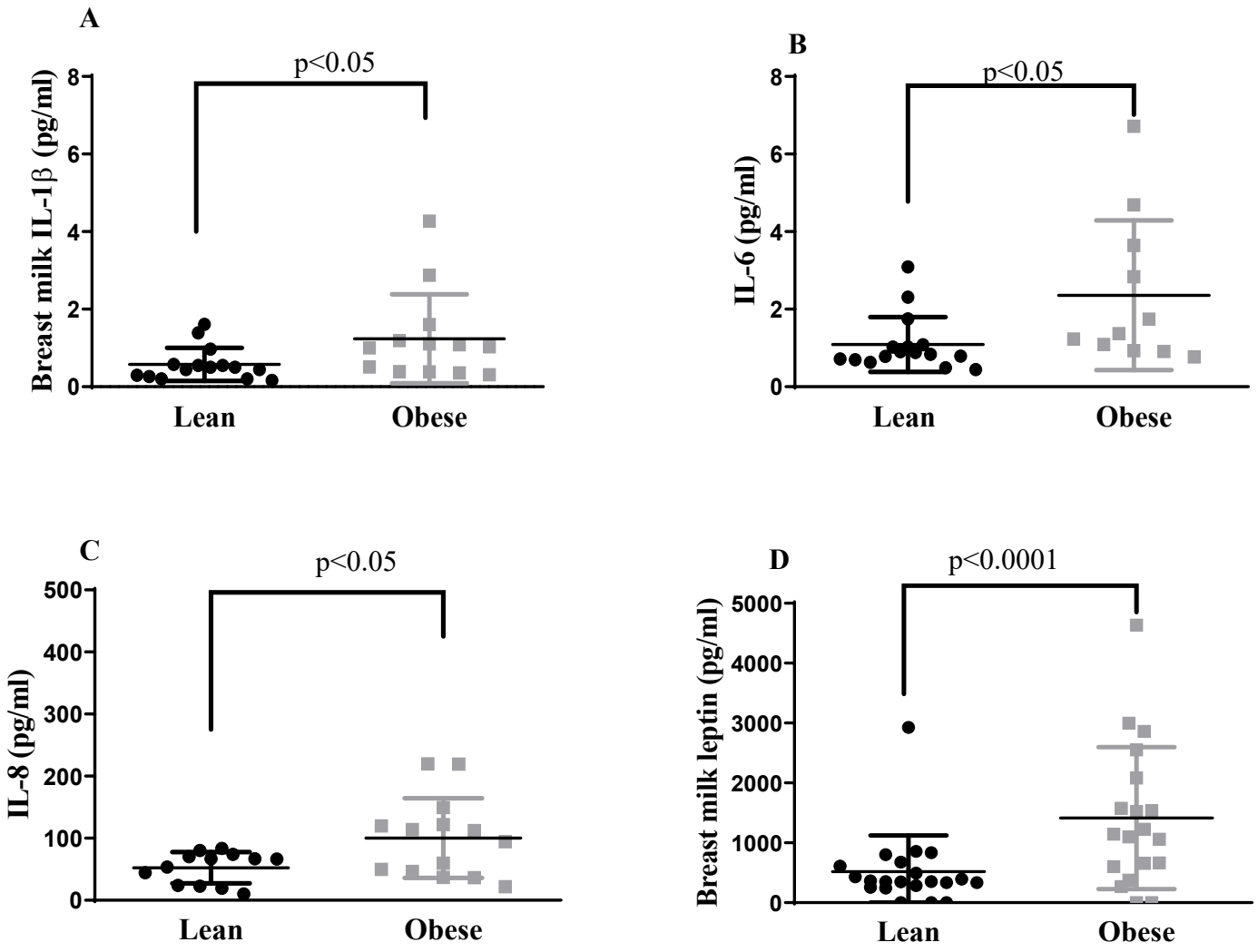
APPENDICES

The data presented in appendix 1, 2, and 3 includes BM cytokine analysis performed by Dr. Sen's research group, and the correlation of the BM cytokines with n-6:n-3 PUFA. These data are important to provide further insights into other components of BM (apart from the n-6 and n-3 fatty acids) that could potentially affect adipogenesis in 3T3-L1 cells.

