DIETARY omega-3 AND omega-6 FATTY ACIDS AND NEONATAL LIVER METABOLISM

by

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ABSTRACT

It is well-known that the n-3 fatty acids are important regulators of fat and glucose metabolism in adult liver; however, to date most research on the importance of n-3 fatty acids in early development has focused on the brain, with little consideration of effects on other organs. This research addressed the importance of the essential n-3 and n-6 fatty acids for the liver during early development. A series of studies were conducted to address the impact of the amount, balance, and types of n-6 and n-3 fatty acids in the maternal diet and infant milk diet on lipids, protein abundance, gene expression, and relevant metabolites in the developing liver. Using milk-formula fed piglets, the first study demonstrated that the supply of n-6 and n-3 fatty acids impacts infant liver fatty acids, with high dietary n-6 fatty acids decreasing hepatic n-3 fatty acids in a pattern similar to n-3 fatty acid deficiency. Using the rat to address the impact of maternal fatty acid nutrition in gestation and lactation on the infant liver, the second study showed that adding n-3 fatty acids to the maternal diet lead to higher long chain n-3 fatty acids in neonatal liver, and this was associated with higher expression of enzymes of fatty acid oxidation and lower expression of enzymes of glycolysis and amino acid catabolism, with altered amino acid patterns when compared to n-3 fatty acid deficiency. In the third study, providing long chain n-3 fatty acids in the maternal diet led to marked increase in long chain n-3 fatty acids in milk and in the liver of the milk-fed rat pups, and this was associated with lower gene expression for enzymes of fatty acid synthesis and glycolysis and higher gene expression for an enzyme of ketogenesis in the neonatal liver. These studies provide new knowledge to show that the amount, types and balance of n-3 and n-6 fatty acids in the maternal and infant diet are relevant to hepatic metabolic regulation in the early postnatal period. Nutrition support of young infants should consider the needs and functions of n-3 fatty acids beyond the brain.
PREFACE

This dissertation was prepared according to the University of British Columbia Faculty of Graduate Studies requirements.

A version of Chapter 2 has been published. [Novak EM, Dyer RA, Innis SM. High dietary omega-6 fatty acids contribute to reduced docosahexaenoic acid in the developing brain and inhibit secondary neurite growth. Brain Res. 2008, 1237:136-145]. I was responsible for all data analysis and interpretation, and I wrote the manuscript together with my supervisor Dr Sheila Innis. Animal care and lipid analysis were done by members of the Innis Lab. Neuronal cell culture studies were done by Roger Dyer.

A version of Chapter 3 has been submitted for publication [Novak EM, Keller BO, Innis SM. Metabolic development in the liver and the implications of the n-3 fatty acid supply]. I conceived the study design and objectives together with Dr Innis. I prepared the experimental diets and was responsible for all aspects of animal care and sample collection. I developed methods for 2D gel separation of proteins in our laboratory, carried out all protein, gene expression and metabolite analyses and assisted with lipid and fatty acid analysis. Analysis of the 2D gels was done with assistance from Erin Lee. Identification of proteins by MALDI-TOF mass spectrometry was done by Dr Bernd Keller and Erin Lee, and HPLC analysis of amino acids was done by Dr Keller and Josephine Cheng. Lipid analysis was done with assistance from members of the Innis Lab, including Janette King, Benny Chan and Michael George. I was responsible for all data analysis and I wrote the manuscript together with Dr Keller and Dr Innis.

A version of Chapter 4 has been accepted for publication [Novak EM, Innis SM. Impact of maternal dietary n-3 and n-6 fatty acids on milk medium chain fatty acids and the implications for neonatal liver metabolism. Am J Physiol Endocrinol Metab. 2011]. I conceived the study design and objectives, together with my supervisor Dr Innis. I prepared the experimental diets
and was responsible for all aspects of animal care and sample collection, gene expression experiments, and analysis of plasma triglycerides and insulin. Human milk collection and lipid analysis were done with assistance from members of the Innis Lab, including Janette King, Benny Chan, Michael George and Shalu Duggal. I was responsible for all data analysis and I wrote the manuscript together with Dr Innis.

Ethics approval to conduct this research was obtained by the University of British Columbia Animal Care Committee; certificate number A01-0189 for studies in Chapter 2 and A06-0368 for studies in Chapters 3 and 4.
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LIST OF ABBREVIATIONS

2D: 2 dimensional
Acaca: acetyl CoA carboxylase
Acly: ATP citrate lyase
Acox1: acyl CoA oxidase
AI: adequate intakes
AMDR: acceptable macronutrient distribution range
ANOVA: analysis of variance
ATP: adenosine triphosphate
cDNA: complementary deoxyribonucleic acid
CFSII: Continuing Survey of Food Intakes by Individuals
ChREBP: carbohydrate response element binding protein
Cpt1a: carnitine palmitoyl transferase
E: embryonic
DNA: deoxyribonucleic acid
EAR: estimated average requirements
ELISA: enzyme-linked immunosorbent assay
EPG: ethanolamine phosphoglycerides
FAO: Food and Agriculture Organization of the United Nations
Fads2: fatty acid desaturase 2 (Δ 6)
Fasn: fatty acid synthase
FBP1, Fbp1: fructose 1,6 bisphosphatase
G6pdx: glucose-6-phosphate dehydrogenase
GLC: gas liquid chromatography
HDL: high-density lipoprotein
HMG: 3-hydroxy-3-methyl-glutaryl
Hmgcr: HMG-CoA reductase
Hmgcs2: HMG-CoA synthase 2 (mitochondrial)
HPLC: high performance liquid chromatography
IL: interleukin
IPG: immobilized pH gradient
ISSFAL: International Society of the Study of Fatty Acids and Lipids
kcal: kilocalorie
LC: long chain
LDL: low-density lipoprotein
LXR: liver X receptor
MALDI-TOF: matrix-assisted-laser-desorption-ionization time of flight
MCFA: medium chain fatty acid
MLX: max-like factor
mRNA: messenger ribonucleic acid
NADP: nicotinamide adenine dinucleotide phosphate
PAGE: polyacrylamide gel electrophoresis
PC: phosphatidylcholine
PCR: polymerase chain reaction
Pcx: pyruvate carboxylase
PE: phosphatidylethanolamine
Pfk1: phosphofructokinase
PI: phosphatidylinositol
Pklr: pyruvate kinase
PPAR: peroxisome proliferator activated receptors
Pppara: PPARα
Ppargc1a: PPARγ coactivator 1-α
PS: phosphatidylserine
RDA: recommended dietary allowance
RNA: ribonucleic acid
rpm: revolutions per minute
Scd1: stearoyl CoA desaturase
SD: standard deviation
Sds: serine dehydratase
SEM: standard error of the mean
Shmt1, SHMT1: serine hydroxymethyltransferase
Slc25a1: mitochondrial citrate transporter
sn: stereo-specifically numbered
SREBP: sterol regulatory element binding protein
VLDL: very-low-density lipoprotein
WHO: World Health Organization
COMMON NAMES OF FATTY ACIDS

12:0 lauric acid
14:0 myristic acid
16:0 palmitic acid
18:0 stearic acid
18:1n-9 oleic acid
18:2n-6 linoleic acid
18:3n-3 α-linolenic acid
20:4n-6 arachidonic acid (ARA)
20:5n-3 eicosapentaenoic acid (EPA)
22:4n-6 adrenic acid
22:5n-6 docosapentaenoic acid
22:6n-3 docosahexaenoic acid (DHA)
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CHAPTER 1: Introduction

1.1 Background and rationale

The omega (ω) or n-3 and n-6 fatty acids are essential in the diet and the amounts consumed are believed to be important for cardiovascular, immune and mental health (1-4). During development, the accumulation of n-3 and n-6 fatty acids in fetal and infant tissues is influenced by maternal intakes of n-3 and n-6 fatty acids and varies widely (5, 6). The n-3 and n-6 fatty acids are known to be important for infant brain and retina development (3, 7, 8), but the physiological effects of variability in n-3 and n-6 fatty acid accumulation in other tissues, including the liver, remains poorly understood.

The liver plays a central role in macronutrient metabolism through balancing the supply of macronutrients received from the diet or other tissues with endogenous synthesis, and also exporting triglycerides, glucose and amino acids to meet the needs of other organs and maintain plasma glucose homeostasis. The ability of the liver to adapt to changes in the macronutrient supply is likely to be of key importance in the switch from in utero nutrition, in which the fetus receives a continuous supply of glucose via the placenta, to postnatal nutrition in which the infant receives a milk diet that provides approximately 50% of energy from fat and a lower proportion of energy from glucose and amino acids than in utero (9). The neonatal liver must be capable of adapting to the high fat supply, while preventing hypoglycemia and accumulation of excess fat. In the adult, n-3 and n-6 fatty acids regulate pathways of fatty acid and glucose metabolism in the liver through regulation of gene expression for key enzymes of fatty acid and glucose metabolism (10). Whether the supply of n-3 and n-6 fatty acids received via the placenta or in milk is important in metabolic regulation in the infant liver is not known.

The overall objective of this dissertation was to gain understanding of how maternal and infant dietary fatty acids influence the accumulation of n-3 and n-6 fatty acids in the infant liver,
and to address the potential implications of differences in infant liver n-3 and n-6 fatty acids, particularly with respect to metabolic regulation. This research is important to understand dietary requirements for n-3 and n-6 fatty acids that promote optimal development, with consideration of effects beyond the roles of n-3 fatty acids in brain development.

1.2 Literature review

The literature review begins with a brief introduction to fatty acids and their metabolism, followed by a review of n-3 and n-6 fatty acids, which includes definitions and dietary sources, historical and current recommendations, the relationship between n-3 and n-6 fatty acid metabolism, and current understanding of the functional roles of n-3 and n-6 fatty acids in adults. This is followed by a review of fatty acid transfer from mother to fetus, secretion of fatty acids in milk, accumulation of n-3 and n-6 fatty acids in fetal and infant tissues, and current understanding of the roles of n-3 and n-6 fatty acids during early development. The final section reviews the unusual metabolic state of the fetus and neonate with respect to the supply of energy substrates and the adaptations in hepatic metabolism that occur at birth.

1.2.1 Fatty acids

Fatty acids are carboxylic acids that have the following basic structure:

\[ \text{Methyl end} \rightarrow \text{H}_3\text{C}[\text{CH}_2]_n\text{COOH} \leftarrow \text{Carboxyl end} \]

Fatty acids are often grouped into classes based on the number of double bonds in the carbon chain. The saturated fatty acids, as illustrated above, have no double bonds; monounsaturated fatty acids have one double bond; polyunsaturated fatty acids have two or more double bonds. A common convention for naming fatty acids is to state the number of carbons in the fatty acid chain followed by a colon, then the number of double bonds. For unsaturated fatty acids, the position of the carbon at which the first unsaturated bond occurs counting from the
methyl end of the fatty acid chain is then denoted after the ω or n symbol. Using this convention, the saturated fatty acid, palmitic acid, is denoted as 16:0, indicating that there are 16 carbon atoms and no double bonds. The unsaturated fatty acid, linoleic acid, is designated as 18:2n-6 with 18 carbons in the fatty acid chain, two double bonds and the first double bond between the sixth and seventh carbon atoms from the methyl end of the fatty acid chain. Alternatively, although less commonly used, fatty acids can be named by the position of the double bond relative to the carboxyl end, using the designation delta (Δ) to signify the position of the double bonds. Using this format, linoleic acid, which has 18 carbons and two double bonds is 18:2Δ⁹,¹². Although this system is less often used to identify fatty acids, the Δ designation is widely used to name the enzymes that insert double bonds between carbons in a fatty acid. For example, Δ9 desaturase adds a double bond between carbons 9 and 10 from the carboxyl end on a fatty acid.

In cells, levels of unesterified (free) fatty acids are low and most fatty acids are found esterified in neutral and polar lipids. The major storage form of fat is triglyceride (triacylglycerol), which is comprised of three fatty acids esterified to a glycerol molecule (11). The major structural lipids found in membranes surrounding cells and intracellular organelles are the phospholipids, which are defined by the presence of a phosphate group, and these include the glycerophospholipids and sphingomyelin. Glycerophospholipids are comprised of two fatty acids bound to the stereo-specifically numbered (sn)-1 and sn-2 positions of a glycerol molecule and a phosphatidic acid bound to the sn-3 position. The phosphatidic acid may be bound to a number of compounds, resulting in the different species of glycerophospholipids, the most abundant of which are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (12). Other types of phospholipids, include sphingomyelin, for which the backbone is ceramide not glycerol (11). Fatty acids may also be esterified to cholesterol, and whereas free cholesterol is found mainly in cell membranes, esterified cholesterol is located primarily in the cytoplasm of cells. Numerous other lipid classes exist,
including additional forms of sphingolipids, and complex lipids bound to carbohydrates, for example the glyceroglycolipids. More detailed reviews of the lipid classes and functions are available (11-13).

Fatty acids can be obtained from the diet, or they can be synthesized *de novo* in the liver, mammary gland and some other organs using acetyl CoA derived from the oxidation of glucose, amino acids or fatty acids. The initial and committed step in fatty acid synthesis is the carboxylation of acetyl CoA to form malonyl CoA, catalyzed by acetyl CoA carboxylase (14). Additional units of acetyl CoA are added to form a fatty acid through a series of condensation, dehydration and reduction reactions, requiring (nicotinamide adenine dinucleotide phosphate) NADPH, and these are catalyzed by the multi-enzyme complex fatty acid synthase (14). The usual product of fatty acid synthase in the liver is 16:0. In the mammary gland, in contrast to the liver, the major product of fatty acid synthesis is 14:0, explained by the presence of a mammary specific enzyme, thioesterase II (15, 16), and this will be discussed further in Chapter 4. Longer chain saturated and unsaturated fatty acids can be synthesized from 16:0 through elongation and desaturation reactions (14). Humans and other animals have Δ9, Δ6, and Δ5 desaturases, and these enzymes enable synthesis of monounsaturated and n-9 polyunsaturated fatty acids, as well as further desaturation of dietary derived n-6 and n-3 fatty acids (17, 18). However, the Δ15 and Δ12 desaturase enzymes needed to insert a double bond at the n-3 or n-6 position of an 18 carbon fatty acid are only present in plants and single cell organisms, thus a source of n-6 and n-3 fatty acids must be provided in the diets of humans and other animals.

In the diet, most fatty acids are obtained in the form of triglyceride, with smaller amounts in dietary phospholipids and cholesterol esters (19). A brief review of the digestion and absorption of fatty acids from dietary triglycerides and their transport in plasma is included here. More detailed reviews are available elsewhere (20-26). Digestion of triglyceride begins in the
stomach with the action of lingual and gastric lipases that specifically hydrolyze fatty acids at the sn-3 position of triglycerides, generating free fatty acids and sn-1,2 diglycerides. In the intestine, the hydrophobic products of gastric hydrolysis are emulsified by bile salts secreted from the gall bladder. Further hydrolysis occurs by pancreatic lipase, which cleaves fatty acids at the sn-1 and sn-3 position of triglycerides, generating mainly free fatty acids and sn-2 monoglycerides. Some of the sn-2 monoglycerides may be further rearranged into sn-3 monoglycerides and then completely hydrolyzed to release free fatty acids and glycerol (20). In addition to pancreatic lipase, carboxyl ester lipase, also known as bile salt stimulated lipase, releases fatty acids from triglycerides, phospholipids and cholesterol esters (27). Carboxyl ester lipase is also secreted into milk by the mammary gland and, because pancreatic lipase activity is low at birth, the carboxyl ester lipase secreted in milk may be important in facilitating fat digestion in the infant (28). After digestion, fatty acids and monoglycerides enter the enterocyte by passive diffusion or a protein carrier mediated transport system, although the contribution of the carrier transport system to total fatty acid absorption in humans is still under debate (29). Most of the short and medium chain (<14 carbons) unesterified fatty acids absorbed from the intestinal lumen enter the portal vein for transport directly to the liver bound to albumin (20). The remaining unesterified fatty acids are re-esterified with the sn-2 monoglycerides into triglycerides, then assembled into chylomicrons, which are comprised of a triglyceride and cholesterol-ester core surrounded by phospholipids, unesterified cholesterol and apolipoproteins, including apoB48 (20). The chylomicrons are then secreted into the lymph and enter the circulation via the thoracic duct (22). At the endothelial cell surface of capillaries, the chylomicron triglycerides are hydrolyzed by lipoprotein lipase to release free fatty acids and monoglycerides that are then taken up by cells (24). Chylomicron remnants, deplete of triglycerides, are taken up by the liver through endocytosis (24). Triglycerides synthesized in the liver are secreted in very-low-density-lipoproteins (VLDL), which contain apoB100 in addition to other lipoproteins (23, 31). As the
triglycerides in the VLDL particles are hydrolyzed by lipoprotein lipase, similar to chylomicron metabolism, some of the VLDL become intermediate-density-lipoproteins (IDL) and then low-density-lipoproteins (LDL) (24). LDL particles are taken up by endocytosis in cells throughout the body via the LDL-receptor, which recognizes apoB100, and this represents the major pathway for delivery of cholesterol to cells (26, 31). One of the best-known roles of high-density-lipoproteins (HDL) is in the reverse cholesterol transport pathway (25). HDL particles are secreted from the liver and intestine as immature lipid-poor HDL particles containing phospholipids and apoAI, and acquire cholesterol and apolipoproteins from chylomicron and VLDL particles (24). Mature HDL particles can be taken up by the liver, where the cholesterol is hydrolyzed to unesterified cholesterol and may be used as a substrate for bile acid synthesis (25).

In addition to transport in lipoproteins, unesterified fatty acids, mainly those derived from adipose tissue lipolysis (32) are transported in the plasma bound to albumin (33). The unesterified fatty acids are taken up by muscles and oxidized as a source of energy, or taken up by the liver, where they can then be re-secreted in VLDL.

Fatty acid uptake into cells is believed to involve fatty acid transport proteins and, to some extent, simple diffusion (34). In the cell, fatty acids combine with co-enzyme A to form fatty acyl-CoA. The fatty acyl-CoA may be re-esterified into neutral or polar lipids, or depending on the fatty acid type, they may be desaturated and elongated to form longer chain fatty acids prior to esterification, or they may undergo β-oxidation in the mitochondria or peroxisomes. Multiple forms of intracellular fatty acid binding proteins with tissue specific and diverse functional roles, as well as multiple types of acyl CoA synthetases have been identified (35, 36), and these may influence the fate of fatty acids within cells. Fatty acids have long been recognized as an important energy source from a dietary perspective, as they provide a concentrated source of energy. The oxidation of one molecule of palmitate (16:0) can generate 129 adenosine triphosphate (ATP), whereas the oxidation of one molecule of glucose generates
only 38 ATP. There is also increasing recognition that fatty acids, particularly the n-3 and n-6 fatty acids derived from dietary intake, or released from storage and structural lipids have important roles as components of cell signaling molecules, including anandamides and acyl-amino acids, as precursors for the eicosanoids, and in the regulation of gene and protein expression and protein activity (2, 10, 37, 38).

1.2.2 The n-3 and n-6 fatty acids

1.2.2.1 Definitions and dietary sources of n-3 and n-6 fatty acids

The n-3 and n-6 fatty acids are fatty acids containing two or more double bonds with the first double bond at the third (n-3) or sixth (n-6) carbon from the methyl end of the carbon chain. As mentioned, humans and other animals lack the Δ15 and Δ12 desaturase enzymes needed to insert a double bond at the n-3 or n-6 position of an 18-carbon fatty acid chain and, therefore, a source of n-3 and n-6 fatty acids must be obtained from the diet. The most abundant forms in the human diet are, for the n-3 series α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), and for the n-6 series, linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) (19). Both 18:3n-3 and 18:2n-6 are considered essential in the diet. The richest dietary sources of 18:3n-3 include flax oil, canola oil, soybean oil and English walnuts, and rich sources of 18:2n-6 include corn oil, soybean oil, safflower oil, sunflower oil, almonds and peanuts. The longer chain n-3 and n-6 fatty acids can be obtained in the diet from animal lipids or synthesized in humans and other animals from 18:3n-3 and 18:2n-6, respectively. Dietary sources of the long chain n-3 fatty acids, 20:5n-3 and 22:6n-3 include fish and eggs, with the richest source being fatty fish, such as salmon and sardines. Dietary sources of the long chain n-6 fatty acid, 20:4n-6, include ruminant meats, pork, poultry, eggs and fish. The pathway for the synthesis of long chain n-3 and n-6 fatty acids from the 18 carbon fatty acids is outlined in Figure 1.1. The first step of the pathway, Δ6 desaturation of 18:3n-3 or 18:2n-6 is the rate limiting reaction for desaturation and elongation of polyunsaturated fatty acids. After Δ6
desaturation, two carbons are added by elongase followed by Δ5 desaturation, resulting in 20:5n-3 or 20:4n-6. The currently accepted pathway for synthesis of 22:6n-3 and 22:5n-6 involves two steps of elongation, then Δ6 desaturation resulting in the intermediate fatty acids 24:5n-6 and 24:6n-3, which are translocated from the endoplasmic reticulum to the peroxisome for partial β-oxidation to form 22:5n-6 and 22:6n-3 (39). Importantly, the desaturase and elongase pathway is shared between the n-3 and n-6 fatty acids, and this may lead to competition for desaturation and elongation between these two series of fatty acids. This will be discussed further in section 1.2.2.4. Stable isotope tracer studies have estimated that less than 10% of a dose of 18:3n-3 is recovered in 20:5n-3, while less than 1% of a dose of 18:3n-3 is recovered in 22:6n-3 (40), but these types of studies are difficult to interpret. The amount of a substrate converted into a product depends on how much of the product is needed, and how much substrate is given. For example, if the requirement for 22:6n-3 is 100 mg per day and the diet has 2 g of 18:3n-3, then only 5% of the dietary 18:3n-3 needs to be converted to fulfill needs of 22:6n-3; if the diet supplies 1 g 18:3n-3, then 10% of the dietary 18:3n-3 will be converted. To date, the amount of 22:6n-3 needed each day to replace 22:6n-3 lost during membrane lipid turnover, or to support new membrane lipid synthesis during growth is not known.
1.2.2.2 Historical perspectives of research and dietary recommendations for essential fatty acids

The recognition of n-3 and n-6 fatty acids as essential nutrients began with the discovery by Burr and Burr in 1929 that rats fed a fat-free diet developed deficiency symptoms that included an abnormal, scaly condition of the skin and diminished growth (41). This suggested that fat or a component of fat was a required nutrient. Burr and Burr later showed that 18:2n-6, but not saturated fatty acids reversed the symptoms of deficiency in rats (42), and this led to the recognition of 18:2n-6 as an essential dietary nutrient in animals. The requirement for essential fatty acids in humans was first reported in 1962 in infants who were fed fat-free skim milk diets.
and developed skin lesions and failure-to-thrive (43). In the 1970’s, similar skin lesions and an elevated plasma ratio of 20:3n-9/20:4n-6, known as the triene/tetraene ratio resulting from increased metabolism of the n-9 fatty acids were reported in hospitalized adults given intravenous nutrition with fat-free amino acid and dextrose solutions (44). These clinical studies provided evidence that n-6 fatty acids are dietary essential nutrients for both infants and adults.

The requirement and specific metabolic functions of the n-3 fatty acids was not recognized until somewhat later, beginning with the work of Wheeler, Benolken and Anderson (45) who, in 1975, showed that the altered electroretinogram responses of rats fed fat-free diets or diets supplemented with 18:1n-9 were improved by the addition of 1-2% 18:3n-3 to the diet. Later studies by Neuringer and Reisbick reported altered electroretinogram responses, impaired visual acuity (46), polydypsia (47) and altered stereotyped behavior (48) in offspring of monkeys fed diets containing safflower oil, which provides 18:2n-6 but not 18:3n-3, compared to soybean oil, which provides both 18:2n-6 and 18:3n-3. These, together with similar studies in rats (49), suggested a critical role for n-3 fatty acids in neural development.

Although n-6 fatty acids were recognized as essential nutrients in animals as early as 1930 (42), their inclusion as required nutrients in dietary recommendations in Canada and the United States did not occur until the 1960’s. In 1963, the Dietary Standard for Canada published by Health and Welfare Canada (50), recommended 1% energy from 18:2n-6. This recommendation was primarily based on the need for essential fatty acids in infants, although a need for essential fatty acids for adults was also recognized. In 1975, a revised Dietary Standard for Canada recommended 2% energy from 18:2n-6 for infants and 1-2% energy 18:2n-6 to meet the needs of all other age groups (51). In 1983, the Recommended Nutrient Intakes, which replaced the Dietary Standard for Canada, suggested an essential fatty acid intake of 3% energy (52), and in 1990 this was revised to state 3% energy as 18:2n-6 (53).
In the United States, 18:2n-6 was first recognized as an essential nutrient for infants in the 1964 Recommended Dietary Allowances report (54) published by the Institute of Medicine of the National Academy of Sciences. The 1964 report recommended that 1-3% energy should be provided as 18:2n-6 to meet the needs of most infants. The 1968 Recommended Dietary Allowances report (55) recommended that infant formulas contain 3% 18:2n-6 to prevent essential fatty acid deficiency. Recognition of the need for essential fatty acids in adult diets first appeared in the United States in the 1974 Recommended Dietary Allowances report (56), after n-6 fatty acid deficiency had been observed in humans fed experimental fat-free diets and in hospitalized patients fed fat-free intravenous liquids. The suggested intake for infants and adults was 1-2% energy as 18:2n-6.

While the initial recommendations for essential fatty acids were low and based on preventing deficiency symptoms, other dietary recommendations emerged and this focused on replacing dietary saturated fatty acids with polyunsaturated fatty acids, with the goal of lowering serum cholesterol. This message was also included in the 1968 Recommended Dietary Allowances report (55) and reports published by the National Heart Lung and Blood Institute in the United States, as reviewed in (57). Justification for higher intakes of polyunsaturated fatty acids was based, in part, on the studies of Keys (58) that showed that replacing saturated fat with polyunsaturated fatty acids decreased serum cholesterol levels, believed at that time to be an important risk factor for coronary heart disease. In 1977, dietary guidelines published by the United States Senate Select Committee on Nutrition and Human Needs, as reviewed in (57), suggested that polyunsaturated fatty acids should comprise about 10% of dietary energy, with 10% from monounsaturated fatty acids and up to 10% from saturated fatty acids. Following this, the 1980 Recommended Dietary Allowances report (59) suggested that adults consuming a higher-fat diet (up to 35% energy from fat) should consume 8-10% of energy as polyunsaturated fatty acids as a means to improve blood lipid profiles and decrease risk of coronary heart disease.
Thus, the Recommended Dietary Allowances reports in 1980 suggested a nearly 10-fold higher recommendation for polyunsaturated fatty acids than that of 1974 (56, 59), and this was driven by the hypothesis that increasing polyunsaturated fatty acid intake would decrease the risk of coronary heart disease.

Up until 1980, recommendations for essential fatty acids in both Canada and the United States recognized only the n-6 fatty acids, as these fatty acids prevented classic clinical and biochemical signs of essential fatty acid deficiency. The potential importance of dietary 18:3n-3 for the brain and retina was first recognized in the 1983 Recommended Nutrient Intake Report of Canada (52) and the 1980 Recommended Dietary Allowances report of the United States (59), although recommended intake levels were not set in either report. Recommended intakes were first stated in Canada in the 1990 Recommended Nutrient Intake report which suggested an intake of 0.5% energy as 18:3n-3. In the United States, however, the 1989 Recommended Dietary Allowances report (60) did not include recommended intakes for n-6 or n-3 fatty acids, as it was stated that essential fatty acid deficiency was not observed in healthy adults.

1.2.2.3 Contemporary dietary recommendations for essential fatty acids

The Recommended Dietary Allowance from the United States and the Recommended Nutrient Intake reports of Canada have been replaced by the Dietary Reference Intakes, published by the Institute of Medicine of the United States National Academy of Sciences. The Dietary Reference Intakes now provide dietary recommendations for both the United States and Canada. Nutrient recommendations in the Dietary Reference Intakes are provided as estimated average requirements (EAR), which are set to meet the requirements of 50% of healthy individuals, and the recommended dietary allowance (RDA), which are set to meet the requirements of 97% of healthy individuals (19). For nutrients for which there is insufficient evidence to set an EAR or RDA, recommendations are reported as adequate intakes (AI), which
for most nutrients represents the median or mean nutrient intake among apparently healthy individuals in the United States (19). For macronutrients, an acceptable macronutrient distribution range (AMDR) is also provided, taking into account that energy requirements can be met through carbohydrate, fat or protein and, thus, requirements for each of the macronutrients depends on intakes of the other macronutrients. The AMDR are considered to reflect the range of macronutrient intakes that are associated with reduced risk of chronic disease while providing adequate amounts of essential nutrients associated with that macronutrient (19).

The Dietary Reference Intakes (19) stated a lack of scientific evidence from which to set requirements for n-3 and n-6 fatty acids, therefore, recommendations for all stages of the lifespan were reported as AI. For adults, the AI represents the median intakes of n-3 and n-6 fatty acids of healthy men and women in the United States. The AI for 18:3n-3 for adults aged 19-50 years is 1.6 g/day for men and 1.1 g/day for women, and for 18:2n-6 the AI is 17 g/day and 11 g/day for men and women, respectively. For pregnant and lactating women, the AI represents the median intakes of n-6 and n-3 fatty acids of apparently healthy pregnant and lactating women, respectively, in the United States. The AI for both pregnancy and lactation was set at 1.3 g 18:3n-3/day and 13 g 18:2n-6/day (19). In addition, an AMDR was set for n-3 and n-6 fatty acids for adults, including pregnant and lactating women and children over 12 months-of-age. The AMDR for the n-3 fatty acids is 0.6-1.2% energy, with the lower boundary equivalent to the intake needed to meet the AI based on median energy requirements, and the upper boundary set at the highest intake of n-3 fatty acids observed in individuals in the United States. The Dietary Reference Intakes also state that about 10% of the AMDR for the n-3 fatty acids can be obtained from the long chain n-3 fatty acids, 20:5n-3 and 22:6n-3. The AMDR for the n-6 fatty acids is 5-10% energy. The lower boundary of the AMDR for the n-6 fatty acids approximates the AI. The upper boundary approximates the highest intakes of 18:2n-6 for individuals in the United States and was set due to the stated lack of evidence of the long-term safety of diets with more than
10% energy 18:2n-6, and concern that high levels of 18:2n-6 in cell membranes may lead to a pro-oxidant state. Importantly, the AMDR for n-6 fatty acids greatly exceeds the early recommendations of 1-2% energy 18:2n-6, which was considered to meet requirements for n-6 fatty acids (50-56).

The Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) recommend an n-3 fatty acid intake of 0.5-2% energy and 250-2000 mg 20:5n-3 and 22:6n-3 per day (61). These recommendations were based on the beneficial role of 18:3n-3, 20:5n-3 and 22:6n-3 in preventing cardiovascular disease. The FAO/WHO also suggested an acceptable macronutrient distribution range for 18:2n-6 of 2.5-9% energy, with the lower end set to meet requirements for 18:2n-6 and the upper end set based on evidence to show that 18:2n-6 reduces LDL cholesterol and, therefore, may reduce risk of cardiovascular disease.

Many other groups have also published recommendations for n-3 and n-6 fatty acid intakes, particularly for adults. The International Society of the Study of Fatty Acids and Lipids (ISSFAL), which is a scientific society, published a report stating a “healthy intake of 18:3n-3 is approximately 0.7% energy” and “2% energy from 18:2n-6 is adequate for healthy human adults” (62). The recommendation for 18:3n-3 was derived from studies looking at primary and secondary prevention of cardiovascular disease. The ISSFAL report also recommended a minimum intake of 500 mg of 20:5n-3 and 22:6n-3 for prevention of cardiovascular disease in healthy adults (62). The position paper on dietary fatty acids of the American Dietetic Association and Dietitians of Canada recommended 3-10% energy from 18:2n-6, 0.6-1.2% energy from 18:3n-3 and 500 mg/day of long chain n-3 fatty acids, 20:5n-3 and 22:6n-3 (63). The position paper, however, emphasized the need for a food-based approach to dietary recommendations, which translates to “a diet high in fruits and vegetables, whole grains, legumes, nuts, lean protein (i.e., lean meats, poultry, and low-fat dairy products), fish (especially
fatty fish high in n-3 fatty acids), together with the use of non-hydrogenated margarines and oils” (63). The American Heart Association published a statement paper supporting the Dietary Reference Intake recommendation of 5% to 10% of energy from n-6 fatty acid, and in the report also suggest that reducing n-6 fatty acid intakes “would be more likely to increase than to decrease risk for coronary heart disease” (64). The American Heart Association Dietary Guidelines also recommend consuming two servings of fish (about 8 oz) per week, particularly fatty fish. Recommendations to consume two servings of fatty fish per week, which provides approximately 500 mg of 20:5n-3 and 22:6n-3 per day, have also been made by other professional organizations, including the American Dietetic Association and Dietitians of Canada (63), FAO/WHO (61, 65), and the National Academy of Sciences (66), and are also included in Canada’s Food Guide for Healthy Eating (67).

Specific recommendations for long chain n-3 fatty acids during pregnancy and lactation have also been made, based on potential beneficial effects of 20:5n-3 and 22:6n-3 in infant development. The Institute of Medicine report “Seafood choices: Balancing benefits and risks” (66) suggested that pregnant and lactating women aim for two 3 oz (85 g) servings of fish per week, as a source of long chain n-3 fatty acids. Similarly, Health Canada recommends that pregnant and lactating women and women of childbearing age aim to consume 150 g (two 75 g servings) of fish per week (68). The FAO/WHO suggest an intake of 300 mg 20:5n-3 + 22:6n-3 per day, of which 200 mg should be 22:6n-3 (69). The FAO/WHO recommendation for pregnancy was based on the adult recommendations for prevention of cardiovascular disease (250 mg/day), adjusted for the higher estimated energy requirements of pregnancy. The requirement for lactation was based on the intake of 22:6n-3 required to achieve a level of 0.32 g 22:6n-3/100 g fatty acid in breast milk, which has been estimated to be the average 22:6n-3 found in studies of human milks reported from different countries (70). The International Society for the Study of Fatty Acids also recommended at least 200 mg/day 22:6n-3 for pregnant and
lactating women, which as stated in their report, can be achieved through two meals of fish per week (71).

Nutrient recommendations for infants, for most nutrients, are based on the assumption that breast-milk from healthy mothers provides the best nutrition for the growing infant. Using this approach, the Dietary Reference Intake recommendations for fatty acids for infants were based on the average intake of milk by full-term exclusively breast-fed infants and the average fatty acid composition of breast-milk from healthy mothers (19). The AI for 18:3n-3 and 18:2n-6 for infants 0-6 months are 0.5 g/day and 4.4 g/day, respectively, based on an average intake of 0.78 L/day of milk with an average n-3 fatty acid content of 0.63 g/L (1.58% of total fatty acids) and an average n-6 fatty acid content of 5.6 g/L (14% of total fatty acids). For infants 7-12 months of age, the AI for 18:3n-3 is 0.5 g/day and for 18:2n-6 the AI is 4.6 g/day, and this is based on the average intake of n-3 and n-6 fatty acids from breast-milk and complementary foods, as consumed in the United States (19). Similar to the Dietary Reference Intakes, the FAO/WHO recommendations for fatty acids for infants are based on the average amounts of fatty acids provided in breast milk.

It is important to note that while early recommendations for essential fatty acids were based on preventing deficiency, the current Dietary Reference Intakes are based on the median intakes of n-3 and n-6 fatty acids in the United States and, for infants, the average amounts of n-3 and n-6 fatty acids provided in breast-milk. The recommendations for 18:2n-6 are still largely driven by messages to prevent cardiovascular disease by replacing saturated fatty acids with n-6 fatty acids (primarily 18:2n-6). Current recommendations, however, do not consider the potential consequences of high intakes of n-6 fatty acids in antagonizing the need for n-3 fatty acids, or whether this is appropriate during pregnancy and lactation, or infancy. This will be discussed further in the next section.
1.2.2.4 Competition between n-3 and n-6 fatty acids

Dietary intakes of 18:2n-6 have increased dramatically in the past few decades. Studies from the 1940’s estimated the mean intake of 18:2n-6 at about 2.5% energy (72), whereas the Continuing Survey of Food Intakes by Individuals (CFSII, 1994-1996 and 1998) reported mean intakes of 18:2n-6 of about 6% energy among adults in the United States (19). The 95th percentile of 18:2n-6 intake among adults in the CFSII was 20-25 g per day, which based on the reported average energy intakes translates into about 9-12.5% energy from 18:2n-6. In Canada, mean intakes of polyunsaturated fatty acids, 90% of which is 18:2n-6, are about 5-6% energy (73). A study of pregnant women in Vancouver, British Columbia reported a mean and 95th percentile intake of 18:2n-6 of 4% and 9.5% energy, respectively (74). The increase in 18:2n-6 intakes has likely occurred due to the widespread use of vegetable oils high in 18:2n-6 and their addition to many new products, such as salad oils and crackers (75), as well as the replacement of animal fat with unsaturated fats, as a result of initiatives to reduce saturated fatty acid intake (57). The increase in 18:2n-6 intake has led to an increase in the dietary n-6/n-3 fatty acid ratio from historical estimates of about 1-2:1 to current levels often exceeding 10:1 (19, 76). The n-6 and n-3 fatty acids share the same desaturase and elongase enzymes necessary to make the long chain polyunsaturated fatty acids (Figure 1.1), and are both acylated into the sn-2 position of phospholipids, for which there is a finite number. This, therefore, raises concern over the possibility of competition between the n-6 and n-3 fatty acids. Because of this the Dietary Reference Intakes suggest that the ratio of 18:2n-6/18:3n-3 be maintained at 5-10:1 (19). Results of animal studies, however, estimate that long chain n-3 fatty acid levels decrease with 18:2n-6 intakes greater than 2% energy (77, 78), thus, 5% energy from 18:2n-6, the lower boundary of the AMDR (19), and 18:2n-6/18:3n-3 ratios of 5:1 or higher may be too high to support desaturation and elongation of 18:3n-3 to long chain n-3 fatty acids.
Reports of competition between the n-6 and n-3 fatty acids can be dated back to the 1960’s. Early studies by Mohrhauer and Holman (79, 80) and Rahm and Holman (81) showed that increasing dietary intakes of 18:2n-6 decreases long chain n-3 fatty acid levels in tissue lipids of the rat, while increasing 18:3n-3 decreases long chain n-6 fatty acid levels. Animal studies have consistently shown that high intakes of n-6 fatty acids decrease tissue levels of 20:5n-3, although the effect on 22:6n-3 is less clear (77, 78, 82, 83).

Understanding whether or not high intakes of 18:2n-6 are relevant for human n-3 fatty acid accretion is complicated by the variability among diets and presence of preformed 20:5n-3 and 22:6n-3 in some diets. There is limited clinical data on the effect of decreasing 18:2n-6 on long chain n-3 fatty acid accumulation in adults, particularly with low intakes of 18:2n-6. Liou and Innis (84) showed that decreasing dietary 18:2n-6 from 10.5% energy (range 7-15.8%) to 3.8% energy (range 2.9-5.2%) with a constant 1% energy from 18:3n-3 for four weeks increased mean levels of 20:5n-3 in plasma phospholipids from 0.58 to 0.93 g/100 g fatty acid, but had no detectable effect on the mean levels of 22:6n-3 in healthy adult men. Similarly, Chan et al. (85) showed that decreasing 18:2n-6 from about 12% energy to about 5% energy with 18:3n-3 held constant at about 1.7% energy led to an increase in 20:5n-3 from 0.3 to 0.8 g/100 g fatty acid in plasma phosphatidylcholine and 0.3 to 0.9 g/100 g fatty acid in phosphatidylethanolamine, but had no detectable effect on 22:6n-3 levels in any fraction. These studies indicate that intakes of about 4-5% energy 18:2n-6 support higher levels of 20:5n-3 in plasma, but not 22:6n-3, when compared to higher intakes of 18:2n-6; however, it should be noted that the circulating lipid levels of 20:5n-3 and 22:6n-3 needed to support optimal tissue functions are not known.

Infants exclusively fed formula, for which the nutrient composition is known, offer a unique opportunity to evaluate the effects of specific nutrient intakes and nutrient interactions. The effect of lowering the amount of 18:2n-6 in infant formulas has provided some insight into
the relevance of high 18:2n-6 intakes on circulating n-3 fatty acid levels in human infants. Clark et al. (86) fed infants formulas containing, as a percent of energy, A. 7.5% 18:2n-6 and 0.4% 18:3n-3, B. 7% 18:2n-6 and 1.8% 18:3n-3, or C. 2% 18:2n-6 and 0.6% 18:3n-3, all with no long chain polyunsaturated fatty acids, for 10 weeks, then measured infant plasma and erythrocyte fatty acid levels. Increasing 18:3n-3 in formula B compared to formula A led to increased 20:5n-3 in both plasma and erythrocytes, with a small but significant increase in 22:6n-3 in erythrocyte lipids. Decreasing 18:2n-6 in formula C compared to formula A, resulted in increased 20:5n-3 in plasma and erythrocyte lipids and higher 22:6n-3 in infant erythrocyte lipids. Interestingly, the magnitude of increase in 20:5n-3 was greater in infants fed formula C with low 18:2n-6 and 0.6% energy 18:3n-3 than in those fed formula B, with 1.8% 18:3n-3, but 7% 18:2n-6. After 10 weeks, the erythrocyte lipids of breast-fed infants had 6.5 g 22:6n-3/100 g fatty acid, while the infants fed formulas A, B, or C had 3.5, 4.8, and 4.0 g 22:6n-3/100 g fatty acid, respectively. Thus, even with manipulation of 18:2n-6 and 18:3n-3 in the infant formulas, the levels of 22:6n-3 in erythrocyte lipids from infants fed formula were lower than in breast-fed infants. However, the breast-fed infants had received preformed 22:6n-3 in their mother’s milk. A number of other studies have shown that increasing 18:3n-3 in formula increases infant plasma 20:5n-3, but levels of 22:6n-3 do not reach those of breast-fed infants consuming 22:6n-3 in milk (87, 88). An interpretation of these studies could be that conversion of 18:3n-3 to 22:6n-3 does not enable accretion of 22:6n-3 to match those of an infant fed a diet with preformed 22:6n-3 (i.e. breast-milk). An alternative explanation is that the 18:2n-6 in the infant formulas is too high to enable conversion of 18:3n-3 to 22:6n-3. Most infant formulas provide 18:2n-6 at about 7-8% energy, but animal studies have shown that the desaturase enzymes are saturated at intakes of about 2-3% energy 18:2n-6 (77, 78). In the study by Clark et al. (86), although 18:2n-6 was reduced to 2% energy, the formula contained only 0.6% energy from 18:3n-3. To date, no studies have investigated the effect of decreasing intakes of 18:2n-6 to 2% energy or less while also
maintaining a dietary 18:2n-6/18:3n-3 ratio of 2:1 or lower. This dissertation addresses this gap in knowledge using piglets fed milk replacer formulas with low (less than 2% energy), or higher (10% energy) amounts of 18:2n-6, with a constant 1% energy from 18:3n-3 as a model to investigate the effects of 18:2n-6 on long chain n-3 fatty acid accretion in tissues, including the liver, brain and heart.

1.2.2.5 Functional roles of n-3 and n-6 fatty acids

Fatty acids vary in chain length and degree of unsaturation and these properties confer unique functionality to each of the n-3 and n-6 fatty acids. Levels of 18:3n-3 are relatively low in most tissue lipids (89), and this may contribute to a perception that 18:3n-3 does not play an important role in cell function. The low levels of 18:3n-3 in tissue phospholipids may be explained, in part, by the preferential oxidation of this fatty acid when compared to most other dietary fatty acids (90, 91). Radioisotope tracer studies of in vivo fatty acid oxidation in rats reported that recovery of $^{14}$CO$_2$ from $^{14}$C 18:3n-3 over 24 hours was greater than when $^{14}$C was fed in 18:1n-9, 18:2n-6, or 20:4n-6 (90). In humans, recovery of $^{13}$CO$_2$ from $^{13}$C methyl or carboxyl labeled fatty acids over 10 hours was greatest for 12:0, followed by 18:3n-3, then in decreasing order, trans 18:1n-9, cis 18:1n-9, 18:2n-6, 16:0 and 18:0 (91), suggesting greater oxidation of 18:3n-3 when compared to the other unsaturated and saturated 18 carbon chain fatty acids. The rate-limiting step for mitochondrial fatty acid oxidation is fatty acid transfer into the mitochondria by carnitine palmitoyl transferase 1. Acyl-carnitine formation was reported to be highest for 18:3n-3 compared to 18:2n-6, 18:1n-9 or 16:0 (92), thus preferential oxidation of 18:3n-3 may be due to a higher affinity for carnitine palmitoyl transferase 1, although the specific mechanisms have not been elucidated. Oxidation of 18:3n-3 generates nine units of acetyl CoA per 18:3n-3 molecule. The acetyl CoA can be completely oxidized to CO$_2$ in the citric acid cycle, used to synthesize ketones, or exported to the cytosol in the form of citrate for de novo synthesis of fatty acids and cholesterol. Following administration of a dose of $^{13}$C-
18:3n-3, $^{13}$C was detected in saturated fatty acids, monounsaturated fatty acids and cholesterol in the brain, adipose tissue and liver in both developing and adult rats and non-human primates (93-95), as well as in saturated and monounsaturated fatty acids in plasma phosphatidylcholine and triglycerides in humans (96). The labeled carbons from $^{13}$C-18:3n-3 have also been detected in saturated and monounsaturated fatty acids in animals fed a diet high in saturated and monounsaturated fatty acids (95), as well as in rat pups from dams fed an n-3 fatty acid deficient diet (97). These findings suggest that oxidation and recycling of carbons may represent an important function of 18:3n-3.

A more well recognized role of 18:3n-3 is as a precursor for synthesis of the long chain n-3 fatty acids. As described previously, the proportion of 18:3n-3 converted to 22:6n-3 has been estimated to be low. The apparent low proportion of a stable isotope dose of 18:3n-3 appearing in 22:6n-3 (40) may be explained by rapid β-oxidation of 18:3n-3, leaving low amounts of 18:3n-3 available for desaturation and elongation. Because 18:3n-3 shares a common desaturation and elongation pathway with 18:2n-6, it is possible that high intakes of 18:2n-6 “saturate” the desaturase enzymes and block conversion of 18:3n-3 to the longer chain n-3 fatty acids. Whether the requirement for 18:3n-3 is explained only through the need for synthesis of longer chain n-3 fatty acids, or if 18:3n-3 has other roles, for example as a source of acetyl CoA for de novo synthesis of sterols, fatty acids and ketones, is still unclear. The unique metabolism of 18:3n-3 when compared to other fatty acids, such as 18:2n-6, suggests the function of 18:3n-3 requires further investigation, and should be considered when determining dietary needs for n-3 fatty acids.

Levels of 18:2n-6 are found in higher amounts, relative to 18:3n-3 in cells and tissues, both in phospholipids and in storage triglycerides in adipose tissue (98-100). This may simply reflect higher intakes of 18:2n-6 and the need to store fatty acids in tissue lipids, or perhaps this
reflects retention of 18:2n-6 due to its “essential” nature. Early studies showed that one of the symptoms of dietary 18:2n-6 deficiency was scaly skin (41, 43), and since then studies have revealed an important role for 18:2n-6, as well as its long chain metabolites, 20:3n-6 and 20:4n-6, in maintaining the epidermal water barrier system in the skin and preventing epidermal hyperproliferation (101). Linoleic acid (18:2n-6) is also the primary fatty acid used in esterification of plasma cholesterol by lecithin cholesterol acyltransferase (102), and it is a precursor for the long chain n-6 fatty acids, 20:3n-6, 20:4n-6 and 22:4n-6.

The long chain fatty acids, 20:5n-3, 22:6n-3, 20:3n-6, 20:4n-6, and 22:4n-6 are considered the physiologically essential forms of n-3 and n-6 fatty acids. On a molecular level, the functions of these fatty acids may be explained by their roles as components of membrane phospholipids, where they influence membrane fluidity, permeability and membrane-associated protein function (103, 104), as precursors or components for cell signaling molecules (2, 37, 38, 105, 106), and as regulators of gene expression (10, 107). The distribution of the n-3 and n-6 fatty acids differs among tissues and organs. For example, in the liver, 22:6n-3, 20:5n-3, 20:4n-6 and 18:2n-6 are the prominent polyunsaturated fatty acids in phospholipids (77, 100, 108, 109), whereas membrane phospholipids of the brain of animals and humans are enriched in 22:6n-3, 22:4n-6 and 20:4n-6, but low in 20:5n-3 and 18:2n-6 (109-112). The different membrane phospholipid levels of n-3 and n-6 fatty acids may reasonably be suggested to reflect their different functional roles in each tissue. In the liver, an organ important for coordinating macronutrient metabolism, n-3 and n-6 fatty acids appear to be particularly important in regulating gene and protein expression for enzymes of metabolic pathways (10); whereas in the brain, which sends and receives nerve signals to peripheral tissues, membrane lipid n-3 and n-6 fatty acids appear to be particularly important for signal transduction and in lipid-protein interactions, such as the role of 22:6n-3 with rhodopsin (113, 114). The following paragraphs provide a brief overview of our current understanding of some of the roles of the long chain n-6
and n-3 fatty acids in the brain, immune and cardiovascular system, and this is followed by a more detailed review of the n-6 and n-3 fatty acids in the liver.

Numerous observational and intervention studies have investigated the role of dietary 22:6n-3 in early brain development both in the maternal diet, as fish, fish oils or algal source 22:6n-3, or in infants or animals fed milk formulas with and without 22:6n-3. Although there is still considerable debate in this area, some studies have shown that lower dietary or blood levels of 22:6n-3 are associated with poorer performance on tests of mental, visual and motor development in young infants (7, 115-117). At the other end of the lifespan, some epidemiological evidence has suggested that lower intakes of 22:6n-3 are associated with an increased risk of depression and age-related cognitive decline (4, 118, 119); however, more research is required to determine the causal role of n-3 fatty acid intakes or altered n-3 fatty acid metabolism in these disorders. The mechanisms by which lower 22:6n-3 may influence brain development and function may include impaired neurogenesis (120), altered metabolism of neurotransmitters (121), and altered membrane receptor and protein activities and gene expression (78, 122). Arachidonic acid (20:4n-6) is also important in membrane lipids of the nervous system. It is a precursor for prostaglandin E2, which is required for normal neural function and long-term potentiation (123), and it is a component of arachidonylethanolamides, also known as anandamide, and arachidonylglycerol, which are ligands for the cannabinoid receptors that are important in the regulation of food intake and numerous other physiological processes (106). Multiple other fatty amides, including arachidonylglycine and docosahexaenoylglycine have recently been characterized and their role in the brain and peripheral tissues is being increasingly investigated (37, 124).

A considerable body of research has been dedicated to understanding the role of the long chain n-3 and n-6 fatty acids in the immune system and inflammatory response. Fatty acids
released from immune cell membranes are precursors for the inflammatory mediators, eicosanoids and docosanoids, which play an important role in the initiation, propagation and resolution of inflammation (2, 38, 105). The eicosanoids generated from 20:4n-6 include the series 2 prostaglandins and thromboxanes, and series 4 leukotrienes, and these are generally considered to have pro-inflammatory properties, although some of the 20:4n-6 generated eicosanoids, i.e. PGE$_2$, have both pro-inflammatory and anti-inflammatory properties. Arachidonic acid (20:4n-6) is also a precursor for lipoxins that play key roles in the active termination of inflammation (2, 105). Eicosanoids generated from 20:5n-3 include the series 3 prostaglandins and series 5 leukotrienes, and these are usually, but not always, anti-inflammatory or have less potent pro-inflammatory actions than the 20:4n-6-derived eicosanoids (2, 105). Eicosapentaenoic acid (20:5n-3) is also a precursor for the E-series resolvins, and 22:6n-3 is a precursor for the D-series resolvins, protectins and maresins, all of which are important in the resolution of inflammation (38). The n-3 fatty acids, 20:5n-3 and 22:6n-3 may also inhibit gene expression for pro-inflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (2). In summary, the metabolites of 20:4n-6 have both anti-inflammatory and pro-inflammatory properties, whereas the metabolites and effects of the n-3 fatty acids are generally weakly inflammatory, anti-inflammatory, or resolving. Based on this, it has been suggested that the balance of n-6 to n-3 fatty acids in phospholipids of immune cells may be important for chronic inflammation and in inflammation-related diseases, including rheumatoid arthritis, inflammatory bowel disease and cardiovascular disease (105, 125, 126).

Epidemiological and intervention studies have shown that lower intakes of fish or fish oils, which are rich sources of 20:5n-3 and 22:6n-3, are associated with an increased risk of death from cardiovascular disease (127). The protective effects of n-3 fatty acids may be due to direct effects of n-3 fatty acids on ventricular fibrillation and energy metabolism in the heart (128), secondary effects involving changes in immune and inflammatory function via eicosanoid
metabolism (2, 38), or some other aspect of lipid metabolism, including indirect effects in the liver or other organs. It is well-known that dietary 18:2n-6 fatty acids lower LDL cholesterol, whereas higher intakes of n-3 fatty acids lower circulating triglyceride levels (58, 129, 130), both of which when elevated are risk factors for cardiovascular disease. These effects could, in part, be explained by n-3 and n-6 fatty acid regulation of liver lipid metabolism, which will be described in more detail in the next section.

1.2.2.6 Essential fatty acids and regulation of liver metabolism and gene expression

The liver plays a central role in integrating the supply of macronutrients from the diet and fatty acids released from adipose tissue with the demands of the body for glucose, amino acid and fatty acids, and must respond to the fluctuating macronutrient composition of the diet and changes in substrate supply in the fed and fasted state. Macronutrient metabolism in the liver is regulated by hormones, including insulin and glucagon, and the dietary macronutrient supply and composition. Mechanisms for metabolic regulation include allosteric regulation of enzyme activity, posttranslational modification of enzymes, including phosphorylation or protein splicing, or regulation at the level of enzyme gene transcription. It is now generally accepted that the n-3 and n-6 fatty acids suppress de novo fatty acid synthesis and promote fatty acid β-oxidation in the liver and that this occurs through regulation of gene expression (10, 107).

Studies by Gottlicher and colleagues in 1992 (131) first showed that fatty acids, including 18:2n-6, 20:4n-6, 18:1n-9 and 12:0 were ligands for peroxisome proliferator activated receptors (PPAR). PPAR are nuclear receptor ligand-activated transcription factors. Three subtypes of PPAR have been identified, including PPARα, β/δ and γ (132). PPARα is predominantly expressed in the liver and is thought to be responsible for fatty acid induced changes in the expression of genes of hepatic lipid and carbohydrate metabolism (133, 134). Activated PPARα binds deoxyribonucleic acid (DNA) as a heterodimer with retinoid-X-receptor (RXR) (135) on
peroxisome proliferator response elements (PPRE) in the promoters of target genes and initiates subsequent recruitment of co-activators and gene transcription (132, 133). In vitro, ligands for PPARα include multiple unesterified unsaturated fatty acids and eicosanoids, and this has been confirmed by molecular and structural analysis (136), although their “strength” as regulators of PPAR activity differs. One determinant of the strength of PPAR activators is the usual abundance of ligands within the cell. Pawar and Jump (137) found that addition of 18:1n-9, 18:2n-6, or 20:4n-6 to cells did not influence the unesterified pool of these fatty acids, and they did not influence PPARα activity, whereas addition of 20:5n-3 resulted in a marked increase in the concentration of free 20:5n-3 and subsequent activation of PPARα. Similar, although less strong effects were found for 22:6n-3. Thus, one hypothesis is that 20:5n-3 is the most potent regulator of PPARα (138), although the relative potency of ligands for PPARα in vivo and among different organs is still unclear. Target genes of PPAR include those encoding proteins involved in fatty acid oxidation and transport and glucose metabolism, thus, through PPAR, the n-3 and n-6 fatty acids upregulate gene expression for enzymes of fatty acid oxidation. PPAR response elements have been identified in the promoters of Acyl CoA oxidase (Acox) (139), which encodes a regulatory enzyme of peroxisomal fatty acid oxidation, cytochrome P450 IV family (140) and glucose transporters (141). PPARs have also been shown to regulate carnitine palmitoyl transferase (Cpt1a), acyl CoA synthetase, fatty acid transport protein and uncoupling proteins in adipose tissue (107).