Appendix I

Leptin and cytokines levels in BM

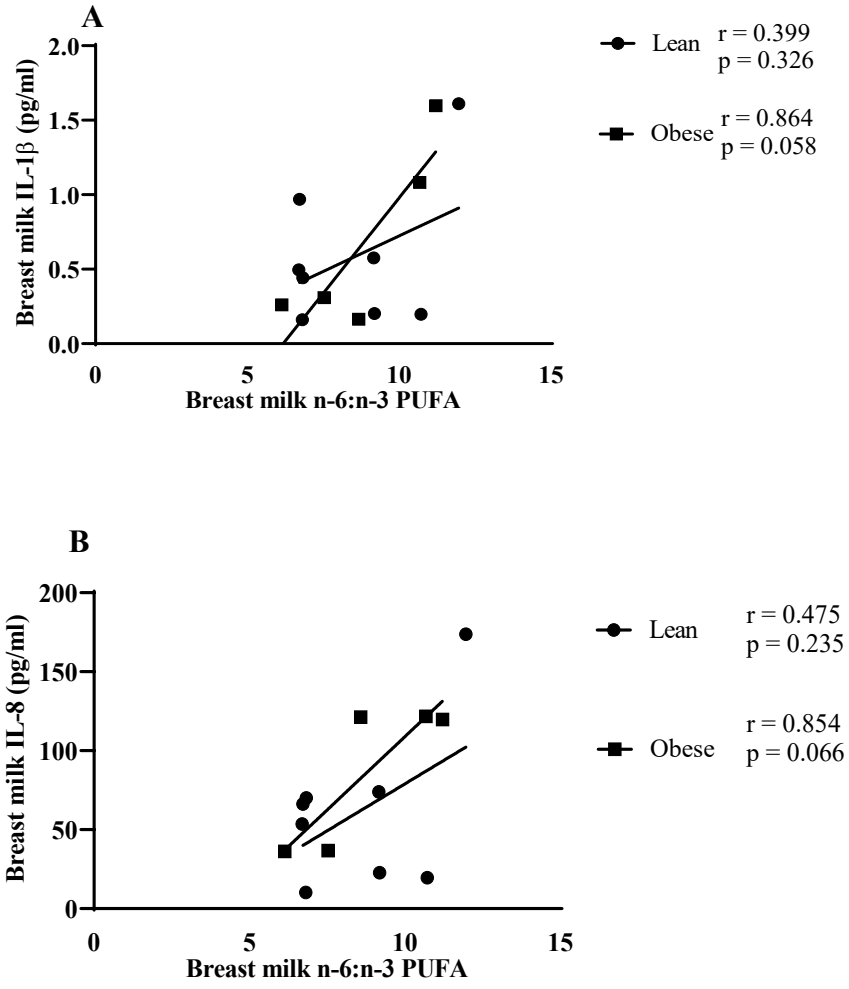
Leptin was measured individually and, cytokines (IL-1β, IL-6, and IL-8) were measured as a multiplex in the laboratory of Genital Tract Biology (Fichorova Laboratory, Brigham and Women's Hospital, Boston, MA) using electrochemiluminescence (ECL) detection technology on the Meso Scale Discovery (MSD) Sector imager S600 platform (MSD, Gaithersburg MD) as previously described (Fichorova et al., 2008; Vicier et al., 2019). All samples were assayed neat in duplicate with an analytical range of 100,000 pg/mL to 137.2 pg/mL for leptin, where the lower limit of detection (LLD) was 272 pg/mL. For IL-1β, the range was 2,356 pg/mL to 0.143 pg/mL, with an LLD of 0.029 pg/mL. For IL-6, the range was 2,884 pg/mL to 0.18 pg/ml, with an LLD of 0.18 pg/mL. For IL-8, the range was 2,212 pg/mL to 0.14 pg/mL, where the LLD was 0.084 pg/mL. A quality control pool comprised of split aliquots of the same sample was tested on each assay plate to assess inter-assay variation. The coefficient of variation for inter-assay variability of the quality control pool samples was 9 % for Leptin, 12 % for IL-1β, 16 % for IL-6 , and 10 % for IL-8.