In contrast to genes for enzymes of fatty acid oxidation, gene expression for enzymes of fatty acid synthesis and glycolysis are inhibited by polyunsaturated fatty acids, and this is thought to occur through the transcription factors sterol regulatory element binding protein (SREBP) and carbohydrate response element binding protein (ChREBP) (142-144). SREBP are helix-loop-helix basic leucine zipper transcription factors, which include SREBP1c, SREBP1a and SREBP2 (145). SREBP2 is primarily involved in regulation of sterol synthesis, whereas...
SREBP1 is responsible for insulin-induced up-regulation of genes involved in fatty acid synthesis and glycolysis (145). SREBP1c is the predominant SREBP expressed in rodent and human liver (146). SREBP1c is synthesized as a 125 kDa precursor protein and is inserted into the endoplasmic reticulum. Cleavage of the protein causes the release of the mature 65 kDa protein that moves to the nucleus to bind sterol response elements (SRE) in genes for fatty acid synthesis, including fatty acid synthase, acetyl CoA carboxylase, citrate lyase, malic enzyme and stearoyl CoA desaturase (142, 143, 145). Studies by Yahagi et al. (142) showed that dietary 20:5n-3 or 20:5n-3 plus 22:6n-3 fed as fish oil led to decreased expression of genes involved in fatty acid synthesis in wild-type mice, but not in transgenic mice over expressing the mature form of SREBP1c, suggesting that n-3 fatty acids inhibit processing of the precursor SREBP1c to the mature nuclear form. Subsequent studies also showed that n-6 and n-3 fatty acids decreased Srebp1c gene transcription (147). This may be explained by liver X receptor (LXR), which binds to the promoter of the Srebp1c gene to enhance Srebp1c transcription, but is also inhibited by n-3 and n-6 fatty acids (148, 149). The precise role of LXR in Srebp1c regulation is still under debate (150). Of the n-3 and n-6 fatty acids, 18:2n-6, 20:4n-6, 20:5n-3 and 22:6n-3 have all been shown to decrease SREBP1c activity by decreasing proteolytic processing of precursor SREBP1c to the mature form (150), decreasing transcription of Srebp1c gene (147), as well as reducing Srebp1c messenger ribonucleic acid (mRNA) stability leading to less precursor SREBP1c (151). In addition, 22:6n-3 has been shown to uniquely increase proteasomal degradation of the nuclear form of SREBP1c, and it has, therefore, been suggested to be the most potent repressor of SREBP1c (152).

ChREBP is constitutively expressed in liver and is translocated to the nucleus under conditions of high glucose, where it binds carbohydrate response elements (ChRE) to promote transcription of glycolytic and lipogenic genes (153). The currently accepted mechanism for the translocation of ChREBP involves dephosphorylation of cytosolic ChREBP by protein
phosphatase 2. Under high glucose conditions, xylulose-5-phosphate, a metabolite of the pentose phosphate pathway, increases and this activates protein phosphatase 2, which then dephosphorylates ChREBP and enables translocation to the nucleus. ChREBP forms heterodimers with max-like-factor (MLX) and binds to ChREBP response elements on the promoters of target genes (154). The n-3 and n-6 fatty acids have been shown to inhibit ChREBP translocation from the cytosol to the nucleus and promote Chrebp mRNA decay, which leads to decreased transcription of genes including pyruvate kinase, fatty acid synthase and acetyl CoA carboxylase (144). The n-6 and n-3 fatty acids may also regulate levels of MLX (155), although the mechanisms for fatty acid regulation of ChREBP and/or MLX are still unclear. Fatty acid regulation of ChREBP appears to be less specific than other transcription factors, with 18:2n-6, 20:5n-3 and 22:6n-3 equally repressing ChREBP activity (144).

The n-6 and n-3 fatty acids have also been suggested to regulate liver X receptor (LXR), nuclear factor κβ (NFκβ) and hepatocyte nuclear factor-4-alpha (HNF-4α) (107, 138, 156). Many of the genes regulated by n-6 and n-3 fatty acids in the liver encode enzymes of lipid and carbohydrate metabolism, but n-3 and n-6 fatty acids have also been shown to regulate genes involved in the oxidative stress response, cell proliferation and apoptosis.

It is important to note that most of the evidence to show fatty acid regulation of gene expression has been established through in vitro cell systems or through animal models that involve feeding specific diets, but which also involve feeding the same diets and, thus, fatty acids at each meal, every day. The fat and fatty acid content of usual human diets varies widely from meal-to-meal and from day-to-day. In humans consuming their usual diets, the importance of n-6 and n-3 fatty acids in regulating expression of genes involved in fatty acid and glucose metabolism is still not clear. Some evidence has been provided by studies in human hepatoma cells (HepG2) and biopsy analyses. Addition of 20:4n-6, 20:5n-3 or 22:6n-3 to HepG2 cells led
to a significant decrease in *Srebp1c* mRNA and SREBP1c protein expression, as well as a decrease in stearoyl CoA desaturase mRNA (157). In one study, lower levels of 20:5n-3 and 22:6n-3 in liver from obese patients compared to healthy weight adults was associated with lower hepatic gene expression of *PPARA* and carnitine palmitoyl transferase 1, and higher gene expression of *SREBP1C* and fatty acid synthase (158). An interpretation of this is that 20:5n-3 and 22:6n-3 influence *PPARA* and *SREBP1C* expression in human adult livers, as in rodents and cell models. On the other hand, lower 20:5n-3 and 22:6n-3 may be explained by a higher proportion of triglyceride in the livers of obese subjects, diluting the apparent concentration of 20:5n-3 and 22:6n-3. In this case, the changes in gene expression may be a result of altered fat metabolism and triglyceride accumulation in the liver, rather than direct effects of 20:5n-3 and 22:6n-3.

In summary, the n-3 and n-6 fatty acids are essential in the diet and have unique functional roles in the body. In animal and cell culture models, n-3 and n-6 fatty acids alter gene expression for enzymes regulating glucose and lipid metabolism, which leads to a shift in metabolism towards increased oxidation of fatty acids, with decreased oxidation of glucose for energy and decreased *de novo* synthesis of fatty acids. Whether n-6 and n-3 fatty acids regulate metabolic pathways in development and the implication for infant health is not known, but this is of concern as intakes of n-6 fatty acids have changed dramatically over the past few decades and optimal requirements for early development have not been established.

### 1.2.3 Essential fatty acids in development

#### 1.2.3.1 Placental transfer of essential fatty acids

Before birth, the fetus receives all of its essential nutrients, including essential fatty acids via the placenta. How fatty acid transfer across the placenta occurs and is regulated is still unclear, but the potential mechanisms are becoming understood. Lipoproteins do not cross the
placenta, but placental cells express lipoprotein receptors and have lipoprotein lipase, phospholipase A2 and intracellular lipase activity, all of which enable uptake and hydrolysis of lipoproteins to release unesterified fatty acids for transfer to the fetus (159). The human placenta also expresses placental plasma membrane fatty acid binding protein, fatty acid translocase, fatty acid transport protein, and cytosolic fatty acid binding proteins, enabling membrane uptake and translocation of unesterified fatty acids (160). From the placenta, unesterified fatty acids are released to the fetal circulation where they bind α-fetoprotein and are transported directly to the liver for further processing (159).

Considerable evidence exists to show that levels of n-3 and n-6 fatty acids in fetal umbilical cord blood are directly correlated with maternal plasma and erythrocyte phospholipid levels of the same n-3 and n-6 fatty acid (161-164), and these in turn are influenced by maternal dietary intakes of n-3 and n-6 fatty acids (74, 163, 165). For example, in a study by Elias and Innis (161), in same time point analysis, correlations between maternal plasma phospholipid fatty acid and infant plasma phospholipid fatty acid at birth were r = 0.48 for 20:4n-6, r = 0.48 for 22:6n-3 and r = 0.73 for 20:5n-3, with \( P<0.001 \) for all correlations. The importance of the maternal diet on fetal transfer of fatty acids is also clearly illustrated in studies involving maternal supplementation with fish oils containing 20:5n-3 and 22:6n-3. In these studies, maternal supplementation with fish oil leads to higher 20:5n-3 and 22:6n-3 in maternal plasma and erythrocyte phospholipids and higher 20:5n-3 and 22:6n-3 in umbilical plasma and erythrocyte fatty acids (166-169).

Regardless of maternal diet, fetal plasma is relatively enriched in 22:6n-3 and 20:4n-6 when compared to maternal plasma. For example, Elias and Innis (161) reported levels of 22:6n-3 and 20:4n-6 in maternal plasma phospholipids of 5.03 g/100 g total fatty acid and 8.72 g/100 g fatty acid, with corresponding infant cord plasma phospholipids levels of 7.62 g/100 g fatty acid.
and 17.7 g/100 g fatty acid, respectively. In contrast to the enrichment of 22:6n-3 and 20:4n-6 in fetal plasma, the levels of 18:3n-3 and 18:2n-6 were 0.36 g/100 g fatty acid and 20.8 g/100 g fatty acid in maternal plasma phospholipids, but only 0.05 g/100 g fatty acid and 7.49 g/100 g fatty acid in infant cord plasma phospholipids. This enrichment in the relative proportions of 22:6n-3 and 20:4n-6 in fetal (newborn infant) plasma has led to the hypothesis that there is preferential transport of these fatty acids from mother to fetus. In support of this hypothesis, placental membrane fatty acid binding proteins that have a higher affinity for 22:6n-3 and 20:4n-6 compared to other fatty acids have been identified (170), and studies using perfused human placenta (171) or administration of $^{13}$C labeled 22:6n-3 to pregnant women (172) have also yielded data consistent with selective transport of 22:6n-3 compared to other fatty acids across the human placenta; however, this is complex. The esterified lipids in fetal plasma are likely to originate from fetal liver synthesis and secretion, not the placenta, and the major lipoprotein in fetal circulation is HDL, with very low VLDL and chylomicron (173). In the maternal circulation, higher VLDL and esterified lipids of intestinal origin (i.e. chylomicron) could contribute to differences in the circulating lipid pools, and lower the percent of 22:6n-3 and 20:4n-6. Furthermore, the total quantity of fatty acids in fetal plasma is much lower than in the mother, meaning that comparison of the relative quantities of fatty acid in fetal compared to maternal plasma may be misleading. For example, Otto et al. (162) reported that maternal plasma phospholipid levels of 22:6n-3, 20:4n-6 and 18:2n-6 from women in the Netherlands were 3.87, 8.67 and 20.68 g/100 g fatty acid, respectively, whereas levels in umbilical plasma phospholipids were 6.49, 17.1, and 7.29 g/100 g fatty acid, respectively; however, measured in mg/L plasma, the maternal plasma phospholipid levels of 22:6n-3, 20:4n-6 and 18:2n-6 were 68.0, 152.5, and 364 mg/L plasma, respectively, and in umbilical plasma phospholipids levels were 37.1, 97.5, and 41.6 mg/L, respectively. It is clear then that when expressed as mg/L
plasma, levels of 22:6n-3 and 20:4n-6 may not be higher in the fetus than the mother, while levels of 18:2n-6 may be lower in the fetus.

Two additional pieces of evidence seem to challenge the hypothesis of “preferential” transfer of fatty acids across the placenta. First, as mentioned, umbilical cord fatty acids are correlated with the same fatty acids in maternal plasma and influenced by maternal dietary intake (161-164). This suggests fatty acid transfer down a concentration gradient rather than preferential transfer of specific fatty acids. Second, infant umbilical cord plasma fatty acids are variable, which suggests there is no selective mechanism to prevent low transfer of specific fatty acids, such as 22:6n-3, in the event of a low maternal n-3 fatty acid intake. It is clear from levels of umbilical cord plasma phospholipid n-6 and n-3 fatty acids among women from different geographical regions (Table 1.1) that the n-6 and n-3 fatty acids in cord plasma phospholipids vary among populations, with population means ranging from 2.1-7.62 g 22:6n-3/100 g and 13.6-18.3 g 20:4n-6/100 g umbilical cord plasma phospholipid fatty acid (161, 162, 164). The umbilical cord plasma levels of n-3 and n-6 fatty acids also vary widely among individuals. In the study by Elias and Innis (161), levels of 22:6n-3 in umbilical cord plasma phospholipids ranged from 4.35 to 11.7 g/100 g fatty acid, levels of 20:4n-6 ranged from 13.8 to 23.0 g/100 g fatty acid and levels of 18:2n-6 ranged from 5.04 to 10.7 g/100 g fatty acid. Similar variability in n-6 and n-3 fatty acids was observed in fatty acids in the triglyceride and cholesterol ester fractions of umbilical plasma.

In summary, n-3 and n-6 fatty acid transfer across the placenta varies. Regardless of whether there are “preferential” transport mechanisms, or mechanisms that select against fatty acids, such as 18:2n-6, it is clear that fatty acid transfer is influenced by the maternal n-3 and n-6 fatty acid status. Thus, maternal diets that are low in n-3 fatty acids or high in n-6 fatty acids may compromise the transfer of potentially important n-3 fatty acids to the developing fetus.
Table 1.1 Levels of major n-6 and n-3 fatty acids in fetal umbilical cord plasma phospholipids from different geographical regions.

<table>
<thead>
<tr>
<th>Country</th>
<th>n</th>
<th>Umbilical Cord Plasma Phospholipid Fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canada</td>
<td>70</td>
<td>18:2n-6: 7.49 (1.25) 20:4n-6: 17.7 (2.01) 20:5n-3: 0.37 (0.17) 22:6n-3: 7.62 (1.67)</td>
</tr>
<tr>
<td>England</td>
<td>43</td>
<td>6.99 (1.64) 16.64 (3.11) 0.51 (0.19) 6.62 (1.46)</td>
</tr>
<tr>
<td>Holland</td>
<td>50</td>
<td>7.29 (1.65) 17.1 (3.99) 0.23 (0.07) 6.49 (1.80)</td>
</tr>
<tr>
<td>Finland</td>
<td>50</td>
<td>7.01 (1.93) 15.21 (3.04) 0.56 (0.45) 6.96 (1.28)</td>
</tr>
<tr>
<td>Hungary</td>
<td>50</td>
<td>6.95 (1.73) 18.3 (3.75) 0.08 (0.04) 5.23 (1.53)</td>
</tr>
<tr>
<td>Ecuador</td>
<td>22</td>
<td>8.34 (3.10) 15.65 (3.72) NR (0.04) 6.33 (1.36)</td>
</tr>
<tr>
<td>India</td>
<td>132</td>
<td>(NR) (NR) (NR) (NR) (NR)</td>
</tr>
</tbody>
</table>

Values are means (standard deviation, SD). NR, not reported. From references 161 for Canada, 162 for England, Holland, Finland, Hungary and Ecuador, and 164 for India.

1.2.3.2 Essential fatty acids in milk

After birth, the infant receives essential n-3 and n-6 fatty acids in mother’s milk, infant formula, or other modified milks, and these vary widely in fatty acid composition. Current recommendations for n-3 and n-6 fatty acids for infants are based on the average fatty acid content of human milk and the average intake of milk by the breast-fed infant. Infant formula is designed to match the average composition of protein, fat and carbohydrate in human milk. Human milk has 3.5 - 4.5% fat by weight and provides the infant with 40-60% dietary energy from fat (Table 1.2, 174). For reference, Table 1.2 also provides the average macronutrient content of rat milk. Notably, rat milk is more energy dense, has a higher protein/energy ratio and lower carbohydrate than human milk. It should be noted that while Table 1.2 provides the
average fat content of milk, the macronutrient content of human milk varies with the stage of lactation and within a feed, and the composition of fatty acids also varies with the maternal diet. The fat is secreted in the milk in globules, comprised mainly of triglycerides, but also containing phospholipids, cholesterol, cholesterol esters and free fatty acids (174, 175, Table 1.3). The fatty acids secreted in milk are derived from de novo synthesis in the mammary gland, which contributes primarily saturated fatty acids with chains of 14 carbons or less (medium chain fatty acids), and by uptake of fatty acids from plasma, which in turn can originate from dietary sources, fatty acids secreted in VLDL from the liver, or from fatty acids released from the maternal adipose tissue (176).

As the n-3 and n-6 fatty acids cannot be synthesized de novo in mammals, all of the n-3 and n-6 fatty acids in milk must be derived from n-3 and n-6 fatty acids in maternal plasma, which in turn must originate from the maternal diet. Maternal dietary intakes of n-3 and n-6 fatty acids differ, and this leads to a wide range of n-3 and n-6 fatty acid levels in milk. To illustrate this, Table 1.4 provides the levels of n-3 and n-6 fatty acids in breast-milk reported from studies with women in different geographic locations. Table 1.4 shows that although the mean levels of 20:4n-6 show little variation, levels of 22:6n-3 and 18:2n-6 vary widely among groups of lactating women from different geographic locations, and this reflects differences in the average dietary intakes of 22:6n-3 and 18:2n-6 among women in these regions (70). The range of n-6 and n-3 fatty acids in milk from a large group of women in British Columbia is provided in Table 1.5, and this shows the wide inter-individual variability in milk fatty acids from women in the same geographic location. This variability is largely explained by differences in dietary intake, but also includes variability due to genetic variation in genes encoding enzymes of fatty acid desaturation among women (177), and perhaps other factors.
Table 1.2 Macronutrient composition of human and rat milk.

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat</td>
<td>38</td>
<td>88</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>82</td>
<td>38</td>
</tr>
<tr>
<td>Protein</td>
<td>10</td>
<td>81</td>
</tr>
</tbody>
</table>

% energy

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>47.6</td>
<td>61.4</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>46.7</td>
<td>12.3</td>
</tr>
<tr>
<td>Protein</td>
<td>5.7</td>
<td>26.3</td>
</tr>
</tbody>
</table>

Adapted from Jensen, 1995 (174).

Table 1.3 Composition of human milk lipids.

<table>
<thead>
<tr>
<th></th>
<th>Percent of total lipid by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>98.8</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>0.81</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>0.08</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Adapted from Bitman, 1983 (175).
**Table 1.4** Levels of the major polyunsaturated fatty acids in breast-milk from women in different geographic locations.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Canada</th>
<th>USA</th>
<th>China</th>
<th>Japan</th>
<th>Philippines</th>
<th>Mexico</th>
<th>Chile</th>
</tr>
</thead>
<tbody>
<tr>
<td>22:6n-3</td>
<td>0.17</td>
<td>0.17</td>
<td>0.35</td>
<td>0.99</td>
<td>0.74</td>
<td>0.26</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>(0.07)</td>
<td>(0.14)</td>
<td>(0.14)</td>
<td>(0.57)</td>
<td>(0.37)</td>
<td>(0.20)</td>
<td>(0.21)</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.22</td>
<td>1.05</td>
<td>2.02</td>
<td>1.33</td>
<td>0.43</td>
<td>1.05</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>(0.35)</td>
<td>(0.35)</td>
<td>(0.42)</td>
<td>(0.36)</td>
<td>(0.15)</td>
<td>(0.41)</td>
<td>(0.49)</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.37</td>
<td>0.45</td>
<td>0.45</td>
<td>0.40</td>
<td>0.39</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>(0.07)</td>
<td>(0.14)</td>
<td>(0.14)</td>
<td>(0.07)</td>
<td>(0.07)</td>
<td>(0.07)</td>
<td>(0.14)</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>11.5</td>
<td>14.8</td>
<td>14.9</td>
<td>12.7</td>
<td>7.90</td>
<td>16.05</td>
<td>17.75</td>
</tr>
<tr>
<td></td>
<td>(2.91)</td>
<td>(2.73)</td>
<td>(2.97)</td>
<td>(1.79)</td>
<td>(1.76)</td>
<td>(3.46)</td>
<td>(4.10)</td>
</tr>
</tbody>
</table>

Values are means (SD). Adapted from Yuhas et al., 2006 (70).

**Table 1.5** Major n-6 and n-3 fatty acids in milk collected at one-month postpartum from women in Vancouver.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>g/100 g milk fatty acid</strong></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.27</td>
<td>0.06-0.89</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.08</td>
<td>0.01-0.34</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.51</td>
<td>0.11-3.69</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.41</td>
<td>0.18-0.72</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>13.3</td>
<td>7.70-27.4</td>
</tr>
</tbody>
</table>

Adapted from Xie and Innis, 2008 (177), n = 175.
Although it is known that maternal diet is an important factor contributing to the composition of fatty acids secreted in milk, the process involved in fatty acid secretion by the mammary gland is complex and incompletely understood. Several studies have demonstrated that increasing the maternal dietary intake of 22:6n-3 increases the secretion of 22:6n-3 in human milk (178-180). For example, Makrides et al. (178) randomized breast-feeding mothers to a supplement with 0, 0.2, 0.4, 0.9 or 1.3 g 22:6n-3 per day from five days to 12 weeks postpartum. After 12 weeks of supplementation, the levels of 22:6n-3 in breast-milk had increased in a dose-dependent manner with 0.21, 0.35, 0.46, 0.86 and 1.13 g 22:6n-3/100 g breast-milk fatty acid for the five groups, respectively. In another study, women were randomized to take daily 230 mg 22:6n-3 from algae, 170 mg 22:6n-3 from eggs, 260 mg 22:6n-3 from fish oil, or a placebo with no 22:6n-3 from two to six weeks postpartum (180). After four weeks of supplementation, levels of 22:6n-3 in breast-milk were 0.44, 0.29, 0.39, and 0.19 g/100 g fatty acid in women assigned to take 230, 170, 260 or 0 mg 22:6n-3 per day. Similarly, levels of 22:6n-3 in human milk increased from 0.29 to 0.37 g/100 g fatty acid after two weeks of supplementation with 200 mg 22:6n-3 per day (179). Dietary supplementation with 18:3n-3 does not appear to increase the secretion of 22:6n-3 in human milk. For example, in a study by Francois et al. (181) lactating women were given 10.7 g 18:3n-3/day as flax seed oil for four weeks; for reference, the AI for 18:3n-3 for lactating women is 1.3 g/day (19). Although 18:3n-3 increased from 1.0 ± 0.3 g/100 g fatty acid at baseline to 7.7 ± 1.0 g/100 g milk fatty acid after supplementation, there was no change in levels of 22:6n-3 in milk; however, in this study, if the desaturation pathway was already fully saturated with 18:2n-6, then additional 18:3n-3 supplementation may not be able to increase the synthesis and, hence, the circulating maternal plasma levels of 22:6n-3.

Levels of 22:6n-3 in human milk appear to have decreased over time. Studies on Canadian breast-milk in the 1980s found mean 22:6n-3 levels of about 0.4 g/100 g fatty acid
(182), while mean levels of 22:6n-3 in breast-milk in Canada are now 0.14-0.2 g/100 g fatty acid (70, 177, 183, 184). Similarly, studies dating back to the 1970’s in Britain reported 22:6n-3 levels in milk of 0.59 g/100 g of milk fatty acid, with a range of 0.24-1.22 g/100 g fatty acid (185), whereas levels of 22:6n-3 in milk reported by the same investigators in 1992 were 0.37 g/100 g of milk fatty acid (186). It is possible that dietary intakes of 22:6n-3 have decreased due to changing dietary patterns, for example a decline in egg or fish consumption. Animal feeding practices with grains rich in 18:2n-6 have also led to lower 22:6n-3 in animal meats (187), which could contribute to lower 22:6n-3 in milk of women who consume meat. Notably, however, the decline in 22:6n-3 in human milk has occurred concurrent with the increase in 18:2n-6 in milk, which reflects an increase in 18:2n-6 intakes in the population. For example, in studies in the United Kingdom over 30 years ago, levels of 18:2n-6 in milk were about 6.9 g/100 g fatty acid with a range 5.6-9.1 g/100 g fatty acid (185), whereas the mean level of 18:2n-6 in milk from women in Britain in 1992 were 10.9 g/100 g fatty acid. The mean levels of 18:2n-6 in milk from women in Canada, the United States and Europe are now in the range of 10–14 g/100 g fatty acids (125). Based on this data it seems possible that increasing dietary intakes of 18:2n-6 have contributed to lower levels of 22:6n-3 in milk. The physiological importance of variable, and in particular, high 18:2n-6 in human milk or infant formulas for the infant remains unclear. There is some evidence that higher 18:2n-6 in infant formula leads to lower plasma cholesterol in formula-fed infants. Infants fed formulas and complementary foods providing 16.3% energy as 18:2n-6 had lower levels of plasma total, HDL and LDL cholesterol at 12 months of age than infants consuming 7.1% energy from 18:2n-6 (188). However, as for 18:2n-6, the implications of the alterations in plasma cholesterol by diet in young infants are poorly understood.

In summary, fatty acid recommendations for infants are based on the composition of human milk and the average intake of milk by the breast-fed infant; however, the fatty acid composition of human milk is variable and influenced by maternal diet. Maternal dietary fat
varies widely and has changed over time. Developing requirements for mothers and infants will require a better understanding of the functions of n-3 and n-6 fatty acids in infant growth and development.

1.2.3.3 Tissue accretion and functions of essential fatty acids during fetal and infant development

Maternal dietary n-3 and n-6 fatty acids influence fatty acid transfer across the placenta and the composition of fatty acids secreted in breast milk and these, in turn, alter the fatty acid composition of developing tissues in the fetus and infant. Studies in rats have shown that maternal diets low in 18:3n-3 lead to lower long chain n-3 fatty acid levels in the fetal brain, intestine and liver (108, 120, 189), whereas addition of 22:6n-3 to the maternal diet leads to higher 22:6n-3 in fetal brain and liver (189-191). The 18:3n-3, 18:2n-6 and 22:6n-3 content of the maternal diet has also been shown to influence the n-3 and n-6 fatty acid composition of tissues, including the brain, liver, intestine, heart, adipose and kidneys, after birth (78, 192, 193). This may reflect differences in fatty acids accumulated in utero or differences in the n-3 and n-6 fatty acids supplied in milk. Studies in rats, piglets and baboons fed milk formulas with added 20:4n-6 and 22:6n-3, as in current infant formulas, have shown that addition of 22:6n-3 to the formula diet leads to higher 22:6n-3 in the brain and liver, whereas supplementary 20:4n-6 increases 20:4n-6 levels in the liver, but does not always increase 20:4n-6 levels in the brain (194-196).

There is limited published information on the effect of maternal dietary fatty acids on fatty acid accretion in utero or after birth in humans. Autopsy data showed that infants fed human milk (which contains 20:4n-6, 20:5n-3 and 22:6n-3) or milk formula providing either 1.5% energy 18:3n-3 or <1% 18:3n-3, but with no 22:6n-3, had 1.99, 1.01 and 0.49 g 22:6n-3/100 g liver fatty acid, respectively, 17.7, 13.4, and 11.6 g 22:6n-3/100 g fatty acid, respectively, in frontal cortex phosphatidylethanolamine, and 23.5, 19.3 and 14.4 g 22:6n-3/100
g fatty acid, respectively, in frontal cortex phosphatidylserine (197, 198). These data clearly show that the fatty acid composition of the early diet, including the 18:3n-3 content of the formula, influences infant tissue n-3 fatty acids.

There has been considerable research on the importance of n-3 and n-6 fatty acids in fetal and infant development, particularly with respect to the role of dietary 22:6n-3 (and 20:4n-6) in the maternal or infant diet on brain and retina development (3), as well as infant growth and body composition (199-204), immune system development (205-207), risk of allergic disease (208, 209), and endpoints related to cardiovascular health (210-212).

Accumulation of 22:6n-3 is high in specific phospholipids of the brain and retina (213, 214). In epidemiological studies, lower fish, and thus 22:6n-3 intakes during pregnancy have been associated with poorer performance on tests of cognition (215), verbal intelligence, fine motor skills and social behavior in children (216). Intervention studies have shown both positive and null effects of maternal supplementation with 22:6n-3 or fish oils in pregnancy and lactation on tests of infant visual, motor and mental development (169, 199, 217-224). Multiple studies have addressed the role of supplemental 22:6n-3 (often with 20:4n-6) on neural development in infants, particularly in the context of infant formula, and the findings for these studies are also mixed. Meta-analyses of randomized controlled trials have, thus, concluded that addition of 22:6n-3 and 20:4n-6 to infant formula likely does not benefit physical, visual or neural development of preterm or term infants (115, 116). Although there is some evidence for a beneficial role of 22:6n-3 in infant visual, mental and motor skill development, the importance of providing preformed 22:6n-3 in maternal and infant diets is still under debate.

Studies investigating the effects of n-3 and n-6 fatty acid status on infant growth and body composition have also reported mixed results. Dirix et al. (200) reported a positive association between maternal plasma phospholipid 22:6n-3 at delivery and infant birth weight,
and Lauritzen et al. (201) found a positive association between maternal erythrocyte 22:6n-3 at four months postpartum and infant body mass index at 2.5 years of age. On the other hand, Bergmann et al. (202) reported lower body mass index in 21 month-old infants of mothers supplemented with fish oil compared to infants of mothers that did not receive fish oil. In addition, infants fed formula with 22:6n-3 and 20:4n-6 did not differ in body weight or growth compared to infants fed formula without 22:6n-3 and 20:4n-6 in a combined analysis of 901 infants from four randomized clinical trials (203). A recent meta-analysis also found no effect of long chain n-3 fatty acid supplementation in pregnancy and lactation on infant body composition (225); however, there are few studies in this area.

Although there is considerable published research on the roles of n-3 and n-6 fatty acids in adult immune function and cardiovascular health, only a few studies have considered the role of n-3 and n-6 fatty acids in infant immune and cardiovascular development. Studies by Field et al. (205, 206) have shown that the addition of 22:6n-3 and 20:4n-6 to infant formulas influences T-cell maturation and cytokine production in preterm and term infants, but the impact of the early dietary fatty acid supply on later immune health is still unclear. Damsgaard et al. (210) reported that infants who were given fish oil daily for 3 months had lower systolic blood pressure at 12 months of age compared to infants who were not given fish oil. In a subsequent study, the same group reported that the boys who received fish oil between 9 and 12 months of age had longer heart-rate intervals compared to infants who did not receive fish oil, although the effect was not found for girls (211). Forsyth et al. (212) also reported that infants fed formula with 22:6n-3 and 20:4n-6 had lower diastolic blood pressure at six-years-of-age compared to children that were fed formula without 22:6n-3 and 20:4n-6 as infants. The relevance of these findings for later cardiovascular health remains to be determined.
There is very little known about the role of n-3 and n-6 fatty acids in the developing liver. The n-3 fatty acids are known to accumulate in the fetal liver (213, Table 1.6). Clandinin et al. (226) estimated that 3.76 mg n-3 fatty acids per week are accumulated in fetal liver from 22 weeks gestation to birth, but this likely varies with maternal dietary intakes of n-3 and n-6 fatty acids. There is considerable research on the role of n-3 and n-6 fatty acids in regulation of liver metabolism in adult animals (10, 107). Whether fetal and infant n-6 and n-3 fatty acid status influences liver metabolic development, or later risk of disease is not known.

In summary, maternal intakes of n-3 and n-6 fatty acids influence accretion of these fatty acids in fetal and infant tissues, but research investigating the functions of n-3 and n-6 fatty acids in development have focused mainly on the needs for 20:4n-6 and 22:6n-3 for the brain, immune system and possible effects on infant growth. The function and requirements for n-3 and n-6 fatty acids for the liver and implications for metabolic development are not yet known.
Table 1.6 Accretion of n-3 fatty acids during the last trimester of development.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>n-3 fatty acid accretion rate (mg/week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>3.76</td>
</tr>
<tr>
<td>Brain</td>
<td>21.8</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.37</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.014</td>
</tr>
<tr>
<td>White adipose</td>
<td>367</td>
</tr>
<tr>
<td>Other(^1)</td>
<td>34.1</td>
</tr>
<tr>
<td>Total/week</td>
<td>469 mg</td>
</tr>
<tr>
<td>Total/day</td>
<td>67 mg</td>
</tr>
</tbody>
</table>

Adapted from Clandinin et al. (213). Values represent mg/organ/week and were estimated from autopsy data of infants from 22 weeks gestation to 120 days of age. Accretion rates are based on a birth weight of 3500 g at term. \(^1\)The remaining body tissues were estimated to have a total fat content and fatty acid composition similar to human skeletal muscle with 4% of wet tissue weight as fat and about 1% n-3 fatty acid.

1.2.4 Adaptations in macronutrient metabolism at birth

In utero, the fetus receives a constant supply of nutrients from the mother through the placenta and umbilical vein, and the gastro-intestinal functions and the enzymatic and hormonal regulation associated with enteral feeding are low (9). The exchange of gases, including oxygen and carbon dioxide, and waste excretion also occur through the placenta and umbilical vein (9). After birth, newborn infants must adapt to enteral feeding, with development of the gut, digestive enzymes and hormonal regulation of feeding (9). The neonate must also breathe through the lungs and develop the ability to concentrate and excrete urea and other waste. Outside of the uterus, the neonate must also develop the capacity for thermoregulation and build skeletal muscles needed for movement and resistance to gravity (9).

In addition to the physiological changes occurring at the transition from the intra to extra-uterine environment, the nutrient supply changes dramatically at birth. Before birth, glucose,
which crosses the placenta via facilitated diffusion, is the major energy substrate for the developing fetus (227, 228). Based on estimates of glucose transfer to the fetus, if completely oxidized, glucose would account for approximately 81% of fetal oxygen consumption in the human fetus (228), although it should be noted that the glucose supply is not completely oxidized under normal circumstances. Glucose is also used for de novo synthesis of fatty acid needed for adipose stores, particularly in the final trimester of gestation (159). Proteins are generally not transported to the fetus, with the exception of immunoglobulin, which is transported across the placenta in humans, guinea pigs, rats and rabbits (229). Amino acids are actively transported from the maternal circulation against a concentration gradient to the placenta and then released to the fetal circulation by mechanisms that remain unclear (227, 229). Several lines of evidence suggest that amino acids are catabolized for energy in the fetus. First, there is a net output of ammonia and urea from the fetus (227, 229), indicating catabolism of amino acids. Second, stable isotope tracer studies have shown that the uptake of amino acids by the fetus is much greater than accounted for by amino acid accretion (227). Third, stable isotope tracer studies have shown that about one-third of a dose of labeled leucine is oxidized to carbon dioxide (229). Based on urea excretion by the fetus, amino acids are estimated to provide about 10% of fetal energy requirements (228); however, this is likely to vary depending on availability of glucose. Studies have shown that leucine oxidation increases under conditions of maternal fasting, when maternal glucose levels and, therefore, glucose transport to the fetus would be decreased (227). Lipids, as described in section 1.2.3.1, are believed to cross the placenta as free fatty acids (159). Fatty acid β-oxidation does occur in the fetus and contributes to energy needs, but rates of fatty acid oxidation are estimated to be low (9, 230). Small amounts of glycerol, released from the hydrolysis of triglyceride, also cross the placenta and may contribute to fetal metabolism, either as an energy substrate or as a precursor for gluconeogenesis (228).
Ketogenesis is not active in the fetus, but ketones readily cross the placenta and may be oxidized for energy, or serve as lipogenic precursors (159).

After birth, the neonate no longer receives a constant supply of nutrients and begins feeding at frequent intervals. In contrast to the high and constant glucose supply received in utero, human milk provides approximately 48% energy from fat, 47% energy as carbohydrate and only about 5.7% energy from protein (174). Similarly, rat milk is high in fat, (61% of energy), although compared to human milk it is relatively lower in carbohydrate (12% energy) and higher in protein (26% energy) (174). It is clear that the infant must quickly develop the capacity to oxidize fatty acids for the energy needed for growth and thermoregulation, as well as to prevent excess fatty acid accumulation in the liver or other organs (9). The neonate must also maintain glucose homeostasis between feeds and maintain glucose for the brain, erythrocytes and renal medulla. To accomplish this, the neonate must develop and regulate pathways of gluconeogenesis and glycogenolysis (9). Transitioning to the milk diet, relatively lower in amino acids, the neonate must also spare amino acids for protein and peptide synthesis (9). Thus, successful adaptation to extra-uterine life requires important modifications in fatty acid, glucose and amino acid metabolism.

The liver is of particular interest in this adaptation, as it plays a critical role in integrating the supply and peripheral needs for fatty acids, glucose and amino acids. Because fatty acid oxidation is low during the fetal period, up-regulation of the hepatic mitochondrial and peroxisomal fatty acid oxidation pathways does not occur until late in gestation, or after birth, and this is evident by the increase in carnitine palmitoyl transferase activity, which catalyzes the rate limiting step in mitochondrial ß-oxidation, within the first 24 hours after birth (9). After birth, there is also an increase in the activity of mitochondrial 3-hydroxy-3-methyl-glutaryl-CoA (HMG) CoA synthase, enabling synthesis of ketones from acetyl CoA, and elevated
concentrations of ketones in neonatal plasma (9). Glycogen reserves accumulated in the liver and muscle before birth may also serve as a supply of glucose in the very early postnatal period, but the neonate must up-regulate pathways of gluconeogenesis and decrease hepatic glycolysis to prevent hypoglycemia (9). After birth, there is an increase in the mRNA and in the activity of cytosolic phosphoenolpyruvate kinase, the rate limiting enzyme for gluconeogenesis, and an increase in the activity of glucose-6-phosphatase, which enables release of glucose from the liver for extra-hepatic tissues (9). Together these changes in enzyme expression and activity enable a switch towards increased fatty acid oxidation and ketone synthesis, with decreased hepatic glycolysis and increased gluconeogenesis.

Changes in the activity of metabolic pathways are thought to be regulated by the hormonal milieu at birth. At birth, insulin decreases and glucagon increases in the newborn, and this is thought to be initiated in response to the increased levels of catecholamines, as well as the brief hypoxia and hypoglycemia the neonate experiences immediately after birth (9). The n-3 and n-6 fatty acids are known to be potent regulators of gene expression with effects leading to increased fatty acid oxidation and lower glycolysis in the liver (10, 107), and the n-3 and n-6 fatty acid supply to the fetus and to the neonate via milk varies with maternal dietary fat composition (5, 6). Whether the composition of fatty acids provided to the fetus and neonate may be important in the metabolic transition to feeding at birth has not been explored.

1.2.5 Summary

The n-3 and n-6 fatty acids are known to be important for brain and retina development, but their role in the development of other organs has received very little attention. Changes in the modern food supply and recommendations based on adult heart health have led to dramatic changes in n-6 fatty acid intakes over the past few decades, which may interfere with n-3 fatty
acid metabolism. The effects of the changes in dietary fatty acid intakes over the last century on infant growth and development remain unclear. To understand dietary requirements for n-3 and n-6 fatty acids in early development, we need to understand their functions beyond the brain, retina and immune system.

1.3 Purpose of the research

Maternal to fetal transfer of n-3 and n-6 fatty acids during gestation and secretion of n-3 and n-6 fatty acids in milk show considerable variability that is dependent on the composition of the maternal dietary fatty acids. Dietary n-6 and n-3 fatty acid intakes have changed over the past few decades, and differ widely worldwide. In North America, current recommendations for fatty acid intakes during pregnancy and lactation are based on the average fatty acid intakes of apparently healthy women in the United States, and for infants, based on the average levels of fatty acids received from human milk and complementary foods. Whether current fatty acid intakes also represent the intakes compatible with optimal fetal and infant growth and development are not known. A major limitation to deriving dietary requirements for n-3 and n-6 fatty acids is the lack of understanding of the functions of the different n-3 and n-6 fatty acids in developing tissues. Available autopsy data suggest that relatively high amounts of 22:6n-3 accumulate in the liver before birth, although the function and significance of n-3 or n-6 fatty acids in liver development is not well understood. Following birth, the liver must co-ordinate the transition from in utero nutrition to the high fat milk diet through metabolic adaptation across pathways of fatty acid, glucose and presumably amino acid metabolism. In adult liver, the n-3 and n-6 fatty acids regulate pathways of lipid and carbohydrate metabolism, but whether n-3 and n-6 fatty acids play similar roles in the developing liver is not known. The purpose of the research described here is to determine whether the quantity or balance of the n-3 and n-6 fatty acids provided to the fetal and neonatal liver influences metabolic pathways, with possible
implications for metabolic adaption at birth. The overall goal is to advance knowledge of the metabolic and physiological importance of n-3 and n-6 fatty acids during development.

1.4 Research rationale and objectives

1.4.1 Rationale and objectives for Chapter 2

*Rationale:* The long chain n-3 fatty acid, 22:6n-3, has received considerable attention because of its role in the developing brain and retina. This has led to the inclusion of 22:6n-3 in many infant formulas and a growing awareness of the importance of 22:6n-3 and fish, a rich source of 22:6n-3 and 20:5n-3, for pregnant and lactating mothers. Docosahexaenoic acid (22:6n-3) can be synthesized from 18:3n-3, but the extent to which dietary 18:3n-3 can provide optimal amounts of 22:6n-3, and how this is impacted by the n-6 fatty acid content of the diet is unclear. High intakes of 18:2n-6, as is characteristic of many Western diets, may inhibit conversion of 18:3n-3 to 22:6n-3 and perhaps also compete with the n-3 fatty acids for incorporation into tissue lipids. The first goal of the research described in Chapter 2 was to determine whether altering the balance of 18:2n-6 to 18:3n-3 in diets fed to piglets influences 20:5n-3 and 22:6n-3 accumulation in developing organs, including the liver, brain and heart. In addition, because the inclusion of 22:6n-3, but not 20:5n-3 in infant formula was based largely on the role of 22:6n-3 in the brain and retina, this study also sought to characterize the effects of supplementary 22:6n-3, given with balanced 20:4n-6 as in infant formula, on n-3 and n-6 fatty acid accumulation in the liver and heart.

*Objectives:*

1. To determine the effect of increasing dietary 18:2n-6 at constant 18:3n-3 intake on the n-3 and n-6 fatty acid composition of liver, frontal cortex, heart and plasma of formula fed piglets.
2. To determine if the liver, frontal cortex, heart and plasma n-3 and n-6 fatty acids are the same when supplemental 22:6n-3 and 20:4n-6 are fed to piglets compared to when the fatty acids must be synthesized endogenously from dietary 18:3n-3 and 18:2n-6.

Null hypotheses:

1. The n-3 and n-6 fatty acid composition and ratio in piglet milk replacer (formula) diets will not influence the fatty acid composition of the liver, brain frontal cortex, heart or plasma.

2. Inclusion of supplemental 22:6n-3 and 20:4n-6 in milk replacer diets of piglets will not alter the fatty acid composition of the liver, frontal cortex, heart or plasma.

1.4.2 Rationale and objectives for Chapter 3

Rationale: The composition of fatty acids that accumulate in the developing liver varies with the maternal dietary n-3 and n-6 fatty acid composition. In adult liver, n-3 and n-6 fatty acids are known to regulate the expression of genes involved in fatty acid and glucose metabolism. Whether n-3 and n-6 fatty acids regulate gene or protein expression in developing liver is not known, but it is likely that metabolic pathways and their regulation differ in the developing compared to adult liver. Proteomics enables the study of the complete protein complement in a sample and is a useful technique for detecting changes in protein expression without prior knowledge of potential protein targets. The research reported in Chapter 3 used two-dimensional (2D) gel proteomics combined with targeted gene expression and biochemical analyses to investigate whether gene and protein expression of enzymes regulating glucose, fatty acid and amino acid metabolism are altered in association with differences in neonatal liver n-3 and n-6 fatty acids.
Objectives:

1. To measure the ontogenic expression of key genes for hepatic enzymes of fatty acid oxidation in the rat from embryonic day 19 to postnatal day 10, and to identify the most appropriate age at which to address early developmental differences in gene expression due to altered n-3 and n-6 fatty acids.

2. To establish diets that when fed during pregnancy and lactation will result in a higher or lower proportion of n-3 fatty acids (by weight of total fatty acid) in their offspring liver, without compromising growth.

3. To use 2D polyacrylamide gel electrophoresis (PAGE) combined with mass spectrometry to determine if hepatic protein expression is altered, and identify proteins that are differentially expressed in neonatal livers with higher or lower n-3 fatty acids.

4. To determine if the expression of hepatic genes relevant to fatty acid oxidation and synthesis, glycolysis, gluconeogenesis and amino acid metabolism are altered in neonatal livers with higher or lower n-3 fatty acids.

5. To determine if the concentrations of lipids, including triglyceride, cholesterol and phospholipids, or glucose, glycogen or amino acids are altered in neonatal livers with higher or lower n-3 fatty acids.

6. To determine if glutathione, NADPH and protein carbonyls, as markers of oxidative stress, are altered in neonatal livers with higher or lower n-3 fatty acids.

7. To map the results of the proteomic, gene expression and biochemical studies into metabolic pathways to identify potential differences in metabolism that may occur in neonatal livers as a result of differences in the maternal dietary n-3 fatty acid intakes.

Null hypotheses:

1. Hepatic gene expression for enzymes of fatty acid oxidation will not change from embryonic day 19 to postnatal day 10.
2. Manipulation of the n-3 and n-6 fatty acid content of the maternal diet in pregnancy and lactation will not lead to differences in growth or the proportion of n-3 and n-6 fatty acids (g/100 g fatty acid) in the neonatal offspring liver.

3. Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not lead to differences in protein or gene expression for enzymes of fatty acid, glucose or amino acid metabolism in neonatal liver.

4. Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter the concentration of triglyceride, phospholipid or cholesterol in neonatal liver.

5. Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter glucose, glycogen or amino acid concentrations in neonatal liver.

6. Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter markers of oxidative stress in neonatal livers.

1.4.3 Rationale and objectives for Chapter 4

Rationale: Chapter 3 demonstrated altered gene and protein expression in the livers of young rats exposed to different n-3 and n-6 fatty acid supplies; however, whether the differences in gene expression were specifically due to differences in the n-3 and/or n-6 fatty acids remained unclear, due to concomitant changes in the other fatty acids. Specifically, analysis of the milk fatty acids showed that levels of medium chain fatty acids (MCFA, fatty acids with carbon chains of 14 or less) were lower in milk from rats fed a diet with higher amounts of n-3 and n-6 fatty acids. MCFA are synthesized from acetyl CoA in the mammary gland, and in the liver, n-3 and n-6 fatty acids are known to suppress fatty acid synthesis. Whether n-3 and n-6 fatty acids influence MCFA synthesis and secretion in milk is unclear. The purpose of the research in Chapter 4 was to investigate the interaction of n-6 and n-3 fatty acids with MCFA secretion in
milk, and separate effects of MCFA, n-3 and n-6 fatty acids on regulation of gene expression in the neonatal liver. Studies on human milks were also included to determine whether similar relationships between n-6 and n-3 fatty acids and MCFA occur in human milk.

**Objectives:**

1. To feed lactating rats diets varying in fat and fatty acid content in order to separate the effect of the diet fat content compared to composition on milk MCFA.

2. To determine whether maternal plasma triglyceride and insulin are associated with milk MCFA levels (g/100 g fatty acid) in rats.

3. To determine if differences in the levels of MCFA, n-3 and n-6 fatty acids (g/100 g fatty acid) in milk are associated with differences in the expression of genes relevant to fatty acid and glucose metabolism in the neonatal rat liver.

4. To analyze milk fatty acids from a large group of women and address associations between MCFA and the major n-6, n-3, saturated and monounsaturated fatty acids in milk.

**Null hypotheses:**

1. Levels of MCFA in milk will not differ among rats fed diets differing in fat content and fatty acid composition.

2. Maternal plasma triglyceride and insulin concentrations will show no association to the levels of MCFA in milk.

3. The maternal fat and fatty acid composition will not alter the expression of genes relevant to fatty acid and glucose metabolism in the neonatal rat liver.

4. There will be no associations between MCFA levels and the levels of n-6, n-3, saturated or monounsaturated fatty acids in human milks.
CHAPTER 2: High dietary omega-6 fatty acids contribute to reduced docosahexaenoic acid in the developing brain and inhibit secondary neurite growth

2.1 Chapter synopsis

The research described in this chapter was designed with several objectives aimed to extend understanding of how dietary fatty acids influence n-6 and n-3 fatty acid accumulation in developing tissues. The primary purpose was to investigate whether high dietary 18:2n-6 in the presence of adequate 18:3n-3 impacts n-6 and n-3 fatty acids in developing tissues, in a similar direction to n-3 fatty acid deficiency, thus addressing whether competition between 18:2n-6 and 18:3n-3 is physiologically relevant. The study also addressed whether supplementation of a high 18:2n-6 diet with 22:6n-3 and 20:4n-6 results in the same tissue fatty acid composition to that achieved when the diet has a low amount of 18:2n-6 and adequate 18:3n-3. This question is of practical relevance as it addresses whether supporting the desaturation of 18:3n-3 enables a particular pattern of tissue fatty acids that is not achieved by supplementation with the desaturation products, 22:6n-3 and 20:4n-6. This study used formula fed piglets, a suitable model to address the effects of dietary fatty acids on developing tissue fatty acids. The findings were published with a focus on the brain, due to intense interest in the effects of dietary fatty acids in the developing brain. For this reason, studies with primary cortical neuron cultures were included, and these addressed whether neuronal uptake of 22:6n-3 is specific or if the long chain n-6 fatty acid, 22:5n-6 is similarly accumulated, and the efficiency of 22:6n-3 and 22:5n-6 in supporting neurite outgrowth. Liver, heart, and plasma fatty acids were also analyzed to gain insight into differences among tissues, and this opened the field of research to the developing liver. The studies showed that in the liver, n-3 and n-6 fatty acid accretion was altered by the

1 A version of this chapter has been published:
dietary fatty acid supply; however, the specific types of n-3 and n-6 fatty acids accumulated and the effects of dietary n-3 and n-6 fatty acids differed from the brain. Feeding piglets either an 18:3n-3 deficient diet or a diet with high 18:2n-6 (10% energy) and 1% energy 18:3n-3 led to low 22:6n-3 and high 22:5n-6 and 22:4n-6 in liver phospholipids. A low dietary intake of 18:2n-6 (1% energy), in a 1:1 ratio with 18:3n-3 supported high 22:6n-3, lower 22:5n-6, 22:4n-6, and 20:4n-6, as well as high 22:5n-3 and 20:5n-3. This demonstrated that, in the presence of low 18:2n-6, 18:3n-3 can restrain long chain n-6 fatty acid metabolism and be the substrate for long chain n-3 fatty acid synthesis. Inclusion of 22:6n-3 and 20:4n-6 in the formula diets led to high liver levels of 22:6n-3 and 20:4n-6, but did not enable accumulation of high levels of 20:5n-3 as found when the diet had low 18:2n-6 in a 1:1 ratio of 18:2n-6/18:3n-3. The uniquely different fatty acid composition of the liver compared to the brain, in which 20:5n-3 is consistently low and 20:4n-6 levels were not altered by diet, together with knowledge that n-3 fatty acids regulate gene expression for enzymes of fatty acid and glucose metabolism in the adult liver led to the research in subsequent chapters investigating the implications of n-6 and n-3 fatty acids in the liver for metabolic development.

2.2 Introduction

The non-myelin membranes of the brain contain high levels of the n-3 fatty acid docosahexaenoic acid (22:6n-3) particularly in ethanolamine phosphoglycerides (EPG) and phosphatidylserine (PS) (112). Although the reason for the high 22:6n-3 in brain EPG and PS remains incompletely understood, it is known that decreased 22:6n-3 in the developing brain is associated with impaired neurogenesis, altered metabolism of several neurotransmitters, membrane receptor and protein activities, gene expression, and impaired performance on tasks of learning and memory in animals (111, 113, 120-122, 231-233). Studies of autopsy tissue have shown lower 22:6n-3, but higher proportions of n-6 fatty acids in the brain of infants fed formula
than in infants who were breast fed before death (198, 234), thus providing evidence that early diet has the potential to alter brain 22:6n-3 and n-6 fatty acids in human infants. Some, but not all clinical studies have reported better early mental and motor skill development in infants fed formula containing 22:6n-3 than in infants fed formula without 22:6n-3 (235-239), suggesting that early postnatal fatty acid nutrition is relevant to human brain development.

Docosahexaenoic acid, 22:6n-3 can be obtained directly from the diet as a component of animal tissue lipids, notably fish, or it can be synthesized from its precursor α-linolenic acid (18:3n-3), which is consumed mainly in vegetable oils (39). Metabolism of 18:3n-3 to 22:6n-3 is accomplished by Δ6 desaturation, elongation, and Δ5 desaturation to yield 20:5n-3, which is further elongated, Δ6 desaturated, and chain-shortened to 22:6n-3 (240). However, linoleic acid (18:2n-6) is desaturated and elongated to arachidonic acid (20:4n-6), with further elongation to 22:4n-6 and 22:5n-6 using the same pathway as in the synthesis of 22:6n-3 from 18:3n-3 (240). Early studies demonstrated competitive interaction between 18:2n-6 and 18:3n-3 (77, 81, 241), and showed that n-6 fatty acid requirements are met by as little as 1% energy from 18:2n-6 in animals and infants (78, 242). Increasing dietary 18:2n-6, however, inhibits desaturation of 18:3n-3, reduces 20:5n-3 and 22:6n-3 and increases 22:4n-6 and 22:5n-6 in the brain and other organs (78, 81, 243). Thus, excess desaturation of n-6 fatty acids may result from either inadequate n-3 fatty acids, or excessive intakes of 18:2n-6.

Intakes of 18:2n-6 have increased sharply during the last century, and this has also led to a doubling of 18:2n-6 in human milk (125, 244). The levels of 18:2n-6 in infant formula are based on human milk fatty acids (245), and now also typically provide 8–10% dietary energy from 18:2n-6, with about 0.75–1.3% energy from 18:3n-3. To address possible needs for 22:6n-3, and avoid problems due to suppression of 20:4n-6 synthesis, some infant formulas now contain about 0.15 % energy from 22:6n-3, with addition of 20:4n-6. In the present studies, our
objectives were to address whether high dietary 18:2n-6 reduces 22:6n-3 accretion, and to study the effects of lowering dietary 18:2n-6 or adding 22:6n-3 to a diet high in 18:2n-6 on developing brain fatty acids. We used the colostrum-deprived formula-fed piglet as an appropriate model of the rapidly growing term gestation infant (246, 247). Liver and plasma were analyzed because 22:6n-3 accumulated in the developing brain is derived from hepatic synthesis, via plasma transport (248), and heart lipids were analyzed to give insight into possible mechanisms through which the brain may take up or exclude fatty acids when compared to other organs. Primary cultures of cortical neurons were used to address preferential acylation of 22:6n-3 compared to 22:5n-6, and neurite outgrowth was used as a functional measure of the ability of 22:5n-6 to replace 22:6n-3 in the neuron.

2.3 Experimental procedures

2.3.1 Animals and diets

Male Yorkshire piglets were fed from birth with one of four liquid diets modeled after human and sow milk that were identical in all nutrients except for the composition of the fat (246). The male piglets, each from a separate litter, were randomly assigned to treatment, and hand-fed to appetite every 1.5 hours for the first 10 days, then every 3 hours from 0600 to 2000 from 11 to 30 days of age. The diets contained, as a percent energy, 1.2% 18:2n-6 and 0.05% 18:3n-3 (deficient), 10.7% 18:2n-6 and 1.1% 18:3n-3 (contemporary), 1.2% 18:2n-6 and 1.1% 18:3n-3 (evolutionary), or 10.7% 18:2n-6 and 1.1% 18:3n-3 with 0.3% 20:4n-6 and 0.3% 22:6n-3 (supplemented). We chose the term contemporary to represent the high 18:2n-6 diet as this resembles modern Western diets high in 18:2n-6. The term evolutionary was used to represent the diet with low 18:2n-6 balanced with 18:3n-3 as this resembles early human diets.