Cytokines and leptin levels in the breast milk of lean and obese women at one month postpartum

The data represents (A) Interleukin (IL)-1β, (B) Interleukin (IL)-6, (C) Interleukin (IL-8), and (D) Leptin levels in the breast milk of lean and obese women at one month postpartum. Data are expressed as picogram per milliliters of breast milk and values are expressed as mean ± SD, n = 20. Data were analyzed using Student's t-test to determine the difference between groups, and P<0.05 was considered significant.

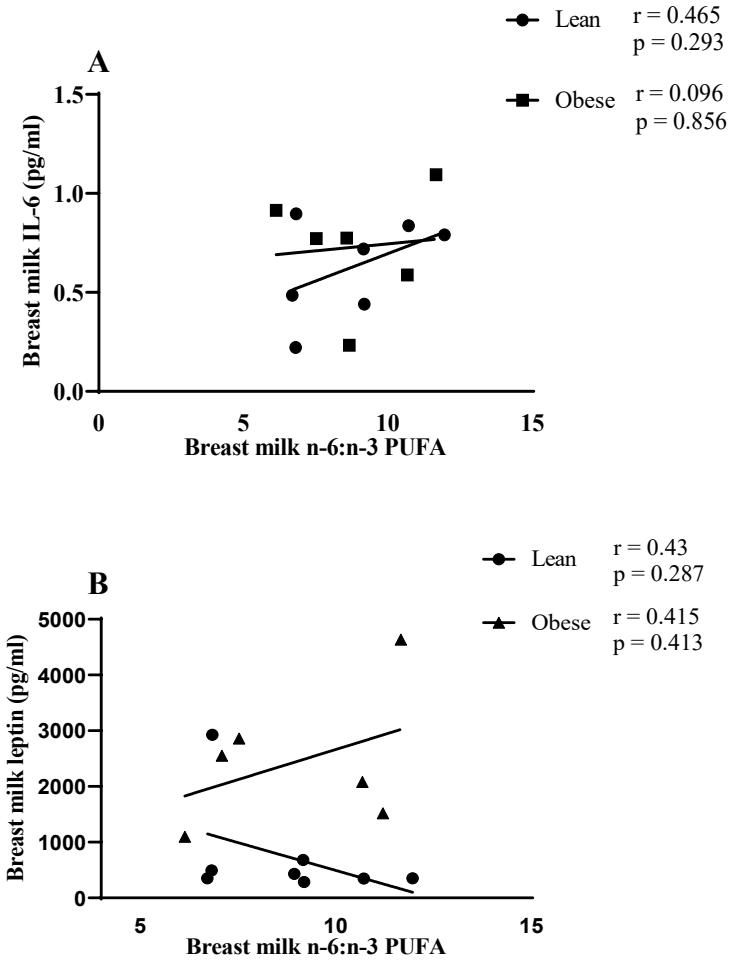
Appendix II



Correlation of n-6:n-3 PUFA with cytokines and leptin in the breast milk of lean and obese women

Correlation of n-6:n-3 PUFA with (A) Interleukin (IL)-1β and (B) Interleukin (IL)-8 in the breast milk of lean and obese women at one month postpartum. Cytokines are expressed as picogram per milliliters of breast milk. Data were analyzed using Pearson correlation. PUFA= polyunsaturated fatty acids; n-3 = Omega-3 PUFA, n-6 = Omega-6 PUFA. P<0.05 was considered significant.

Appendix III



Correlation of n-6:n-3 PUFA with IL-6 and leptin in the breast milk of lean and obese women.

Correlation of the n-6:n-3 PUFA with (A) Interleukin (IL)-6 (B) Interleukin (IL)-8. Cytokines are expressed as picogram per milliliters of breast milk. Data were analyzed using Pearson correlation. PUFA= polyunsaturated fatty acids; n-3 = Omega-3 PUFA, n-6 = Omega-6 PUFA.