2 The complete fatty acid profile as analyzed by GLC in our laboratory is provided in the appendix in Table A2.1.
with lower 18:2n-6 and a ratio of 18:2n-6 to 18:3n-3 between 2:1 and 1:1. The contemporary and supplemented formula diets resemble infant formulas without and with 22:6n-3 + 20:4n-6, respectively. The source of 18:3n-3 was flaxseed oil in the evolutionary diet and soybean oil in the contemporary and supplemented diets. The long chain fatty acids, 20:4n-6 and 22:6n-3 were from single cell triglycerides (Martek Biosciences, Columbia, MD). To maintain a constant amount of fat in the diets, the levels of the monounsaturated fatty acid, 18:1n-9, varied inversely with 18:2n-6. Details of the housing and care of the piglets have been reported previously (232, 246). The protocol and procedures involving the animals in these studies were approved by the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care.

2.3.2 Tissue collection

At 30 days of age, fasted piglets were anesthetized (ketamine hydrochloride, 37.5 mg/kg, MTC Pharmaceuticals, Cambridge, Canada; and xylazine hydrochloride, 3.75 mg/kg, Bayvet Division, Chenango, Etobicoke, Canada), and blood was collected by cardiac puncture, then the animals killed by intracardiac injection of 200 mg/kg pentobarbital. The frontal cortex, heart and liver were rapidly removed, weighed, frozen in liquid nitrogen and stored at −80°C until analyzed. Frontal cortex was analyzed as differences in frontal cortex n-3 and n-6 fatty acids have been associated with altered dopamine metabolism and altered behavioral outcomes in animal studies (232).

2.3.3 Primary neuron cultures

Day 16–17 embryos were obtained from pregnant Sprague Dawley rats by Caesarian section under isoflurane anesthetic. All procedures involving the rats complied with and were approved by the Animal Care Committee of the University of British Columbia. Single cells were dissociated from the whole cerebral cortex and cultured on polylysine coated plates
(0.75 \times 10^6 \text{ viable cells/well}) and incubated with low-glucose Dulbecco's Modified Eagle Medium (Invitrogen-Gibco, Burlington, Ont., Canada) and F12 Nutrient Mixture (Invitrogen-Gibco) supplemented with glucose (1.44 g/L), pyruvate (0.09 g/L), ethanolamine (0.0282 µL/L), β-hydroxybutyrate (0.104 g/L), fumaric acid (0.12 g/L), glycine (0.15 g/L), HEPES (3.57 g/L), sodium bicarbonate (1.2 g/L), creatine (2 mg/mL), and B27 tissue culture supplement (2 mL/100 mL, Invitrogen-Gibco). This media is lipid-free and designed to biochemically select for neurons. For the first 72 hours the media was also supplemented with 1% Penicillin–Streptomycin and 2% fetal bovine serum, to facilitate cell adhesion. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. After 10 days, cells were incubated with varying concentrations of delipidated bovine serum albumin bound 22:6n-3 or 22:5n-6 (249) for 72 hours, with fresh media added to replenish the cultures every 24-48 hours. For analysis of neurite growth, cells were visualized using an Olympus IX51 inverted research microscope (Olympus Canada, Markham, Ont., Canada), and primary and secondary neurite length measured using Image-Pro Plus Software (Mediacybernetics, Bethesda, MD). Analysis was performed on a minimum of 10 cells/well, with three wells/treatment and the entire experiment was repeated three times. For analysis of neuron phospholipids and fatty acids, cells were recovered, rinsed and the fatty acids analyzed as described below. We used two-four separate wells/treatment, with all experiments repeated three times.

2.3.4 Analytical methods

Total lipids were extracted from homogenized tissues, plasma or primary neuron cultures (Appendix A1.1), then lipid classes were separated using a high performance liquid chromatograph (HPLC), equipped with an auto-sampler, evaporative light scattering detector and a fraction collector (249). Lipid classes of interest were recovered, then the fatty acids were converted to their respective methyl esters (Appendix A1.2), separated and quantified by capillary column gas liquid chromatography (GLC) (120, 249).
2.3.5 Statistical analyses

Results are represented as means ± standard error of the mean (SEM) for the diet studies and means ± standard deviation (SD) for neuron cell culture experiments. For diet studies, differences among the groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's test. For the neuron culture experiments, differences were determined using the unpaired t-test. The level of statistical significance was set at $P < 0.05$.

2.4 Results

2.4.1 Effect of dietary 18:2n-6 and the 18:2n-6/18:3n-3 balance on piglet frontal cortex

The brain weight at 30 days of age was not different among the groups (data not shown). While the effects of n-3 fatty acid deficiency in decreasing brain 22:6n-3 with a compensatory increase in 22:4n-6 and 22:5n-6 has been extensively described (250), we sought to determine whether high dietary intakes of 18:2n-6 in a diet containing 18:3n-3 in amounts considered to meet requirements reduces the accumulation of 22:6n-3 in the developing brain. Figure 2.1 provides the results to show the major n-6 and n-3 fatty acids in the piglet frontal cortex after feeding the formula diets for 30 days from birth. Notably, with 1.2% energy from 18:2n-6 in the deficient diet almost devoid of 18:3n-3, the frontal cortex levels of 20:4n-6 were high, and 22:4n-6 and 22:5n-6 were increased, which is the expected response to feeding an n-3 fatty acid deficient diet during early development (250). The high 20:4n-6, 22:4n-6 and 22:5n-6, representing 21.0 ± 0.33, 9.9 ± 0.72 and 6.4 ± 0.75 g/100 g of the fatty acids, respectively in the frontal cortex EPG of piglets fed the deficient diet with 1.2% energy from 18:2n-6 emphasizes that only small amounts of 18:2n-6 are needed in the diet to support desaturation and elongation of n-6 series fatty acids. In piglets fed the contemporary diet with 10.7% energy 18:2n-6 and 1.1% energy from 18:3n-3, we address whether high intakes of 18:2n-6 compromise accretion of
22:6n-3 in the developing brain and promote excess accumulation of long chain n-6 fatty acids. Our results show significantly lower 20:5n-3 and 22:6n-3, with higher 22:4n-6 and 22:5n-6 in the frontal cortex EPG, PS, and phosphatidylcholine (PC) (Figure 2.1), indicating interference with n-3 fatty acid metabolism and excess metabolism of the n-6 series fatty acids compared to the evolutionary diet with only 1% 18:2n-6. The evolutionary diet providing 1.1% energy as 18:3n-3 with a dietary 18:2n-6/18:3n-3 balance close to 1:1 supported substantially higher 22:6n-3 in EPG, PS, and PC, and lower 22:4n-6 and 22:5n-6 in EPG and PS than in piglets fed the n-3 deficient or contemporary diets (Figure 2.1). These results show that competitive interaction between 18:2n-6 and 18:3n-3 for the desaturase enzymes is relevant to accretion of 20:4n-6, 22:4n-6, 22:5n-6 and 22:6n-3 in the developing brain.
Figure 2.1 Major n-3 and n-6 fatty acids in frontal cortex phospholipids.

Values are means ± SEM, n = 5–7 piglets/diet. Within a phospholipid, values with different superscripts are significantly different by ANOVA followed by Tukey's test (P < 0.05). EPG, ethanolamine phosphoglycerides; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol.
2.4.2 Effect of dietary 22:6n-3 and 20:4n-6 on piglet frontal cortex

We next considered the effect of inclusion of 22:6n-3 and 20:4n-6 in the contemporary diet on frontal cortex n-3 and n-6 fatty acids to address whether the same fatty acid composition is achieved by feeding an evolutionary diet with 18:3n-3 and low 18:2n-6, or through addition of preformed 22:6n-3 to a contemporary diet high in 18:2n-6. The contemporary and supplemented diets resemble the infant formula diets that have been the subject of several clinical studies with infants (235, 236, 239). Our results show that feeding 22:6n-3 + 20:4n-6 increased 22:6n-3 and decreased 22:5n-6 in the frontal cortex when compared to piglets fed the contemporary diet without these fatty acids (Figure 2.1). Although the brain EPG levels of 22:6n-3 and 20:4n-6 from piglets fed the supplemented diet were not different from that of piglets fed the evolutionary diet, the evolutionary diet supported higher levels of 20:5n-3 and 22:5n-3, while the supplemented diet led to higher 22:4n-6 in frontal cortex EPG (Figure 2.1). Thus, it is clear that addition of 22:6n-3 + 20:4n-6 to a Westernized diet high in 18:2n-6 does not support the same frontal cortex fatty acid composition as that achieved by an evolutionary diet low in 18:2n-6.

2.4.3 Effects of dietary n-6 and n-3 fatty acids on plasma compared to the brain

Synthesis of 22 carbon chain n-3 and n-6 fatty acids appears to be very low in the brain, and current evidence suggests that the brain takes up 22:6n-3 from circulating plasma fatty acids (250). We, therefore, analyzed plasma phospholipid fatty acids to further understand how circulating fatty acids affect the brain and how fatty acids may be excluded or preferentially taken up. Notably different from frontal cortex in which 18:2n-6 never exceeded 2 g/100 g fatty acid in any phospholipid, 18:2n-6 always exceeded 15 g/100 g fatty acid in the plasma phospholipids (Figure 2.2). Similar to the frontal cortex, the deficient diet and the contemporary diet were associated with low plasma phospholipid 22:6n-3, while the evolutionary diet with balanced n-6/n-3 and the supplemented diet with 22:6n-3 + 20:4n-6 both supported higher 22:6n-3 in plasma phospholipids. Addition of 22:6n-3 + 20:4n-6 to the diet also decreased plasma
phospholipid 22:5n-6 as found in the frontal cortex. Although there was a two-fold decrease in 22:5n-6 in frontal cortex EPG and PS in pigs fed the evolutionary compared to the contemporary diet, there was no difference in 22:5n-6 in plasma phospholipids between these groups. However, levels of 22:5n-6 in plasma phospholipids are low, less than 1 g/100 g fatty acid, and only about 10% of the levels found in the frontal cortex. The intermediate fatty acids, 22:5n-3 and 22:4n-6 in plasma phospholipids, on the other hand, appear to better reflect the increased metabolism of n-3 fatty acids and restraint of n-6 fatty acid metabolism in piglets fed the evolutionary compared to the contemporary and supplemented diets.

**Figure 2.2** Major n-3 and n-6 fatty acids in plasma phospholipids of piglets.

Values are means ± SEM, n = 5–7 non-littermate male piglets/diet. For each fatty acid, values with a different superscript are significantly different by one-way ANOVA followed by Tukey's test (P < 0.05).
2.4.4 Primary neuronal cell uptake of 22:6n-3 and 22:5n-6

Because our results showed frontal cortex EPG 22:5n-6 levels two-fold higher in piglets fed the contemporary compared to the evolutionary diet, but no significant difference in the plasma phospholipid levels of 22:5n-6, we next addressed if neurons lack specificity for acylation of 22:5n-6 compared to 22:6n-3. Brain phosphoglycerides are known to differ in fatty acid composition, with 22:6n-3 selectively enriched in EPG and PS, as shown in Figure 2.1. When incubated with 20 µM 22:6n-3 or 22:5n-6 bound to albumin, primary cultures of cortical neurons took up and acylated 22:6n-3 and 22:5n-6 in EPG and PS, rather than PC or PI (Table 2.1), demonstrating fatty acyl transferase specificity for the 22 carbon chain n-3 and n-6 fatty acids. Notably, the incorporation of 22:5n-6 in cells incubated with 22:5n-6 was similar to incorporation of 22:6n-3 in cells incubated with the same concentration of 22:6n-3, suggesting a lack of specificity for 22:6n-3 over 22:5n-6. We, therefore, next considered competitive acylation into phospholipids through incubation of primary cultures of cortical neurons with albumin-bound 22:6n-3 and 22:5n-6 in ratios provided as 2:1, 1:1 or 1:2 22:6n-3 and 22:5n-6, and determined the fatty acid incorporation into the neuronal total phospholipids. When incubated with 10 µM 22:6n-3 plus 10 µM 22:5n-6, we found higher levels of 22:5n-6 (12.6 ± 0.08 g 22:5n-6/100 g fatty acid) than 22:6n-3 (4.5 ± 0.11 g 22:6n-3/100 g fatty acid) in the phospholipid fatty acids. At two-fold higher concentrations of 22:6n-3 than 22:5n-6, using either 10 µM or 20 µM 22:6n-3 with 5 µM or 10 µM 22:5n-6, respectively, we found concentrations of 5.0 ± 0.3 g 22:6n-3/100 g fatty acid and 10.0 ± 0.8 g 22:5n-6/100 g fatty acid, while with 10 µM or 20 µM 22:5n-6 and 5 µM or 10 µM 22:6n-3, respectively, the neuronal phospholipids had 4.1 ± 0.3 g 22:6n-3/100 g fatty acid and 14.7 ± 1.5 g 22:5n-6/100 g fatty acid. Surprisingly, our results indicate higher uptake and acylation of 22:5n-6 over 22:6n-3. We cannot extend our results to other cells, such as the capillary endothelium or astrocytes, however, it is clear that the neuron will take up and readily acylate 22:5n-6.
Table 2.1  Phospholipid 22:6n-3 and 22:5n-6 concentrations in primary neurons cultured with 20 µM 22:6n-3 or 22:5n-6.

<table>
<thead>
<tr>
<th></th>
<th>EPG</th>
<th>PC</th>
<th>PS</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>22:6n-3</td>
<td>24.9(0.5)</td>
<td>2.0(0.3)</td>
<td>3.5(0.1)</td>
<td>0.3(&lt;0.1)</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>6.9(0.7)</td>
<td>23.0(1.6)</td>
<td>0.2(&lt;0.1)</td>
<td>2.6(0.5)</td>
</tr>
</tbody>
</table>

Values are means with SD in parenthesis of four separate experiments. EPG, ethanolamine phosphoglycerides; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

2.4.5 Effects of 22:6n-3 and 22:5n-6 on neurite outgrowth

Because our results show that neurons take up 22:5n-6 and, in the brain, 22:5n-6 may replace 22:6n-3, particularly when the diet is either deficient in n-3 fatty acids or high in 18:2n-6, we next sought evidence of whether 22:6n-3 has specific effects on neuronal growth not supported by 22:5n-6, using neurite outgrowth as the outcome measure based on previous studies of altered neurogenesis in the embryonic n-3 fatty acid deficient brain (120). Primary neuronal cells were incubated with 20 µM 22:6n-3 or 22:5n-6 and neurite outgrowth was measured after 72 hours. We found no difference in the number of primary neurites, average primary neurite length, or total primary neurite length between cultures incubated with 22:6n-3 or 22:5n-6. However, secondary neurite branching, total secondary neurite length and average secondary neurite length was higher in cultures incubated with 22:6n-3, compared to those incubated with 22:5n-6 (Figure 2.3).
Figure 2.3 Measurement of neurite outgrowth in primary cortical neurons incubated with 20 μM 22:6n-3 or 22:5n-6.

Values are means ± SD. Analysis was performed on a minimum of 10 cells/plate, with 3 plates/experiment. *Significantly different from 22:6n-3 using unpaired t-tests (P < 0.05).

2.4.6 Effects of dietary n-6 and n-3 fatty acids on heart and liver

To further understand brain specific transport and fatty acid uptake mechanisms and to gain better insight into the effects of dietary n-6 and n-3 fatty acids in the whole animal, we also considered the effects of the diets on phospholipids in the heart and liver. This is important for several reasons, including the need for diets to meet the needs of all organs, not only the brain, and because altered metabolism in other organs can impact the brain through secondary mechanisms including changes in hormones or intermediary metabolism. Consideration of the heart and liver EPG and PC fatty acids emphasizes the marked differences in n-3 and n-6 fatty acid accretion compared to frontal cortex (Figure 2.4 and Figure 2.1). Feeding the evolutionary
diet with 1.1% energy 18:3n-3 and low 18:2n-6 resulted in a remarkable 13.4 ± 2.0 g 20:5n-3/100 g fatty acid and 7.5 ± 1.1 g 20:5n-3/100 g fatty acid in heart and liver EPG, respectively, compared to only 0.37 ± 0.05 g 20:5n-3/100 g fatty acid in frontal cortex (Figure 2.4 and Figure 2.1). We also draw attention to 20:4n-6, which was significantly lower in the EPG and PC of piglets fed the evolutionary compared to the deficient, contemporary or supplemented diets. As in plasma, but different from the frontal cortex, inclusion of 20:4n-6 in the supplemented diet increased the concentration of 20:4n-6 in the heart and liver phospholipids (Figure 2.4). While further extension of these results with respect to the heart and liver is beyond the scope of our work related to n-3 fatty acids and the developing brain, it is clear from our results that a diet that meets the needs for accretion of 22:6n-3 in the developing brain, such as the evolutionary and supplemented diets, has remarkably different effects on 20:4n-6 and 20:5n-3 in other organs.
Figure 2.4 Major n-3 and n-6 fatty acids in liver and heart phospholipids of piglets.

Values are means ± SEM, n = 5–7 non-littermate piglets/diet for the deficient, contemporary, evolutionary and supplemented groups, respectively. For each fatty acid, values within a phospholipid with different superscripts are significantly different by one-way ANOVA followed by Tukey's test (P < 0.05). EPG, ethanolamine phosphoglycerides; PC, phosphatidylcholine.

2.5 Discussion

Dietary intakes of the n-6 fatty acid, 18:2n-6, have increased over the last century (76, 125), with unknown implications for n-3 and n-6 fatty acids in the brain. High intakes of 18:2n-6 has led to a doubling of 18:2n-6 in human milk (5, 251), which is extended to infant formulas for which the fatty acid composition is adapted from that of human milk (245). In this chapter, the effects of high intakes of 18:2n-6 in the neonatal diet were addressed using the bottle-fed piglet as an appropriate model for the rapidly growing term gestation infant (247). The results show that contemporary diets providing about 10.7% energy from 18:2n-6, with an 18:2n-6/18:3n-3
ratio of 10:1 compromise the accretion of 22:6n-3 and lead to over-accumulation of n-6 fatty acids in the developing brain. We further show that 1.2% energy from 18:2n-6 both meets n-6 requirements with high tissue levels of 20:4n-6, and when fed in a 1:1 ratio with 18:3n-3 supports high levels of 22:6n-3 in the brain and high 20:5n-3 and 22:6n-3 in other organs. Our results confirm that supplementation of a contemporary diet high in 18:2n-6 with 22:6n-3 provides a remedial approach to achieving high 22:6n-3 in the developing brain, but emphasize that it does not overcome the high levels of intermediate n-6 and low levels of n-3, notably 20:5n-3 in tissues due to feeding a diet high in 18:2n-6. The interpretation of these findings is two-fold; first, human milk in Westernized nations and formula diets seem likely to have an overabundance of 18:2n-6 (245), and secondly, supplementation with long chain fatty acids 22:6n-3 and 20:4n-6 may be a simplistic and incomplete approach to correct the deviations in membrane fatty acids that result from excessive consumption of n-6 fatty acids.

Studies almost 50 years ago showed that 18:2n-6 requirements are very low, in the range of 1–2% energy, in animals and in rapidly growing infants (81, 242), and this was reflected in early recommendations suggesting about 3% energy from 18:2n-6 in infant formulas (55). Regardless, dietary guidelines to promote exchanging 18:2n-6-rich vegetable oils for saturated fats as a strategy to lower plasma cholesterol contributed to marked increases in intakes of 18:2n-6 (57). However, competitive interaction between 18:2n-6 and 18:3n-3 is well-known (252), with available data suggesting that the desaturase pathway is saturated at intakes above 3% energy from 18:2n-6 (77). Furthermore, well-controlled studies involving manipulation of dietary 18:2n-6 and 18:3n-3 have shown that increasing dietary 18:2n-6 decreases 22:6n-3 and increases n-6 fatty acid, particularly 22:5n-6 incorporation into brain and other tissue phospholipids (77, 78, 243). Consistent with the latter, the present studies provide clear evidence that increasing dietary 18:2n-6 from 1.2% to 10.7% energy, while maintaining 18:3n-3 constant at 1.1% energy results in significant reductions in 22:6n-3, as well as the intermediates, 20:5n-3.
and 22:5n-3, with increased accumulation of 22:4n-6 and 22:5n-6 in the developing brain. Although interpretation of the origin of brain fatty acids is not addressed by our study, current evidence suggests that brain synthesis of 22:6n-3 is low and that most brain 22:6n-3 is derived by uptake of 22:6n-3 from plasma unesterified fatty acids, which in a diet not providing 22:6n-3 originates from synthesis of 22:6n-3 in the liver, since the liver is the major site of fatty acid desaturation (248, 253). Thus, it is likely that competition between 18:2n-6 and 18:3n-3 for desaturation and elongation occurs primarily in the liver and decreased brain 22:6n-3 occurs secondary to decreased hepatic secretion of 22:6n-3 and increased secretion of long chain n-6 fatty acids in plasma.

The supply of 22:6n-3 is undoubtedly important for the developing brain and retina (113), and some, although not all studies in term gestation infants have reported lower visual acuity development and lower scores on tests of mental and motor skill development in infants fed formulas without 22:6n-3 when compared to the same formula with supplemental 22:6n-3 or 22:6n-3 plus 20:4n-6 (238). The contemporary and supplemented milk replacement diets used in the present study were modeled after infant formula (235, 236), providing about 50% energy from fat with 10.7% energy 18:2n-6 and 1.1% 18:3n-3 without or with supplemental 22:6n-3 and 20:4n-6 at 0.3% energy each. We provide results to confirm that feeding contemporary formula lacking 22:6n-3 leads to lower accretion of 22:6n-3, with increased amounts of n-6 fatty acids, particularly 22:4n-6 and 22:5n-6 in the developing brain when compared to feeding formula with lower 18:2n-6 or with supplementary 22:6n-3. The frontal cortex EPG levels of 22:6-3 were about 25% lower in piglets fed the contemporary diet compared to the evolutionary diet or 22:6n-3 + 20:4n-6 supplemented diet, with levels of 18.5 g 22:6n-3/100 g fatty acid in the contemporary diet, compared to 23.4 and 24.0 g 22:6n-3/100 g fatty acid in the evolutionary and supplemented diets, respectively. Notably, frontal cortex EPG 22:6n-3 was also about 25% lower in infants fed formula compared to breast-fed infants (198), further suggesting the relevance of
the present study to fatty acid accretion in the developing human brain. Our present study provides a biochemical rationale for the correction of deficits in visual and neural functional outcomes in infants fed formulas when 22:6n-3 and 20:4n-6 are provided in the formula (236, 254).

It is well-recognized that plasma and erythrocyte levels of 22:6n-3 are lower in infants fed formula without 22:6n-3 than in breast-fed infants, or infants fed formula with 22:6n-3 (254-258). However, while 22:5n-6 and 22:4n-6 are increased in erythrocyte EPG of infants fed formula, indicative of over metabolism of n-6 fatty acids, plasma phospholipid levels of 22:5n-6 and 22:4n-6 are low, representing less than 0.4 g/100 g fatty acid, and not increased when compared to breast-fed infants (255). The present study provides important results to show that while elevated plasma 22:5n-6 is a good marker of the increased brain 22:5n-6 that results from dietary n-3 fatty acid deficiency, plasma phospholipid levels of 22:5n-6 do not reflect the excess accumulation of 22:5n-6 in brain of animals fed a diet high in 18:2n-6. Rather, our results suggest that 22:4n-6 is a better marker of excess desaturation and increased levels of n-6 fatty acids in the developing brain secondary to high 18:2n-6 intakes. Of relevance, Innis and Friesen (259) recently showed that maternal red blood cell EPG 22:4n-6 during pregnancy was significantly inversely related to visual acuity in human infants at two months of age, also suggesting that 22:4n-6 is a useful biomarker of disturbances in the n-6 fatty acids in the developing brain.

A compensatory increase in 22:5n-6 in brain of animals fed diets severely restricted in n-3 fatty acids is well-known (111, 120, 246, 260), and high dietary 18:2n-6 also increase 22:5n-6 in the brain (Figure 2.1, 78, 243). However, it is clear from our results that plasma 22:5n-6 is low, not exceeding 0.6 g/100 g plasma phospholipid fatty acid in the present study, yet we found a 10-fold enrichment of 22:5n-6 in brain EPG and PS in piglets fed the deficient and
contemporary diets. In cortical neurons, we show the expected preferential incorporation of 22:6n-3 and 22:5n-6 in EPG and PS, but rather than selectivity for 22:6n-3, our results show an apparent preference for 22:5n-6 over 22:6n-3. We note that in vivo brain fatty acid uptake is complex, involving multiple cell types, lipases and binding proteins (250). However, these results may shed light on the reason for the high 22:5n-6 in the brain EPG and PS of piglets fed the contemporary diet, as well as the high 22:5n-6 in human brain tissue (198, 261). Importantly, 22:6n-3 appears to have a unique role in promoting secondary neurite branching and growth (Figure 2.3). Previous studies have also noted increased total neurite length and number of branches in neurons cultured with 22:6n-3 rather than 22:5n-6 or no fatty acids (262, 263).

Consistent with other studies, our results show that the brain maintains low levels of 18:2n-6, 18:3n-3 and 20:5n-3 (112). In the heart and liver, similar to the brain, feeding an evolutionary diet with balanced 18:2n-6/18:3n-3 or feeding 22:6n-3 in a high 18:2n-6 diet increased phospholipids levels of 22:6n-3. Current evidence suggests that the heart lacks elongase-2 (264), meaning that synthesis of 22:6n-3 from 18:3n-3 does not occur in this organ, with the 22:6n-3 accretion in the heart being explained by uptake of plasma 22:6n-3 derived from hepatic synthesis from 18:3n-3 or by uptake of 22:6n-3 provided in the diet. In contrast to the brain, balanced 18:2n-6 and 18:3n-3 supported a dramatic increase in 20:5n-3 and decreased 20:4n-6 in the heart and liver. The implication of altered 20:4n-6 and 20:5n-3 in organs other than the brain is beyond the scope of this paper focusing on the developing brain. However, these results demonstrate that decisions on dietary n-6 and n-3 fatty acid requirements, if based solely on the brain, because of its unique membrane lipid composition, may lead to marked alterations in other organs, such as the heart and liver, for which the physiological implications have yet to be considered.

In summary, we showed that current high intakes of 18:2n-6 are likely to compromise the
accretion of 22:6n-3 in the developing brain, and that lowering dietary 18:2n-6, or adding preformed 22:6n-3 to the diet supports brain 22:6n-3 accretion. We also show that a dietary source of 22:6n-3, while increasing brain 22:6n-3, does not prevent accumulation of long chain n-6 fatty acids or the loss of intermediate n-3 fatty acids, 20:5n-3 and 22:5n-3, that results from high intakes of 18:2n-6. Several recent studies have provided evidence to show that diets low in n-3 fatty acids from fish, i.e. 20:5n-3 and 22:6n-3, are associated with an increased risk of poor child visual and cognitive development (215, 216, 265-267) and aging related cognitive decline and dementia (119). While higher intakes of 22:6n-3 from fish may reduce risk, our results suggest the need to consider whether the high intakes of 18:2n-6 also compromise brain n-6 and n-3 fatty acid accretion.
CHAPTER 3: Metabolic development in the liver and the implications of the n-3 fatty acid supply

3.1 Chapter synopsis

The objective of the research described in this chapter was to determine whether the amounts of n-3 and n-6 fatty acids supplied to the developing liver are relevant to metabolic regulation in this organ. The rat was used because of the short gestation period, knowledge of the rat’s nutrient requirements, ease of preparation of experimental diets, and the ability to obtain commercially available primers for gene expression studies. Because the rat cannot be bottle-fed and artificial feeding studies do not commence until at least 3-5 days postnatal, dietary fat composition was manipulated in utero and during milk feeding via changes in the mother’s diets.

This research required several background studies due to the limited data available on the role of n-3 and n-6 fatty acids in the developing liver. Initial studies focused on the ontogenic expression of genes for fatty acid oxidation, for the purpose of defining the age at which to study potential changes in gene expression due to differences in the maternal dietary fatty acids. Studies were also done to develop maternal diets that, when fed in pregnancy, had no effect on fetal growth or hepatic triglyceride accumulation, but did alter the liver n-6 and n-3 fatty acids. These preliminary studies concluded that the third day postnatal and maternal diets with 20% energy from fat and either 3.9% energy 18:2n-6 and 1.5% energy 18:3n-3, or 1% energy 18:2n-6 and <0.1% energy 18:3n-3 were appropriate conditions for studies to determine whether the early n-6 and n-3 fatty acid supply influences metabolic regulation in the developing liver.

Next, it was recognized that both the milk diet and many aspects of physiology and endocrinology differ between young milk feeding animals and adults, and thus that the proteins

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3 A version of this chapter has been submitted for publication: Novak EM, Keller BO, Innis SM. Metabolic development in the liver and the implications of the n-3 fatty acid supply.
and genes susceptible to regulation by fatty acids may differ. Therefore, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) coupled with matrix-assisted-laser-desorption-ionization time of flight (MALDI-TOF) mass spectrometry was used to discover which proteins may be regulated by fatty acids at three days postnatal. This method was chosen, because it enables comparison of all proteins with a cell or tissue. In these studies, 10 proteins were identified that consistently differed in the liver of offspring with higher compared to lower levels of long chain n-3 fatty acids. These proteins were targeted to pathways of gluconeogenesis, glycolysis, and amino acid oxidation. In particular, altered abundance of serine hydroxymethyltransferase (SHTM1) was a new finding and this suggested a link to pathways involving non-essential amino acids and methyl metabolism.

For the final studies, gene expression and biochemical analyses were done to build upon the findings of the proteomic studies, and also target pathways known to be altered by fatty acids in adult liver, including pathways of fatty acid oxidation and synthesis. When the findings are integrated, the differences in enzyme expression and metabolite concentrations in neonatal livers with high compared to low levels of long chain n-3 fatty acids are consistent with higher fatty acid oxidation and gluconeogenesis, lower glucose oxidation to pyruvate, sparing of glucose for the pentose phosphate pathway and non-essential amino acid synthesis, and sparing of amino acids for protein and peptide synthesis. These results support a hypothesis that the n-3 fatty acid supply may be important in facilitating metabolic adaptation to feeding with the high fat, lower protein milk at birth.

3.2 Introduction

At birth, nutrient transfer changes abruptly from placental delivery, in which glucose and amino acids represent over 80% of the energy supply, to the postnatal milk diet, which provides about 50% of energy from fatty acids and lower protein (174, 268). Metabolic adaptation of
energy substrate processing, with a decrease in hepatic glycolysis and increase in fatty acid oxidation occurs following birth (9), and is likely important to the maintenance of blood glucose, avoidance of excess hepatic fatty acid accumulation, and efficient utilization of amino acids for protein and peptide synthesis. Early studies showed that the activity and expression of hepatic mitochondrial and peroxisomal enzymes of fatty acid oxidation are low in the fetus and increase rapidly after birth, consistent with the onset of feeding with the high fat milk diet (269, 270). The n-6 and n-3 fatty acids contribute to regulation of fatty acid synthesis and oxidation in adult liver, with the n-3 fatty acids having unique effects that suppress hepatic triglyceride synthesis and secretion (10, 107, 271, 272). Much of the recent interest in fatty acid nutrition during early development has focused on the role of n-3 fatty acids, particularly docosahexaenoic acid (22:6n-3) in the brain and retina (3, 7, 8). Little attention has as yet been given to the possible role of n-3 fatty acids in the neonatal liver faced with adaptation to a high fat milk diet, although accumulation of n-3 fatty acids in the fetal and neonatal liver depends on placental fatty acid transfer in gestation and the milk fatty acids after birth (108, 193, 273, Chapter 2).

Integration of metabolic pathways is a central tenant of healthy development and organ function, with the expectation that failure to balance the supply of fatty acids with endogenous fatty acid synthesis and fatty acid oxidation may lead to lipid accumulation and metabolic stress in the liver and other organs (274-276). However, metabolic control is complex, with the activity of enzyme pathways influenced at multiple levels, through gene expression to post-translational protein modification, with additional developmental regulation of the expression of transcription factors (277, 278). Recently, we described global profiling of the neonatal liver protein complement using 2D PAGE coupled with MALDI-TOF mass spectrometry, and the identification of several key proteins, including serine hydroxymethyltransferase (SHMT1) and fructose-1,6-bisphosphatase (FBP1), not previously known to be altered by n-3 fatty acids (279). Here we extend our proteomic studies to targeted analyses of gene expression and metabolite
concentrations, then integrate the results to provide a novel conceptual framework for the role of n-3 fatty acids in early metabolic regulation, not only of fatty acid metabolism, but also directing carbons towards anabolic pathways, such as the pentose phosphate pathway and the metabolism of non-essential amino acids.

3.3 Materials and methods

3.3.1 Animals and diets

All procedures involving animals were approved and carried out in accordance with the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care. The animals were from Charles River Laboratories and were housed in a temperature-controlled animal facility with a 12 hour light-dark cycle and free access to food and water throughout. In all cases, the livers were rapidly removed, immediately flash frozen in liquid nitrogen and stored at -70°C until analyzed.

First, studies were conducted to characterize the changes in expression of key enzymes of hepatic fatty acid oxidation in the perinatal period in our facility, since it is likely that differences due to fatty acid composition may be masked in the presence of major shifts in gene expression following birth. Studies on the developmental changes in hepatic gene expression were conducted at embryonic (E) day 20 (normal term gestation 21 days), 12-24 and 48-72 hours, and 10 and 17 days postnatal in offspring of dams fed commercial rodent chow (Harlan-Teklad, www.harlan.com). The results showed that expression of gene transcripts for carnitine

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4 Chow was used to reproduce findings of Thumelin et al. (270), who showed low levels of Cpt1a mRNA in fetal rat liver with an increase in Cpt1a expression at birth. The protein, carbohydrate and micronutrient content of the chow diet used for the developmental studies is similar to that in the semi-synthetic diets used for the diet studies. However, it should be noted that laboratory chow is prepared with natural source ingredients, such as ground corn or oat and, therefore, the precise nutrient and non-nutrient composition of the chow diet may differ from the semi-synthetic diets, in which purified nutrients are fed. In addition, the source of oil in the chow
palmitoyltransferase (Cpt1a) and acyl CoA oxidase (Acox1) increased 60 and 20-fold, respectively, from E20 to 12-24 hours postnatal, with a decline at 48-72 hours postnatal then a plateau to 17 days postnatal at levels about 50 and 7-fold higher, respectively, than at E20 (Figure 3.1). Peroxisome proliferator activated receptor alpha (Ppara) increased about 1.5-fold from E20 to 48-72 hours postnatal, with no further change in expression to 17 days postnatal. We, therefore, conducted studies to assess the role of n-3 fatty acids on early hepatic metabolic development at three days (72 hours) postnatal.

**Figure 3.1** Ontogenic expression of genes for enzymes of fatty acid metabolism in rat liver.

![Graph showing ontogenic expression of genes](image)

The dotted line represents the time of birth. Data points represent mean values at one day prior to birth (embryonic day 20), 12-24 hours, 48-72 hours, 10 days and 17 days postnatal for two pups per litter from two-four different litters at each time point. Cpt1a, carnitine palmitoyl transferase; Acox1, acyl CoA oxidase; Ppara, PPARα.

diets is soybean oil, which provides about 9% energy from 18:2n-6 and about 1% energy from 18:3n-3; thus, the chow diet provided higher 18:2n-6, but similar 18:3n-3 to the “n-3 adequate” diet used in the subsequent studies. The extent to which the perinatal expression of hepatic genes differs when the dam is fed chow compared to the semi-synthetic diets is not known.
Our focus is hepatic metabolic regulation by n-3 fatty acids in the absence of confounding effects due to altered fetal growth, or liver triglyceride accumulation. Thus, we next tested several diets to establish maternal dietary conditions that had no effect on litter size, birth-weight, weight at three days of age, or neonatal liver triglycerides, but did alter the neonatal liver fatty acid composition. Nulliparous female rats were fed from two weeks prior to breeding with semi-synthetic diets that provided a constant 4423 kilocalories (kcal) and 200 g protein per kg complete diet, but with varying fat and fatty acid compositions (Table 3.1). Initially, both a low fat (4% energy from fat) and higher fat (20% energy fat) diet with similar n-3 and n-6 fatty acids were fed to determine whether total fat intake influences n-3 and n-6 fatty acids in neonatal liver. The rat milk and offspring neonatal liver fatty acids were not different between the low or higher fat groups, therefore, the low fat group was not included in the final studies, but results are provided in the appendix in Table A3.1 and Table A3.2. The final studies had, as a percent energy, 20% fat and either 1.4% 18:2n-6 and <0.1% 18:3n-3 (n-3 deficient) or 3.9% 18:2n-6 and 1.5% 18:3n-3 (n-3 adequate), n=8-12 per group. An intake of 1% energy 18:2n-6 meets and exceeds the needs for n-6 fatty acids for reproduction and growth, with no signs of essential fatty acid deficiency, also supporting high tissue levels of 20:4n-6 in both rats, human infants and other species (242, 280, Chapter 2). Linoleic acid (18:2n-6) was increased to 3.9% energy in the diet with 1.5% energy 18:3n-3 to avoid the potential loss of hepatic 20:4n-6 when 18:3n-3 represents a similar 1-1.5% energy to 18:2n-6 (Chapter 2). The complete fatty acid composition of the diets is provided in the appendix in Table A2.2. These diets led to differences in 18:3n-3, 20:5n-3 and 22:6n-3, but similar 20:4n-6 in three-day-old liver phosphatidylcholine and phosphatidylethanolamine, the major liver phospholipids (Table 3.2). This dietary model thus enabled consideration of the effects of “high” compared to “low” neonatal liver n-3 fatty acids in

5 The term “n-3 deficient” was used as this diet contained undetectable levels of 18:3n-3. The term “n-3 adequate” was used as this diet provided 18:3n-3 in amounts that meet current dietary recommendations for humans (19) and rodents (338).
the absence of altered fetal growth, hepatic triglyceride accumulation or 20:4n-6. All litters were reduced to a standard 10 pups each on the first day after birth.

### Table 3.1 Nutrient content and oils in the rat diets.

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<th>g/kg complete diet</th>
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<th>n-3 adequate</th>
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<tr>
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<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Starch</td>
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<tr>
<td>Fat</td>
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</tr>
<tr>
<td>Coconut oil</td>
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</tr>
<tr>
<td>High linoleic</td>
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<tr>
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</tr>
<tr>
<td>Canola oil</td>
<td>-</td>
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<tr>
<td>Vitamins⁺</td>
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</tbody>
</table>

The diets provided 4.03 kcal/g and were designed to provide identical protein, carbohydrate, vitamins and minerals per kcal. ⁺Harlan-Teklad #40060, †Harlan-Teklad AIN93
Table 3.2  Litter size, birth-weight, and three-day-old weight, liver triglyceride, phospholipid and phospholipid fatty acids in offspring of rats fed diets differing in fat composition during gestation.

<table>
<thead>
<tr>
<th></th>
<th>n-3 deficient</th>
<th>n-3 adequate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size, n</td>
<td>15 ± 1.0</td>
<td>14 ± 0.67</td>
</tr>
<tr>
<td>Birth-weight, g</td>
<td>6.7 ± 0.42</td>
<td>6.9 ± 0.17</td>
</tr>
<tr>
<td>Weight at 3 days, g</td>
<td>8.1 ± 0.49</td>
<td>9.0 ± 0.20</td>
</tr>
<tr>
<td>3-day-old liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG, µg/mg protein</td>
<td>229 ± 34.9</td>
<td>269 ± 45.6</td>
</tr>
<tr>
<td>PL, µg/mg protein</td>
<td>156 ± 2.67</td>
<td>173 ± 6.86</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>g/100 g fatty acid</td>
<td></td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>1.54 ± 0.14</td>
<td>1.99 ± 0.11</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>22.2 ± 0.99</td>
<td>20.2 ± 0.63</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2.98 ± 0.41</td>
<td>0.84 ± 0.07*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>14.3 ± 0.97</td>
<td>0.06 ± 0.03*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.14 ± 0.03</td>
<td>0.64 ± 0.10*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>10.7 ± 1.52</td>
<td>24.9 ± 0.92*</td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>5.27 ± 0.44</td>
<td>6.29 ± 0.43</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>20.5 ± 1.09</td>
<td>18.9 ± 0.38</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.27 ± 0.25</td>
<td>0.23 ± 0.04*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>5.60 ± 0.43</td>
<td>0.03 ± 0.02*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>&lt;0.01</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.02 ± 0.01</td>
<td>0.58 ± 0.08*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>5.05 ± 0.73</td>
<td>13.0 ± 0.69*</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 6-12 litters/group. PE, phosphatidylethanolamine; PC, phosphatidylcholine. *Significantly different from the n-3 fatty acid deficient group, P<0.05.
3.3.2 Proteomic studies to identify novel protein targets

Two-dimensional PAGE combined with MALDI TOF mass spectrometry and peptide mass fingerprinting were used to identify protein targets susceptible to regulation by n-3 fatty acids in neonatal liver, as recently described in detail (279, Appendix A1.3). The results are included since they guided targeted studies of gene expression and metabolites beyond pathways known to be influenced by n-3 fatty acids in adult liver (10), and enable integration of results concerning changes in protein, gene and metabolite concentrations across multiple metabolic pathways. Protein identification was restricted to only those proteins that consistently differed by at least three-fold in the liver of every neonate with low compared to higher hepatic n-3 fatty acids. Thus, we sought to identify metabolic pathways, but not every protein differing between the groups. Representative spot images and the average spot density of key target hepatic proteins shown to differ between neonates with low and high n-3 fatty acids have been previously published (279) and are shown in Figure 3.2.
Figure 3.2 Average spot densities and representative spot images of proteins shown to differ in abundance by 2D gel proteomics in liver of three-day-old rats born to dams fed diets with deficient or adequate n-3 fatty acids.

Bar graphs show the means ± SD density and in each case *P*<0.05. The proteins were identified by MALDI-TOF-mass spectrometry and are: A. fructose-1,6-bisphosphatase; B. glycerol-3-phosphate dehydrogenase; C. galactokinase; D: elongation factor 1-gamma; E: protein disulfide-isomerase A6; F: catalase; G: cytokeratin 8; H: 60 kDa heat shock protein; I: serine hydroxymethyltransferase; J: argininosuccinate synthase.
3.3.3 Gene expression

Ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (Qiagen, www.qiagen.com) with DNase 1 treatment to digest contaminating genomic DNA, then the RNA integrity was assessed by the presence of 18S and 28S ribosomal RNA bands on agarose gels. RNA (1 µg) was reverse transcribed using the High Capacity complementary DNA (cDNA) Reverse Transcription Kit (Applied Biosystems, www.appliedbiosystems.com). Gene expression was measured by real-time polymerase chain reaction (PCR) using the comparative Ct method (ΔΔCt) of relative quantification with commercially available primers and TaqMan probes specific for the target genes, with actin as the endogenous control (Applied Biosystems). Gene expression studies were based on the results of the proteomic studies, with inclusion of gene targets known to be regulated by n-3 fatty acids in adult liver (10, 107). Targets included, for fatty acid metabolism, Cpt1a, Acox1, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial) (Hmgcs2), ATP citrate lyase (Acly), fatty acid synthase (Fasn), acetyl CoA carboxylase (Acaca), stearoyl (Δ9) CoA desaturase (Scd1), fatty acid desaturase 2 (Δ6) (Fads2), HMG-CoA reductase (Hmgr); glucose and amino acid metabolism, fructose-1,6-bisphosphatase (Fbp1), glucose-6-phosphate dehydrogenase (G6pdx), phosphofructokinase, liver type (Pfk1), liver-type pyruvate kinase (Pklr), pyruvate carboxylase (Pcx), serine dehydratase (Sds), serine hydroxymethyltransferase (Shmt1); and the transcriptional regulators Ppara and PPARγ-coactivator 1α (Ppargc1). The PCR primers are listed in Table 3.3. In each case, diluted cDNA product (5 µL) was incubated with 1X TaqMan Universal PCR mix and 1X TaqMan primer/probe mix in a final reaction volume of 20 µL. The PCR assay was conducted in a 7500 Real Time PCR System (Applied Biosystems) at 50°C for 2 minutes, 95 °C for 10 minutes followed by 50 cycles of 95 °C for 15 seconds and 60°C for 1 minute. Data were analysed using the 7500 System Sequence Detection software, version 1.2.3 (Applied Biosystems). The protocol for gene expression analysis is provided in Appendix A1.4.
**Table 3.3** Gene targets and PCR primers.

<table>
<thead>
<tr>
<th>Gene name (symbol)</th>
<th>PCR primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty acid oxidation:</strong></td>
<td></td>
</tr>
<tr>
<td>carnitine palmitoyltransferase (<em>Cpt1a</em>)</td>
<td>Rn00580702_m1</td>
</tr>
<tr>
<td>acyl CoA oxidase (<em>Acox1</em>)</td>
<td>Rn0056921_m1</td>
</tr>
<tr>
<td><strong>Ketone synthesis:</strong></td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial) (<em>Hmgcs2</em>)</td>
<td>Rn00597339_m1</td>
</tr>
<tr>
<td><strong>Fatty acid synthesis:</strong></td>
<td></td>
</tr>
<tr>
<td>ATP citrate lyase (<em>Acly</em>)</td>
<td>Rn00566411_m1</td>
</tr>
<tr>
<td>fatty acid synthase (<em>Fasn</em>)</td>
<td>Rn00569117_m1</td>
</tr>
<tr>
<td>acetyl CoA carboxylase (<em>Acaca</em>)</td>
<td>Rn00573474_m1</td>
</tr>
<tr>
<td>stearoyl (Δ9) desaturase (<em>Scd1</em>)</td>
<td>Rn00594894_g1</td>
</tr>
<tr>
<td>fatty acid desaturase 2 (Δ6) (<em>Fads2</em>)</td>
<td>Rn00580220_m1</td>
</tr>
<tr>
<td><strong>Cholesterol synthesis:</strong></td>
<td></td>
</tr>
<tr>
<td>HMG-CoA reductase (<em>Hmgcr</em>)</td>
<td>Rn00565598_m1</td>
</tr>
<tr>
<td><strong>Pentose phosphate pathway:</strong></td>
<td></td>
</tr>
<tr>
<td>glucose-6-phosphate dehydrogenase (<em>G6pdx</em>)</td>
<td>Rn00566576_m1</td>
</tr>
<tr>
<td><strong>Glycolysis &amp; gluconeogenesis:</strong></td>
<td></td>
</tr>
<tr>
<td>phosphofructokinase, liver type (<em>PfkI</em>)</td>
<td>Rb99566132_m1</td>
</tr>
<tr>
<td>fructose-1,6-bisphosphatase (<em>FbpI</em>)</td>
<td>Rn00561189_m1</td>
</tr>
<tr>
<td>liver-type pyruvate kinase (<em>Pklr</em>)</td>
<td>Rn00561764_m1</td>
</tr>
<tr>
<td>pyruvate carboxylase (<em>Pcx</em>)</td>
<td>Rn00562534_m1</td>
</tr>
<tr>
<td><strong>Amino acid metabolism:</strong></td>
<td></td>
</tr>
<tr>
<td>serine dehydratase (<em>Sds</em>)</td>
<td>Rn00588631_m1</td>
</tr>
<tr>
<td>serine hydroxymethyltransferase (<em>Shmt1</em>)</td>
<td>Rn01751636_m1</td>
</tr>
<tr>
<td><strong>Transcription regulators:</strong></td>
<td></td>
</tr>
<tr>
<td>PPARα (<em>Ppara</em>)</td>
<td>Rn00566193_m1</td>
</tr>
<tr>
<td>PPARγ-coactivator 1α (<em>Ppargc1</em>)</td>
<td>Rn00594894_g1</td>
</tr>
</tbody>
</table>

1 Primers are from Applied Biosystems.
3.3.4 Biochemical analysis

For lipid analysis, total liver lipids were extracted (Appendix A1.1) then the lipid classes were separated and quantified by HPLC and fatty acids determined by GLC (Appendix A1.2) (249). Glucose was measured using a glucose assay kit (Sigma-Aldrich, www.sigmaaldrich.com) and glycogen was quantified as the difference in glucose following incubation with amyloglucosidase, 50 µg/mL in 50 mM sodium acetate with 0.02% w/v bovine serum albumin for 1 hour (Appendix A1.5). NADPH and NADP were analyzed using commercial reagents (BioVision, www.biovision.com). Protein abundance of serine hydroxymethyltransferase (SHMT1), which catalyzes the reversible conversion of serine to glycine, was increased in neonatal livers with high n-3 fatty acids (Figure 3.2). We, therefore, quantified liver glycine and serine by HPLC coupled with fluorescence detection and electrospray ionization mass spectrometry (ESI-MS) using \(^{13}\)C-labeled glycine and serine (Cambridge Chemical, www.cambridgechemical.com) as internal standards, after derivatization with ortho-phthtalaldehyde (OPA) and 3-mercaptopropionic acid (MPA) based on Terrlink et al. (281, Appendix A1.6). Due to the altered protein abundance of catalase and galactokinase (Figure 3.2), we also quantified glutathione by HPLC with fluorescence detection, and analyzed protein carbonyls (Cayman Chemical, www.caymenchem.com). Protein was assayed according to Lowry (282).

3.3.5 Statistical analysis and data interpretation

Results are presented as means ± standard error (SEM) or standard deviation (SD) as indicated. Differences between groups were determined using unpaired t-tests using the SPSS statistical software package for Windows (version 17; SPSS Inc). Differences were considered significant at \(P<0.05\). The results of the protein, gene expression and biochemical analyses were combined onto metabolic pathways to enable consideration of the potential changes in hepatic
fatty acid, glucose and amino acid metabolism that result when the supply of n-3 fatty acids is altered.

3.4 Results

3.4.1 Maternal dietary fatty acids and offspring liver fatty acids

Neonates of dams fed the n-3 fatty acid adequate diet showed a marked increase in 22:6n-3 compared to offspring of the n-3 fatty acid deficient group, from a mean of 10.7 to 24.9 g /100 g fatty acid and 5.05 to 13.0 g /100 g fatty acid in liver phosphatidylethanolamine and phosphatidylcholine, respectively (Table 3.2). Levels of 18:3n-3 were low in liver phospholipids, increasing to only 0.14 g/100 g fatty acids in phosphatidylcholine and not different between the groups in phosphatidylethanolamine. Regardless of a statistically significant increase in liver phospholipid 20:5n-3 in offspring in the n-3 adequate compared to deficient group, 20:5n-3 remained less than 1 g/100 g fatty acid. The maternal diet fatty acid composition had no significant effect on 18:2n-6 or 20:4n-6 in the offspring liver phospholipids, although the long chain n-6 fatty acids, 22:4n-6 and 22:5n-6 were higher in the n-3 fatty acid deficient group. For the gene expression and metabolite analyses, comparisons are made between the neonatal livers with “high” compared to “low” n-3 fatty acids, reflecting offspring of dams fed the n-3 adequate and deficient diets, respectively, recognizing that differences in the long chain n-6 fatty acids, 22:4n-6 and 22:5n-6, were also present.

3.4.2 Gene expression in neonatal livers

Livers of three-day-old offspring with high n-3 fatty acids showed significantly higher gene expression of Cpt1a and Acox1, and lower Acly and Scd1 (Figure 3.3). Cpt1a and Acox1 encode enzymes of mitochondrial and peroxisomal fatty acid oxidation, respectively, whereas Acly and Scd1 encode enzymes involved in the cleavage of cytosolic citrate to oxaloacetate and
acetyl CoA, and the Δ9 desaturation of fatty acids, respectively. Transcript abundance for *Fasn* and *Acaca*, which are also involved in fatty acid synthesis, *Fads2*, which encodes the Δ6 desaturase, *Hmgcr* and *Hmgcs2*, which encodes the cytosolic HMG CoA reductase involved in cholesterol synthesis and the mitochondrial HMG CoA synthase involved in ketogenesis were not different between the groups (Figure 3.3). Fatty acid oxidation generates acetyl CoA, which requires oxaloacetate for condensation leading to citrate. We, therefore, assessed gene expression of *Pcx*, which encodes the enzyme that carboxylates pyruvate to oxaloacetate, as well as *Sds*, which dehydrates and deaminates serine to form pyruvate; however, we found no difference in expression of these genes in livers with high or low n-3 fatty acids (Figure 3.4). Consistent with the increased *Cpt1a* and *Acox1*, neonatal livers with high n-3 fatty acids had lower liver *Pklr*, which encodes pyruvate kinase, although genes for regulatory enzymes upstream in the glycolytic pathway, *Pfkl* and *G6pdx* were not altered. Notably, *Shmt1* was higher in neonatal livers with high n-3 fatty acids, extending the finding of higher SHMT1 protein abundance (279) to show regulation at the level of gene expression. FBP1, which showed higher protein abundance in neonatal liver with high n-3 fatty acids, also showed a trend to higher *Fbp1* gene expression (*P* = 0.12). We found no difference in expression of the transcriptional regulators, *Ppara* and *Ppargc1* between neonatal livers with high or low n-3 fatty acids (Figure 3.4).
Figure 3.3 Gene expression for enzymes of fatty acid metabolism in three-day-old liver.

Values are means ± SEM, n=5-6 livers/group. * Different from n-3 fatty acid deficient, \( P<0.05 \).
Figure 3.4 Gene expression for enzymes of glucose and amino acid metabolism in three-day-old liver.

![Gene expression graphs](image.png)

Values are means ± SEM, n=5-6 livers/group. * Different from n-3 fatty acid deficient, P<0.05.

3.4.3 Biochemical measures

Quantification of liver glycine and serine by HPLC-mass spectrometry showed significantly lower glycine, although no difference in serine in livers with high n-3 fatty acids (Table 3.4). Liver glucose and glycogen levels were not different between the groups. NADPH
and the NADPH/NADP ratio were higher in neonatal liver with high n-3 fatty acids (Table 3.4). Despite the absence of a difference in *Hmgcr*, liver unesterified and esterified cholesterol were higher in livers with high n-3 fatty acids; however HMG CoA reductase is known to also be regulated by post-translational mechanisms including enzyme degradation and reversible phosphorylation (283). Consistent with higher catalase abundance in neonatal livers with high n-3 fatty acids (279, Figure 3.2), we found higher glutathione in liver with high n-3 fatty acids, although protein carbonyls were not different between the groups (Table 3.4).

**Table 3.4** Metabolites in liver from three-day-old offspring of rats fed diets differing in fatty acid composition during gestation

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>n-3 deficient</th>
<th>n-3 adequate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine, µmol/g tissue</td>
<td>3.32 ± 0.34</td>
<td>2.39 ± 0.04*</td>
</tr>
<tr>
<td>Serine, µmol/g tissue</td>
<td>1.20 ± 0.23</td>
<td>1.38 ± 0.08</td>
</tr>
<tr>
<td>Glucose, µg/mg tissue</td>
<td>4.19 ± 0.99</td>
<td>4.31 ± 0.97</td>
</tr>
<tr>
<td>Glycogen, µg/mg tissue</td>
<td>40.8 ± 5.16</td>
<td>31.0 ± 3.93</td>
</tr>
<tr>
<td>NADPH, ng/mg protein</td>
<td>256 ± 35.8</td>
<td>364 ± 21.5*</td>
</tr>
<tr>
<td>NADPH/NADP</td>
<td>0.71 ± 0.06</td>
<td>1.03 ± 0.06*</td>
</tr>
<tr>
<td>Free cholesterol, µg/mg protein</td>
<td>8.94 ± 0.17</td>
<td>9.67 ± 0.18*</td>
</tr>
<tr>
<td>Cholesterol ester, µg/mg protein</td>
<td>3.53 ± 0.29</td>
<td>6.07 ± 0.81*</td>
</tr>
<tr>
<td>Glutathione, µmol/g tissue</td>
<td>2.31 ± 0.34</td>
<td>3.82 ± 0.17*</td>
</tr>
<tr>
<td>Protein carbonyls, nmol/mg protein</td>
<td>0.59 ± 0.15</td>
<td>0.56 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± SEM for n = 4-8 litters/diet group. *Significantly different from n-3 fatty acid deficient group, *P*<0.05.
3.4.4 Pathway integration

Figure 3.5 combines results of the analyses of gene expression, metabolites and protein abundance on a schematic of key pathways of fatty acid, glucose, and amino acid metabolism. When integrated, these results provide novel evidence that higher n-3 fatty acids in neonatal liver alters not only fatty acid oxidation and the use of glucose as a source of energy, but also intersecting anabolic pathways relating to amino acid metabolism. Beginning with fatty acid metabolism, neonatal livers with high n-3 fatty acids showed higher transcript abundance for Cpt1a and Acox1 and higher glycerol-3-phosphate dehydrogenase, consistent with increased triglyceride catabolism, glycerol entry to glycolysis or gluconeogenesis, and fatty acid oxidation. Acetyl CoA from fatty acid oxidation can enter the citric acid cycle for oxidation to CO₂, be used for synthesis of ketones via mitochondrial HMG CoA synthase 2, or after export from the mitochondria as citrate and cleavage to oxaloacetate and acetyl CoA by citrate lyase, it can be used for cholesterol or fatty acid synthesis. Unexpectedly, despite increased Cpt1a and Acox1, Acly was lower in liver with high n-3 fatty acid, with no evidence of a difference in Hmgcs2. However, the lower Pklr suggests decreased entry of glucose carbons to the citric acid cycle at the level of phosphoenolpyruvate conversion to pyruvate, suggesting no net change in energy metabolism via the citric acid cycle. The results from the proteomic studies to show higher fructose-1,6-bisphosphatase, glycerol-3-phosphate dehydrogenase and galactokinase suggest increased gluconeogenesis. Although consistent with increased triglyceride-glycerol metabolism, this also raises the question of the metabolic fate of glucose carbons when not used for energy. Phosphofructokinase (encoded by Pfkl) also catalyzes a rate-limiting step in hepatic glycolysis, and has been shown to be regulated at the level of gene transcription (284-286), however, we found no evidence of a difference in Pfkl. As shown in Figure 3.5, the lower Pklr, with no difference in Pfkl, points to a redirection of glucose carbons away from the citric acid cycle toward the pentose phosphate pathway and synthesis of serine and glycine, consistent with the
increase in *Shmt1* and NADPH. Notably, both of these pathways require amino acids, directly or as a source of nitrogen. In this regard, the results from the proteomic studies showing lower argininosuccinate synthase protein abundance and increased abundance of proteins involved in protein translation are consistent with increased use of amino acid nitrogen in biosynthetic pathways.
Figure 3.5 Integrated schematic to show sites of altered gene and protein expression, and metabolite differences in livers of three-day-old rats with higher n-3 fatty acids.

Up and down arrows indicate higher or lower levels in liver with high compared to low n-3 fatty acid, as in Table 3.2. Underlined genes and metabolites were measured but not different between the diet groups. Macronutrients are denoted by hexagons to indicate that the balance is determined by hepatic uptake, synthesis, oxidation and secretion. Acaca, acetyl CoA carboxylase; Acly, ATP citrate lyase; Acox1, acyl CoA oxidase; ASS1, argininosuccinate synthase; Cpt1a, carnitine palmitoyl transferase; EEF1G, elongation factor 1-gamma; Fads2, fatty acid desaturase 2; Fasn, fatty acid synthase; FBP1, fructose-1,6-bisphosphatase; G6pdx, glucose-6-phosphate dehydrogenase; GALK1, galactokinase; GPD1, glycerol-3-phosphate dehydrogenase; Hmgcr, HMG CoA reductase; Hmgs2, HMG CoA synthase; Pcx, pyruvate carboxylase; PDIA6, protein disulfide-isomerase A6; Pfkl, phosphofructokinase, liver type; Pklr, liver-type pyruvate kinase, Scd1, stearoyl desaturase; Sds, serine dehydratase; SHMT1, serine hydroxymethyltransferase. *Peroxisomal enzyme.
3.5 Discussion

This chapter describes a novel approach to extend understanding of the role of n-3 fatty acids in metabolic regulation in neonatal liver. We previously used 2D gel proteomics to identify proteins robustly altered in response to changes in the maternal dietary n-3 fatty acids, demonstrating differences in the abundance of proteins in multiple metabolic pathways (279). Here, we further develop the model to targeted studies of gene expression and metabolites, and then integrate the results to provide an overview of the metabolic implications of the neonatal n-3 fatty acid supply. The results show that in the absence of differences in growth, simply altering the long chain n-3 fatty acids in the neonatal liver leads to changes in the abundance of proteins and expression of genes for enzymes regulating fatty acid oxidation, glycolysis, gluconeogenesis, and amino acid metabolism, with changes in the concentrations of NADPH, glycine and glutathione. We suggest that these results are best explained by a role of n-3 fatty acids in facilitating metabolic adaptation to feeding with a high fat milk diet, with higher fatty acid oxidation, and sparing of glucose and amino acids for anabolic pathways of protein and peptide synthesis (Figure 3.5).

Regardless of the considerable interest in the role of n-3 fatty acids in the developing brain (3, 7, 8), very little is as yet known regarding the role of these fatty acids in the liver. The transition from placental nutrient transfer to the milk diet at birth involves an abrupt change in the energy substrate supply for which coordinated changes in hepatic metabolism must occur to enable fatty acid oxidation, prevent excess lipid accumulation in the liver, maintain glucose homeostasis, and support peptides, proteins and other biosynthetic processes. In addition, whereas muscle is the major site of fatty acid oxidation in adults, muscle mass and physical activity are low in the neonate, and thus the liver may play a more central role in fatty acid metabolism than later in life. Notably, excessive hepatic lipid accumulation is problematic in
parenterally fed neonates given intravenous soybean oil emulsions, and this is alleviated by the use of fish oils rich in 20:5n-3 and 22:6n-3 (287), although the mechanisms are not well understood. In the present study, higher Cpt1a, Acox1 and glycerol-3-phosphate dehydrogenase expression, with lower Acly and Scd1 is consistent with increased triglyceride catabolism and lower fatty acid synthesis in liver with high n-3 fatty acids (Figure 3.5). The higher expression of enzymes of fatty acid oxidation was accompanied by lower Pklr, suggesting a reciprocal decrease in glucose oxidation as fatty acid oxidation increased. These findings highlight a key role for n-3 fatty acids in early regulation of fatty acid and glucose metabolism.

Numerous studies in adults have shown that the n-3 fatty acids increase fatty acid oxidation and suppress glycolysis in the liver (10, 107, 288). In humans this translates to the well-known plasma triglyceride lowering effect of long chain n-3 fatty acids (271, 289). In rodents, feeding diets with higher n-3 fatty acids leads to higher hepatic Acox1 and Cpt1a and lower Pklr, Scd1, Fasn and Acaca (142-144, 290-296). Transcript abundance of Scd1 and Srebp1c, which encodes sterol regulatory element binding protein (SREBP) the transcription factor thought to regulate transcription of genes for fatty acid synthesis (142, 143), were also inhibited by 18:2n-6, 20:4n-6, 20:5n-3, and 22:6n-3 in human hepatoma cells (157, 297). While the effects of n-3 and n-6 fatty acids in regulation of fatty acid and glucose metabolism in the adult liver have been described, several transcription regulators, including SREBP and PPAR show distinct developmental profiles (277, 278), and this, together with the high fat milk diet of the neonate set the stage to consider that n-3 fatty acids may have different effects in the infant compared to adult liver. In the present study, higher liver n-3 fatty acids, secondary to increasing maternal dietary n-3 fatty acids led to higher Acox1 and Cpt1a and lower Pklr and Scd1 in the neonatal liver consistent with the effect of n-3 fatty acids in the adult (142-144, 290-295). However, in contrast to studies on the adult liver, we found no evidence that higher n-3 fatty acids led to lower Fasn or Acaca in the neonatal liver. A reasonable explanation is that the high
fat content of the neonatal milk diet is associated with maximum suppression of lipogenic enzymes, such that no additional inhibition by n-3 fatty acids occurred.

Studies on the effects of dietary n-3 fatty acids on fatty acid and glucose metabolism often overlook the essential roles of glucose derived carbons beyond their role as an energy source. Our finding of lower Pklr, but higher Shmt1 and NADPH in neonatal livers with high n-3 fatty acids point to redirection of glucose carbons towards the pentose phosphate pathway and as the carbon backbone for synthesis of the non-essential amino acids, serine and glycine. To the best of our knowledge, ours are the first studies to show higher protein abundance of SHMT1 due to higher liver n-3 fatty acids, confirmed at the mRNA level by targeted real-time PCR in the present study. The perhaps unexpected lower hepatic free glycine with high n-3 fatty acids could reflect increased use of glycine for protein, purine or glutathione synthesis. While the higher NADPH, lower glycine and decreased Pklr gene expression with high n-3 fatty acids are consistent with increased entry of glucose carbons to the pentose phosphate pathway (Figure 3.5), we note that specific data to show this are not provided by our studies.

A role of n-3 fatty acids in regulation of amino acid and protein metabolism is intriguing. In addition to increased SHMT1, our proteomic studies showed higher elongation factor 1γ and protein disulfide isomerase, and lower argininosuccinate synthase, which together suggest decreased amino acid catabolism and increased protein synthesis. Recent studies using stable isotopes in developing and adult animals have reported that feeding 20:5n-3 and 22:6n-3 led to lower oxidation and increased muscle protein incorporation of $^{13}$C labeled phenylalanine (298, 299). Similarly, supplementation with 20:5n-3 and 22:6n-3 increased insulin-stimulated incorporation of phenylalanine into muscle proteins in elderly subjects (300). Clearly, the possibility that n-3 fatty acids influence amino acid metabolism, perhaps directly through
regulation of gene and protein expression (Figure 3.5), or via insulin or other pathways (298-300) and the implications for lean tissue accretion requires further investigation.

In summary, the present study demonstrates that the maternal dietary fatty acids, particularly the n-3 fatty acids, are relevant to neonatal liver metabolism. Our global approach integrating protein, gene, and metabolite measures across multiple pathways offers novel insights into pathways altered by the n-3 fatty acids, extending the focus beyond energy metabolism to biosynthetic pathways. We have emphasized that the neonatal liver is faced with a high fat milk diet and that the metabolic importance of liver and muscle may differ from the adult due to the low muscle mass and physical activity of the newborn. The roles of n-3 fatty acids in directing glucose and amino acids to biosynthetic pathways, and the implications for lean tissue accretion merit further study.
CHAPTER 4: Impact of maternal dietary n-3 and n-6 fatty acids on milk medium chain fatty acids and the implications for neonatal liver metabolism

4.1 Chapter synopsis

The studies in this chapter addressed how the maternal dietary fat content and composition impacts n-3, n-6, and medium chain fatty acids (MCFA) in milk, and also whether the milk fatty acid composition impacts metabolic development in young animals. MCFA were considered for several reasons. The n-3 fatty acid deficient diets used in Chapters 2 and 3 were prepared with coconut oil, which is rich in MCFA, and this raises the possibility that any effects of n-3 fatty acid deficiency were due to or confounded by MCFA. For the infant, MCFA are well-absorbed, transported to the liver as unesterified fatty acid, and rapidly oxidized, thus hepatic metabolism may differ in neonates receiving milks or formulas with different amounts of MCFA. The regulation of MCFA secretion in milk is also complex. MCFA are synthesized from glucose in the mammary gland and their secretion in milk is increased by diets higher in carbohydrate and lower in fat. In humans, n-3 and n-6 fatty acid intakes increase with increasing fat intakes, and the n-6 and n-3 fatty acids are known to decrease fatty acid synthesis, at least in the adult liver.

The studies in this chapter used seven different diets designed to dissociate the importance of the amount of fat and carbohydrate, and the composition of fatty acids in the maternal diet for milk fatty acid composition and neonatal liver metabolism in the rat. The relevance of maternal diet to the milk composition was addressed through measures of the percent milk fat and fatty acids. The relevance of maternal diet to the neonatal liver was addressed through analysis of liver fatty acids and the expression of key genes of glycolysis, fatty acid synthesis and fatty acid oxidation. The results provide novel data to show that maternal

6A version of this chapter has been accepted for publication:
plasma triglycerides are the most strongly related to the MCFA secretion in milk. As such, the diets that lowered maternal triglycerides, which included a high carbohydrate, low fat diet and a high fat diet with high levels of long chain n-3 fatty acids, also led to higher MCFA in milk. The higher MCFA levels in milk, however, had no apparent effects on major pathways of fatty acid synthesis or oxidation, or glycolysis in the neonatal liver. Importantly, high n-3 fatty acids in the neonatal liver of rats fed milk that was high in long chain n-3 fatty acids, led to changes in gene expression consistent with decreased glucose oxidation at the level of pyruvate conversion to acetyl CoA, decreased fatty acid synthesis and increased use of acetyl CoA for ketogenesis. Overall, the studies provide data to support the hypothesis that the fatty acid composition of milk is relevant to neonatal liver metabolism.

4.2 Introduction

Fatty acids in milk provide the infant with a concentrated energy source, and fatty acids for developing membrane lipids and energy storage in adipose triglycerides. The major sources of fatty acids for secretion in milk triglycerides are fatty acids derived by uptake from the maternal plasma and fatty acids synthesized de novo in the mammary gland epithelial cells (176, 244, 301-303). Typically, microsomal fatty acid synthesis leads to the saturated fatty acid palmitic acid (16:0), which can be further elongated to stearic acid (18:0) and desaturated by stearoyl CoA desaturase (SCD) to oleic acid (18:1n-9) (14). Fatty acid synthesis in the mammary epithelial cells, in contrast, is terminated at fatty acid chain lengths of 8 to 14 by the mammary specific enzyme thioesterase II, and this give rise to MCFA (15, 16, 176, 301, 302, 304). The reason why the mammary epithelial cells synthesize MCFA rather than 16:0 is incompletely understood, but may involve the need to secrete milk fat as liquid droplets, a process enabled by higher proportions of MCFA, and benefits to the infant that include well-absorbed fatty acids that can be transported directly to the liver for β-oxidation (15, 305-307).
Large numbers of studies make it clear that human milk levels of MCFA vary widely, from about 6 to 20% of milk fatty acids, with large variability also in milk levels of unsaturated fatty acids (174). While the fatty acid composition of the maternal diet appears to be the major factor contributing to variability in the milk unsaturated fatty acids, 18:1n-9, 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 (174, 244, 245, 251, 308), little attention has been given to the role of the dietary fatty acid composition on secretion of MCFA in milk, with most studies focused on the increased secretion of MCFA among lactating women with high carbohydrate diets (176, 304, 308-310). In the liver, which like the mammary gland has the capacity for fatty acid synthesis and triglyceride secretion, the n-6 and n-3 fatty acids inhibit fatty acid synthesis at the level of gene transcription via interaction with several transcription factors, including sterol regulatory element binding protein (SREBP) (10, 142, 143, 149, 311, 312). Recent studies have shown a central role of SREBP in regulating mammary gland fatty acid synthesis (302), and decreased expression of fatty acid synthase in mammary glands of rats and mice fed diets high in corn oil (313, 314), which is rich in 18:2n-6 but not n-3 fatty acids (315).

Our focus is the effect of maternal diet fat quantity and composition on milk fatty acids and their relevance to hepatic gene expression in the milk-fed neonate. In the present study we addressed whether the maternal dietary energy from fat or carbohydrate, or n-6 and n-3 fatty acids at constant fat intake influence milk MCFA secretion in milk. To accomplish this, we custom prepared diets to enable rigorous control of dietary protein and essential nutrient density relative to dietary energy and combined this with studies to address the relevance of the milk fatty acids for growth, liver fatty acids and hepatic gene expression in the milk-fed pup. Parallel studies addressed associations between MCFA and n-6 and n-3 fatty acids in mature human milk, and give insight into the similarities and differences between milk lipid synthesis in rodents and humans.
4.3 Materials and methods

4.3.1 Animals and diets

All animal procedures were conducted as approved by the Animal Care Committee of the University of British Columbia and conformed to the guidelines of the Canadian Council on Animal Care. Female Sprague Dawley rats (Charles River Laboratories) were housed in a temperature-controlled animal facility with a 12 hour light-dark cycle and free access to food and water. The animals were randomly assigned to one of seven semi-synthetic diets on day 10 of gestation and maintained on the same diet throughout lactation, n=6-7/group. The diets were prepared to provide constant protein, vitamins and minerals per kcal of diet, as described in detail (189) and provided in Table 4.1 and Table 4.2. The diets were designed for three comparisons, with no MCFA in any diet. First, fat and carbohydrate were exchanged by isocaloric substitution to give as a percent of energy, 78% carbohydrate and 4% fat, or 62% carbohydrate and 20% fat, each with a similar 2-3% energy from 18:2n-6. Since replacement of carbohydrate with fat necessitated higher fat, this was accomplished with the monounsaturated fatty acid, 18:1n-9. Hence the high carbohydrate and comparative high fat diet are designated as Low fat and High fat-18:1n-9, respectively. Next, to address the effects of saturated, monounsaturated, n-6 and n-3 fatty acids, an additional three diets with 20% energy from fat were prepared using oils high in 16:0, 18:2n-6, or 18:3n-3 and these are designated as High fat-16:0, High fat n-6 or High fat n-3, respectively. Finally, the effects of long chain (LC) n-3 fatty acids, 20:5n-3 and 22:6n-3 were addressed in diets with 20% energy from fat that included 0.29% energy 20:5n-3+22:6n-3, or 4.3% energy 20:5n-3+ 22:6n-3 (High fat-low LC n-3, High fat-high LC n-3, respectively). The diets with 20:5n-3 and 22:6n-3 had similar 18:2n-6 and 18:3n-3 to the High fat-18:1n-9 diet, thus enabling comparison to a diet with no 20:5n-3 and 22:6n-3. In all cases, fresh diet was provided each day in excess of each animal’s needs. All litters were reduced to a standard 10 pups at birth. Liver and blood from one pup per litter was
taken on postnatal day three for studies of hepatic gene expression and biochemical analysis. Livers were flash frozen in liquid nitrogen, then transferred to storage at -70°C. Blood samples were centrifuged at 3700 rpm for 15 minutes to separate plasma. Approximately 500 µL of milk was collected from each dam at four days postpartum, following intraperitoneal administration of 0.2 mL oxytocin (10 IE/mL) under isofluorane anesthesia. Non-fasting blood samples were collected from rat dams and centrifuged as above to separate plasma. All samples were stored at -70°C until analyzed.
Table 4.1 Nutrient content and oils in the rat diets.

<table>
<thead>
<tr>
<th>g/ complete diet</th>
<th>Low Fat</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>198</td>
<td>198</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>288</td>
<td>229</td>
<td>229</td>
<td>229</td>
<td>229</td>
<td>229</td>
</tr>
<tr>
<td>Starch</td>
<td>576</td>
<td>458</td>
<td>458</td>
<td>458</td>
<td>458</td>
<td>458</td>
</tr>
<tr>
<td>Fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High oleic</td>
<td>7.8</td>
<td>98.3</td>
<td>-</td>
<td>39.1</td>
<td>81.0</td>
<td>93.3</td>
</tr>
<tr>
<td>safflower</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palm oil</td>
<td>-</td>
<td>-</td>
<td>98.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>High linoleic</td>
<td>11.9</td>
<td>-</td>
<td>-</td>
<td>59.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>safflower</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flax seed</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17.3</td>
<td>-</td>
</tr>
<tr>
<td>Fish</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
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<tr>
<td>Cellulose</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Vitamins *</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Minerals †</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>SeO₂</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
</tbody>
</table>

The diets provided 3.70 kcal/g and 4.03 kcal/g for the Low Fat and High Fat diets, respectively, and were designed to provide identical protein, cellulose, vitamins and minerals per kcal.  *Harlan-Teklad #40060, †Harlan-Teklad AIN93
Table 4.2 Carbohydrate, fat and fatty acid content of the rat diets.

<table>
<thead>
<tr>
<th>% kcal</th>
<th>Low Fat</th>
<th>18:1n-9</th>
<th>16:0</th>
<th>n-6</th>
<th>n-3</th>
<th>Low LC n-3</th>
<th>High LC n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>78</td>
<td>62</td>
<td>62</td>
<td>62</td>
<td>62</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Fat</td>
<td>4.0</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturates</td>
<td>0.4</td>
<td>1.6</td>
<td>9.9</td>
<td>1.8</td>
<td>1.7</td>
<td>1.7</td>
<td>3.5</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>1.6</td>
<td>15.8</td>
<td>7.5</td>
<td>8.0</td>
<td>13.6</td>
<td>15.2</td>
<td>6.3</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>2.0</td>
<td>2.6</td>
<td>2.1</td>
<td>10.0</td>
<td>2.7</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>2.0</td>
<td>&lt;0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.16</td>
<td>2.3</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.13</td>
<td>2.0</td>
</tr>
</tbody>
</table>

The complete fatty acid composition of the diets is provided in the appendix in Table A2.3.

4.3.2 Human milk

The protocol and procedures involving the women who provided their milk were approved by the Ethical Review Boards for Research Involving Human Subjects at the University of British Columbia and the British Columbia Children’s and Women’s Hospital. All subjects provided written informed consent prior to participation. Milk samples were obtained from 175 women at one month postpartum and 149 women at two months postpartum, with 131 women providing milk at both one and two months postpartum. All of the women were breast-feeding one full-term gestation infant, all were over 18 years-of-age, and 56% were breast-feeding their first infant. At the time of milk collection, none of the women reported that they were taking any supplemental oils containing n-3 fatty acids, and none followed a vegan diet. All of the women were participants in a prospective study that involved random assignment to 400 mg/day 22:6n-3 or a placebo from 16 weeks gestation until the infant was born (74, 220), with
supplementation ending four to eight weeks prior to milk collection. Milk (60-100 mL) was collected into prepared, labeled vials, frozen on collection and stored at –70°C until analysis.

4.3.3 Milk fatty acid analyses

For analyses of rat and human milk fatty acids, the milks were thawed in ice-cold water, 9:0, 13:0 and 17:0 added as internal standards, then the fatty acids were directly transmethylated (183, 316, 317, Appendix A1.7). The fatty acid methyl esters were separated and quantified by capillary GLC using a SP-2560 capillary column (100 m x 0.25 mm internal diameter, 20-μm film thickness) (Supelco, Bellefonte, PA), which enabled separation of saturated and cis unsaturated fatty acids, as well as conjugated linoleic acid (CLA) (183).

4.3.4 Molecular and biochemical analyses

Rat plasma triglycerides were analyzed in 5 µL plasma using the Triglyceride-SL reagent (Genzyme Diagnostics, www.biopacific.net), insulin was analyzed in 10 µL plasma using a rat specific insulin enzyme-linked immunosorbent assay (ELISA, Alpco, www.alpco.com), and β-hydroxybutyrate was analyzed by enzymatic assay (Cayman chemical, www.caymanchem.com). Neonatal liver fatty acids were analyzed by GLC following extraction of total liver lipids (Appendix A1.1, A1.2, 249). For gene expression analysis, RNA was extracted using the RNeasy Mini Kit (Qiagen, www.qiagen.ca) and 1 μg of RNA reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, www.appliedbiosystems.com). Gene expression by real-time PCR was done using the comparative Ct method (ΔΔCt) of relative quantification, with commercially available primers and TaqMan probes specific for mitochondrial citrate transporter (solute carrier family 25, Slc25a1, Rn00820906_g1), ATP citrate lyase (Acly, Rn00566411_m1), fatty acid synthase (Fasn, Rn00569117_m1), stearoyl CoA desaturase (Scd1, Rn00594894_g1), pyruvate kinase, liver type (Pkdlr, Rn00561764_m1), carnitine palmitoyl transferase (Cpt1a, Rn00580702_m1),
cytosolic serine hydroxymethyltransferase (*Shmt1*, Rn01751636_m1), and 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*, Rn00597339_m1), with actin as the endogenous control (Appendix A1.4). Figure 4.1 provides metabolic pathways to show the site of action of the enzymes for which gene expression was assessed.

**Figure 4.1** Metabolic schematic illustrating enzyme functions of gene expression targets

![Metabolic schematic](image)

*Acly*, ATP citrate lyase; *Cpt1a*, carnitine palmitoyl transferase; *Fasn*, fatty acid synthase; *Hmgcs2*, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2; *Pklr*, pyruvate kinase, liver type; *Scd1*, stearoyl CoA desaturase; *Shmt1*, serine hydroxymethyltransferase; *Slc25a1*, solute carrier family 25 (mitochondrial citrate transporter).

### 4.3.5 Statistical analysis

For the milk fatty acids, results are provided as means ± standard error (SEM). Differences in milk or liver fatty acids, plasma ketones and insulin, and gene expression due to the maternal diet in rats were analyzed by unpaired t-tests for comparison between the Low fat
and High fat-18:1n-9 groups and by one way ANOVA with Fisher’s least significant difference (LSD) for comparisons among the high fat diet groups. Pearson correlations were used to determine relationships between plasma triglycerides or insulin and milk MCFA, and between fatty acids in human milks. Differences were considered significant at $P<0.05$. All analyses were done with SPSS for WINDOWS (version 15; SPSS Inc, Chicago, IL).

4.4 Results

4.4.1 Maternal dietary fat and MCFA levels in rat milk

To address whether the proportion of dietary energy from fat influences MCFA synthesis and secretion in milk, we compared the levels of MCFA in milk from lactating rats fed diets that differed in the proportion of energy from fat and carbohydrate, but not protein or n-6 and n-3 fatty acids (Low fat compared to High fat-18:1n-9 diet). These diets had no effect on the milk total fatty acid concentrations with $21.1 \pm 2.38$ and $21.5 \pm 0.99$ g fatty acid/dL for dams fed the Low fat and High fat-high 18:1n-9 diets, respectively, $P>0.05$. However, the total MCFA (sum of 8:0 to 14:0) was increased almost two-fold in milk from dams fed the Low fat compared to High fat-18:1n-9 diet, $33.5 \pm 4.33$ and $16.8 \pm 2.60$ g/100 g milk fat, respectively, $P <0.001$ (Figure 4.2). Details of the milk fatty acids for rats fed the different diets are in Table 4.3. The offspring weight and growth from day 1 to 15 postnatal were not different between pups from dams fed the Low fat and High fat-18:1n-9 diets ($P>0.05$, Figure 4.3).
Figure 4.2 MCFA in milk from rats fed diets varying in carbohydrate, fat and fatty acids.

Values are means ± SEM. MCFA are significantly higher in the Low fat compared to High fat-18:1n-9 group, $P < 0.05$. Levels of 18:2n-6 are significantly higher in the High fat-n-6 group compared to the other High fat groups, and levels of 18:3n-3 are significantly higher in the High fat-18:3n-3 group compared to the other High fat groups, $P < 0.05$. 
Figure 4.3 Rat pup growth curves from day 1 to day 15 postnatal.

Values are means for six pups/group. High fat-high LC n-3 are significantly different from all other groups at all time points ($P<0.05$).

4.4.2 Maternal dietary fatty acid composition and milk MCFA

Next, to address whether the maternal diet fatty acid composition, at constant fat intake influences milk MCFA, we fed a constant 20% energy from fat, but with different amounts of 16:0, 18:1n-9, 18:2n-6 and 18:3n-3 (Table 4.2). Again, the milk total fat concentration remained constant with 21.5 ± 0.99, 20.1 ± 1.18, 19.9 ± 1.38, 19.8 ± 1.79 g fatty acid/dL in milk for the High fat-18:1n-9, -16:0, -n-6 and -n-3 diets, respectively, $P>0.05$. The dietary fatty acid composition of the lactating rat did, however, have a marked effect on the fatty acid composition of the milk. Thus, 16:0 was highest in milk from rats fed the High fat-16:0 diet, while 18:2n-6 and its metabolites, 20:4n-6, 22:4n-6 and 22:5n-6 were highest in milk from rats fed the High fat n-6 diet, 18:3n-3 and its metabolites, 20:5n-3 and 22:6n-3 were highest in milk from dams fed the High fat n-3 diet, and 18:1n-9 was highest in milk from rats fed the High fat-18:1n-9 diet (Figure 4.2). Despite the marked increase in 18:2n-6 to 20.1 g/100 g of the milk fatty acid in dams fed the High-fat n-6 diet, the milk MCFA levels remained similar to milk with only 4.5 to 6.4 g 18:2n-6/100 g fatty acid (Figure 4.2, Table 4.3). These results show that uptake of high
amounts of 18:2n-6 by the mammary gland, with subsequent secretion in milk, does not suppress secretion of MCFA in milk triglycerides. Rather, suppression of milk MCFA seen in association with a diet rich in 18:2n-6 is best explained by the high fat content rather than the 18:2n-6 content of the maternal diet. The offspring body weight and growth was not different from postnatal day 1 to 15 among pups nursed by dams fed the High fat-16:0, 18:1n-9, n-6 and n-3 diets ($P > 0.05$, Figure 4.3).

4.4.3 Maternal dietary long chain n-3 fatty acids and milk MCFA

Previous studies have shown that feeding the LC n-3 fatty acids in fish oils increases plasma and tissue 20:5n-3 and 22:6n-3, and decreases plasma triglycerides (271, 289). Milk from dams fed the High fat-18:1n-9, High fat-low LC n-3, and High fat-high LC n-3 diets, providing 0, 0.29% or 4.3% energy from 20:5n-3+22:6n-3 had 21.6 ± 0.99, 18.4 ± 2.29 and 18.7 ± 1.35 g fatty acid /dL milk, respectively, $P > 0.05$, showing no triglyceride lowering effect of the LC n-3 fatty acids. However, 20:5n-3 increased from 0.02 ± <0.01 to 0.16 ± 0.01 to 3.48 ± 0.12, and 22:6n-3 increased from 0.26 ± 0.02 to 0.68 ± 0.05 to 5.96 ± 0.26 g/100 g milk fat from dams fed 0, 0.29% or 4.3% energy from 20:5n-3+22:6n-3, respectively, $P < 0.001$ (Figure 4.4). Unexpectedly, the milk MCFA increased almost 200% to 36.5 ± 2.25 compared to 16.8 ± 2.60 g/100 g milk fat from rats fed diets with 4.3% energy compared to rats fed diets without 20:5n-3+22:6n-3 (Figure 4.4, Table 4.3). These results provide an unambiguous demonstration that 20:5n-3+22:6n-3 do not suppress mammary gland fatty acid synthesis, even when fed in high amounts. However, differences in pup growth were also evident, with pups from rats fed the High fat-high LC n-3 diet having a higher body weight on postnatal day 1 and maintaining a higher weight trajectory over the first 10 days postnatal than pups from the low or no LC n-3 fatty acid groups (Figure 4.3, $P<0.05$).
Table 4.3 Fatty acid composition of milk from rats consuming a low or high fat maternal diet.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Low Fat</th>
<th>High Fat</th>
<th>High Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18:1n-9</td>
<td>16:0</td>
<td>n-6</td>
</tr>
<tr>
<td>Dietary variables</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>21.4 ± 1.08*</td>
<td>15.7 ± 0.52a</td>
<td>26.2 ± 0.54b</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>24.5 ± 2.10*</td>
<td>48.1 ± 1.42c</td>
<td>32.9 ± 1.09c</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>5.48 ± 0.41</td>
<td>5.59 ± 0.23b</td>
<td>4.54 ± 0.29a</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.08 ± 0.01*</td>
<td>0.05 ± &lt;0.01a</td>
<td>0.08 ± 0.01ab</td>
</tr>
<tr>
<td>Mammary derived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>33.5 ± 4.33*</td>
<td>16.8 ± 2.60a</td>
<td>22.1 ± 1.72a</td>
</tr>
<tr>
<td>Desaturation products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4n-6</td>
<td>1.13 ± 0.08</td>
<td>1.29 ± 0.09b</td>
<td>1.19 ± 0.09ab</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.61 ± 0.03</td>
<td>0.62 ± 0.07c</td>
<td>0.60 ± 0.04cd</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.24 ± 0.03</td>
<td>0.28 ± 0.04b</td>
<td>0.29 ± 0.02b</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.03 ± 0.01*</td>
<td>0.02 ± &lt;0.01a</td>
<td>0.03 ± &lt;0.01a</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.26 ± 0.03</td>
<td>0.26 ± 0.02a</td>
<td>0.29 ± 0.03a</td>
</tr>
</tbody>
</table>

Values are means ± SEM for n=6-7 animals/diet group. *Different from the same fatty acid in the High fat-18:1n-9 milk by unpaired t-test, P<0.05. Values in the High fat columns with different superscripts are significantly different by ANOVA with LSD for post-hoc analysis, P<0.05.
**Figure 4.4** Fatty acids in milk from rats fed the High fat-18:1n-9, High fat-low LC n-3 and High fat-high LC n-3 diets providing 0, 0.29 or 4.3% energy from 20:5n-3 and 22:6n-3, respectively.

Values are means ± SEM. * Different from High fat-18:1n-9 and low LC n-3, *P* < 0.05.
4.4.4 Maternal plasma triglycerides are associated with differences in milk MCFA

The mammary gland must co-ordinate *de novo* fatty acid synthesis in the mammary epithelial cells with the uptake of fatty acids from plasma in order to maintain a constant milk fat supply. Feeding LC n-3 fatty acids increased the milk MCFA, and LC n-3 fatty acids are known to decrease plasma triglycerides (271, 289), therefore, we next hypothesized that lower maternal plasma triglyceride would be associated with higher MCFA secretion in milk. As expected, lactating rats with higher MCFA in milk also had lower plasma triglyceride concentrations, with 89.4 ± 18.0 and 69.7 ± 15.0 compared to 195 ± 21.1 and 165 ± 18.0 mg triglyceride/dL in rats fed the High fat-18:1n-9 and High fat-low LC n-3 diets compared to the Low fat and High fat-high LC n-3 diets, respectively, *P*<0.05. There was also a significant inverse relationship between the maternal plasma triglycerides and milk MCFA, *r* = -0.580, *P*<0.01 (Figure 4.5). The maternal plasma insulin, on the other hand, was not different among the groups and showed no relationship to the milk MCFA concentration (Figure 4.5). Overall, these results demonstrate that the availability of plasma-derived fatty acids is a primary determinant of MCFA secretion in milk, not the fatty acid composition of the diet,
**Figure 4.5** Association between maternal plasma triglyceride and insulin and MCFA in milk.

![Graph showing correlation between plasma triglyceride and insulin with MCFA in milk](image)

- × Low fat, ◆ High fat 18:1n-9, ● High fat-low LC n-3, ▲ High fat-high LC n-3.

### 4.4.5 Maternal dietary fatty acids influence liver fatty acid composition

To begin to address the potential implications of milk fatty acid composition for the neonate, we next analyzed the neonatal liver fatty acids at three days postnatal. Pups from mothers fed the Low fat diet had higher liver 16:0 and lower 18:1n-9 compared to pups from mothers fed the High fat-18:1n-9 diet (Figure 4.6). Neonatal livers of pups fed by dams in the high fat groups showed similar relative amounts of fatty acids to those in the milk (Figure 4.6). The retention of high levels of 20:4n-6 and 22:6n-3 in neonatal liver relative to the small amounts of these fatty acids in milk is particularly notable. Specifically, 20:4n-6 never exceeded 3 g/100 g milk fatty acid, but reached 10-18 g/100 g of neonatal liver fatty acid, and 22:6n-3 represented less than 1 g/100 g milk fatty acid in the Low fat and High fat-18:1n-9, 16:0, n-6, n-3 and low LC n-3 groups, with retention in the neonatal liver to levels ranging from 7-12 g/100 g liver fatty acid. An increase in the milk 22:6n-3 to 6 g/100 g fatty acid in rats fed the High fat-high LC n-3 diet led to a mean of 28 g 22:6n-3/100 g fatty acid in neonatal liver.
Figure 4.6 Rat milk and offspring liver fatty acids at three days postnatal.

Values are means ± SEM for (from left to right) the Low fat, High fat-18:1n-9, 16:0, n-6, n-3, low LC n-3 and high LC n-3 groups. *Different from High fat-18:1n-9. For the high fat diet groups, within a sample type, milk or liver, bars with different superscripts are significantly different, P<0.05.
4.4.6 Maternal dietary fatty acids influence offspring hepatic gene expression

We next sought to understand whether the differences in milk MCFA, n-6 or n-3 fatty acids are relevant to metabolic development in the neonatal liver through analysis of genes expression for enzymes relevant to the expected effects of high MCFA, n-6 or n-3 fatty acids provided in milk. In this regard, MCFA absorbed from the intestine are preferentially transported directly to the liver and may undergo rapid β-oxidation to acetyl CoA (305), which is a key intermediate for biosynthesis of other fatty acids and ketones. Oxidation of MCFA may also spare glucose and oxidation of other fatty acids. The n-6 and n-3 fatty acids are known to increase fatty acid β-oxidation and decrease fatty acid synthesis in adult liver through mechanisms that involve regulation of several transcription factors (10, 142, 143, 149, 311, 312). Therefore, to elucidate potential effects of altered milk MCFA, n-6 or n-3 fatty acids, we probed gene expression of enzymes of fatty acid, glucose and acetyl CoA metabolism in neonatal liver. In addition, to address potential effects extending to amino acid metabolism, we also determined expression of Shmt1, which encodes the enzyme that catalyzes the reversible conversion of serine to glycine. Our results show no effect of higher MCFA in milk on expression of any of the target genes in three-day-old pup liver (Figure 4.7). In contrast, the higher long chain n-3 fatty acids in neonatal liver of rats born to dams fed the High fat-high LC n-3 diet was associated with lower gene expression of Pklr, suggesting lower glycolysis, although with no effect on Cpt1a, the rate limiting enzyme of fatty acid oxidation. Neonates with higher long chain n-3 fatty acids also had lower Acly, the enzyme that cleaves cytosolic citrate to generate acetyl CoA, as well as lower Fasn and Scd1, which encode enzymes involved in fatty acid synthesis, and higher Hmgcs2, which is involved in ketogenesis. Together these results suggest that n-3 fatty acids direct acetyl CoA away from fatty acid synthesis and towards increased ketone synthesis. We found no difference in Slc25a1, which encodes the citrate transporter, or Shmt1. Because differences in gene expression were apparent in offspring of rats fed the high LC n-3 diet, but not
those born to mothers fed the High fat-18:1n-9 or n-6 diets, the results suggest that the n-3 fatty acids have uniquely different effects from 18:2n-6 on hepatic gene expression in the milk-fed neonate.

**4.4.7 Differences in neonatal liver gene expression are not associated with differences in plasma triglyceride or ketones**

We next determined whether the changes in neonatal liver *Achy*, *Fasn*, and *Hmgcs2* expression were associated with differences in plasma triglycerides or ketones (Figure 4.8). Plasma triglyceride concentrations were lower in pups in the Low fat compared to the High fat-18:1n-9 diet group. However, despite differences in liver *Hmgcs2* expression, there was no significant difference in plasma β-hydroxybutyrate concentrations among the three-day-old pups.
**Figure 4.7** Gene expression of enzymes involved in fatty acid and glucose metabolism in three-day-old liver.

Values are means ± SEM. * Different from High fat-18:1n-9 and High fat n-6, $P<0.05$. 
Figure 4.8 Plasma triglyceride and β-hydroxybutyrate in three-day-old rats.

Values are means ± SEM. *Different from High fat-18:1n-9, P<0.05.

4.4.8 Human milk unsaturated fatty acids are inversely associated with MCFA

The total MCFA content of human milk was not different at one and two months postpartum, with 11.8 ± 0.27 (range 4.34 – 26.7, n = 175) and 11.2 ± 0.25 (range 4.25 – 25.2, n = 149) g/100 g milk fatty acid, respectively (P > 0.05). Lauric acid (12:0) and 14:0 were the most abundant and together represented 90% of the total milk MCFA, with a significant correlation between 12:0 and 14:0, r = 0.562, P<0.001, n = 324. We also found no difference in the milk MCFA between women assigned to 22:6n-3 and the placebo during pregnancy (P >0.05), thus results for all the women were combined for further analysis. To assess the relationship between MCFA and other fatty acids in milk, we grouped the milks by quartile of total MCFA at one month postpartum, shown in Table 4.4, and two months postpartum, provided in Table 4.5. As the milk MCFA increased, the levels of 18:1n-9, 18:2n-6 and 18:3n-3 decreased. The sum of MCFA, 18:1n-9, 18:2n-6 and 18:3n-3 in the milk was remarkably constant representing about 60% of total fatty acids, further showing the reciprocal changes of MCFA derived from
mammary gland synthesis with the 18 carbon chain unsaturated fatty acids (Figure 4.9). We found no evidence of any significant relationship between milk MCFA and 20:5n-3 ($r = 0.088, P = 0.248$) or 22:6n-3 ($r = -0.022, P = 0.773$) at one month or two months postpartum ($r = 0.025, P = 0.766$ and $r = -0.111, P = 0.179$, respectively). However, the 95th percentile content of 20:5n-3 and 22:6n-3 was relatively low, at 0.16 and 0.53, respectively at one month, and 0.18 and 0.48 g/100 g fatty acid, respectively, at two months postpartum.
Table 4.4  Major fatty acids in human milk at one month postpartum separated by quartiles of milk MCFA.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Quartiles of Milk MCFA</th>
<th>g/100 g fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.31-9.99</td>
<td>10.0-12.5</td>
</tr>
<tr>
<td>MCFA</td>
<td>n = 51</td>
<td>n = 67</td>
</tr>
<tr>
<td>18:1n-7*</td>
<td>2.19 ± 0.05</td>
<td>1.91 ± 0.04</td>
</tr>
<tr>
<td>18:1n-9*</td>
<td>37.4 ± 0.39</td>
<td>35.0 ± 0.43</td>
</tr>
<tr>
<td>18:2n-6*</td>
<td>13.8 ± 0.36</td>
<td>13.9 ± 0.41</td>
</tr>
<tr>
<td>18:3n-3*</td>
<td>1.63 ± 0.07</td>
<td>1.64 ± 0.08</td>
</tr>
<tr>
<td>Σ 18 unsat*</td>
<td>55.0 ± 0.66</td>
<td>52.5 ± 0.58</td>
</tr>
<tr>
<td>16:0</td>
<td>20.0 ± 0.37</td>
<td>20.0 ± 0.34</td>
</tr>
<tr>
<td>18:0</td>
<td>6.10 ± 0.17</td>
<td>6.20 ± 0.16</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.34 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.41 ± 0.01</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.07±&lt;0.01</td>
<td>0.07 ±&lt;0.01</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.02±&lt;0.01</td>
<td>0.02 ±&lt;0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.07±&lt;0.01</td>
<td>0.09 ±0.01</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.27 ±0.01</td>
<td>0.28 ±0.02</td>
</tr>
<tr>
<td>CLA</td>
<td>0.27 ±0.01</td>
<td>0.27 ±0.01</td>
</tr>
<tr>
<td>Other†</td>
<td>9.21 ± 0.32</td>
<td>8.58 ± 0.27</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The 5th to 95th percentile of milk MCFA was divided into four equal quartiles. *Highest and lowest quartiles are significantly different by unpaired t-test, *P*<0.05. CLA: conjugated linoleic acid. †Other includes 16:1, 18:3n-6, 18:4n-3, 20:0, 20:1, 20:3n-9, 20:2n-6, 22:0, 22:1n-11, 22:1n-9, 22:5n-3, 24:0, 24:1 and trans fatty acids.
Table 4.5  Major fatty acids in human milk at two months postpartum separated by quartiles of milk MCFA.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Quartiles of Milk MCFA</th>
<th>g/100 g fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.25-9.99</td>
<td>10.0-12.5</td>
</tr>
<tr>
<td></td>
<td>n = 52</td>
<td>n = 54</td>
</tr>
<tr>
<td>MCFA</td>
<td>8.26 ± 0.20</td>
<td>11.1 ± 0.11</td>
</tr>
<tr>
<td>18:1n-7*</td>
<td>2.06 ± 0.05</td>
<td>1.94 ± 0.04</td>
</tr>
<tr>
<td>18:1n-9*</td>
<td>37.9 ± 0.47</td>
<td>35.6 ± 0.36</td>
</tr>
<tr>
<td>18:2n-6*</td>
<td>15.2 ± 0.44</td>
<td>13.7 ± 0.38</td>
</tr>
<tr>
<td>18:3n-3*</td>
<td>1.77 ± 0.09</td>
<td>1.60 ± 0.07</td>
</tr>
<tr>
<td>Σ 18 unsat*</td>
<td>56.9 ± 0.62</td>
<td>52.8 ± 0.51</td>
</tr>
<tr>
<td>16:0</td>
<td>19.1 ± 0.37</td>
<td>20.1 ± 0.35</td>
</tr>
<tr>
<td>18:0*</td>
<td>6.19 ± 0.15</td>
<td>6.42 ± 0.16</td>
</tr>
<tr>
<td>20:3n-6</td>
<td>0.30 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.36 ± 0.01</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.05 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.02 ± &lt;0.01</td>
<td>0.02 ± &lt;0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.22 ± 0.01</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>CLA</td>
<td>0.24 ± 0.01</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>Otherj</td>
<td>8.23 ± 0.26</td>
<td>8.24 ± 0.26</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The 5th to 95th percentile of milk MCFA was divided into four equal quartiles. *Highest and lowest quartiles are significantly different by unpaired t-test, P<0.05. CLA: conjugated linoleic acid. jOther includes 16:1, 18:3n-6, 18:4n-3, 20:0, 20:1, 20:3n-9, 20:2n-6, 22:0, 22:1n-11, 22:1n-9, 22:5n-3, 24:0, 24:1, and trans fatty acids.
4.5 Discussion

These studies began with the knowledge of the large variability in human milk MCFA, ranging from 4 to about 25% of milk fatty acids (174, 244). MCFA are synthesized from glucose in the mammary gland (15, 16, 176, 301-304), whereas unsaturated fatty acids, particularly the n-6 and n-3 fatty acids, are taken up from plasma (174, 244). The mammary gland must integrate the supply and synthesis of fatty acids with the need for fatty acids for secretion of milk triglycerides. Our results convincingly demonstrate that the availability of plasma lipids for the mammary gland epithelial cell, not the fatty acid composition itself, is a pivotal factor contributing to regulation of MCFA synthesis and secretion in milk.

Early studies on MCFA synthesis and secretion in human milk reported an increase in MCFA with high dietary carbohydrate intakes (304, 309, 310), and implicated insulin and glucose stimulation of mammary gland fatty acid synthesis. More recent studies have revealed a potential role for 18:2n-6 in decreasing MCFA synthesis in the mammary epithelial cell, possibly through mechanisms involving SREBP. Srebp and fatty acid synthase gene expression were
lower in the mammary glands of rats fed 23% compared to 4.8% energy from corn oil (314), which is rich in 18:2n-6 (315). Lower citrate lyase, acetyl CoA carboxylase and fatty acid synthase protein abundance was also reported in mammary glands of mice fed 46% energy as corn oil compared to standard rodent chow with 18% energy from fat as soybean oil (313). However, the specific effect of 18:2n-6 remained unclear, as altering the 18:2n-6 content of the diet by changing fat intake also involved inverse changes in the proportion of energy from carbohydrate. Therefore, we designed diets to dissociate the effects of n-6 and n-3 fatty acids from total fat and carbohydrate intake on mammary gland secretion of MCFA in milk. Our results show that at constant fat and carbohydrate intakes, changing the dietary fatty acid, 16:0, 18:1n-9, 18:2n-6 or 18:3n-3, had no effect on the milk MCFA, even when 18:2n-6 was increased from 4.5 to 20 g/100 g of the milk fatty acids by a maternal diet rich in this fatty acid (Table 4.3, Figure 4.2). Based on this, we conclude that the apparent inhibition of MCFA synthesis by diets high in 18:2n-6 are explained by the higher fat, rather than 18:2n-6 in the diet. Similarly, the concomitant secretion of high 20:5n-3 and 22:6n-3 and high MCFA in milk of lactating dams fed the high LC n-3 diet shows that dietary long chain n-3 fatty acids do not suppress MCFA secretion in milk. However, lower maternal plasma triglycerides, either due to a high carbohydrate-low fat diet, or a high fat diet rich in 20:5n-3 and 22:6n-3 led to a remarkable almost two-fold increase in milk MCFA (Figure 4.4 and Figure 4.5). Overall, the results provide evidence that MCFA secretion in milk is determined by the availability of plasma fatty acid for uptake by the mammary gland. Here we note that the inverse association of 18:2n-6 and MCFA in human milk could also be interpreted as evidence that unsaturated fatty acids influence mammary gland fatty acid synthesis in humans. However, since the levels of 18:1n-9, 18:2n-6 and 18:3n-3 in human milk increase with increasing maternal dietary intakes of these fatty acids (244, 318), higher levels of unsaturated fatty acids in human milk may simply reflect higher maternal fat intakes. No association was evident between 20:5n-3 or 22:6n-3 and MCFA levels
in human milk; however, in our study median (5th to 95th percentile) dietary intakes of 20:5n-3 and 22:6n-3 were 65 (10-228) and 105 (10-430) mg/day, respectively (259), which is below the amounts associated with significant triglyceride lowering in normolipemic individuals (271). Similarly in lactating rats, the low LC n-3 diet provided 0.29% kcal as 20:5n-3 and 22:6n-3, equivalent to about 640 mg 20:5n-3 and 22:6n-3 in a 2000 kcal diet, and this had no effect on the MCFA levels in rat milk (Figure 4.4).

The implications of variability in milk fatty acids for metabolism in the neonatal liver has received little attention, although several studies have reported that n-3 fatty acids regulate gene expression for enzymes of fatty acid synthesis and oxidation and glycolysis in adult rodent liver (10). Our results show that the fatty acid composition of the milk diet had a marked effect on the accumulation of fatty acids in the neonatal liver (Figure 4.6), consistent with previous reports (78, 193, Chapter 2). In the present study, higher hepatic long chain n-3 fatty acids, specifically 20:5n-3 and 22:6n-3, secondary to maternal feeding with the High LC n-3 diet resulted in altered hepatic gene expression in the neonate, with lower Pkhr, Acly, Fasn and Scd1 and higher Hmgcs2. The absence of a difference in Cpt1a expression may simply reflect that all neonates were receiving a milk diet, which is high in fat (174). The lower Fasn, Acly, Fasn and Scd1, and higher Hmgcs2 together suggest that higher LC n-3 fatty acids are associated with decreased export of acetyl CoA as citrate from the mitochondria, decreased fatty acids synthesis and increased use of acetyl CoA for generation of ketones. Interestingly, ketones have been proposed to play an important role as a carbon source during early brain development (319). At three days of age no significant effects of the milk fatty acid composition on neonatal plasma triglycerides or ketones were apparent, but whether these appear after longer milk feeding is unknown.

In summary, our results show that within the range of usual diets, the quantity, not composition of fatty acid available for uptake by the mammary gland determines MCFA
secretion in milk. While the liver and mammary gland are both able to synthesize and secrete triglycerides, the liver integrates the energy supply to maintain normoglycemia and directs excess energy towards fatty acid synthesis for storage in adipose tissue, whereas the mammary gland must coordinate fatty acid synthesis and fatty acid uptake from plasma to maintain triglyceride secretion in milk, which, if altered, has implications for the dietary energy density and potential growth of the infant. In this context, although the n-6 and n-3 fatty acids regulate SREBP and lipogenesis in the liver (10, 142, 143, 311), it is reasonable to expect and consistent with our results, that the dietary fatty acid composition does not impact fatty acid secretion in milk. The implications of the variability in maternal dietary fatty acid composition for the developing infant liver, however, merits further understanding.
CHAPTER 5: Conclusion

5.1 Summary

The functional roles of the n-3 and n-6 fatty acids in early development are not well understood, particularly with respect to the potential importance of n-3 and n-6 fatty acids for liver metabolic development. In this dissertation, I have shown that n-3 and n-6 fatty acid accumulation in piglet liver is altered by the fatty acid composition of the milk replacement diet (Chapter 2) and the n-3 and n-6 fatty acid composition of the maternal diet (Chapters 3 and 4). Higher accumulation of 20:5n-3 and 22:6n-3 in infant rat livers was associated with higher gene and protein expression for enzymes of fatty acid oxidation, gluconeogenesis, and ketogenesis, and lower expression of genes and proteins for enzymes of glycolysis, lipogenesis, and the urea cycle. My interpretation of these findings is that the early supply of n-3 fatty acids may be important in facilitating the transition from the in utero environment to feeding with the high fat, low protein milk at birth. The n-6 fatty acids may also be relevant as their metabolism is related to and influences metabolism and accumulation of n-3 fatty acids. Although the relevance of early changes in the expression of enzymes for fatty acid, glucose and amino acid metabolism, and implications for later health of the offspring remain uncertain, my results highlight the need to consider liver metabolic development when deriving recommendations for n-3 and n-6 fatty acids for pregnant and lactating women and infants. The specific hypotheses and outcomes for Chapter 2, 3 and 4 are summarized in Table 5.1 – 5.3.
Table 5.1 Hypotheses and summary of major findings for Chapter 2.

**Null hypothesis #1:** The n-3 and n-6 fatty acid composition and ratio in piglet milk replacer (formula) diets will not influence the fatty acid composition of the liver, brain frontal cortex, heart or plasma.

**Outcome:** Changing the n-3 and n-6 fatty acid content and ratio in the milk diets of piglets led to dramatic differences in the liver, frontal cortex, heart and plasma n-3 and n-6 fatty acids. Both a diet with low 18:3n-3, and a diet with high 18:2n-6, but potentially adequate 18:3n-3, led to lower levels of 22:6n-3 and 20:5n-3 and higher long chain n-6 fatty acids, 22:4n-6 and 22:5n-6 in the liver, frontal cortex, heart and plasma of piglets. Because the fatty acid composition of the high 18:2n-6 formula was similar to infant formulas, this study suggests that infant formulas may put infants at risk for low accumulation of n-3 fatty acids, such as 20:5n-3 in the liver and heart, and 22:6n-3 in all tissues.

**Null hypothesis #2:** Inclusion of supplemental 22:6n-3 and 20:4n-6 in milk replacer diets of piglets will not alter the fatty acid composition of the liver, frontal cortex, heart or plasma.

**Outcome:** Addition of 22:6n-3 and 20:4n-6 to the milk diet led to higher 22:6n-3 in the brain frontal cortex, liver, heart and plasma compared to piglets fed the same formula without 22:6n-3 and 20:4n-6. In the frontal cortex, addition of 22:6n-3 and 20:4n-6 to the formula led to n-3 and n-6 fatty acid levels similar to piglets fed 1% energy from 18:2n-6 in a 1:1 balance with 18:3n-3. However, addition of 22:6n-3 and 20:4n-6 to the milk diet high in 18:2n-6 also led to higher 20:4n-6 and did not enable similar accumulation of 20:5n-3 in the liver and heart to that achieved when the diet provided only 1% energy from each of 18:2n-6 and 18:3n-3.

**Conclusion:** Tissue accumulation of n-3 and n-6 fatty acids depends on the amounts and types of both n-3 and n-6 fatty acids in the diet, and thus, recommendations for n-3 and n-6 fatty acid intakes should not be considered independently. Accumulation of n-3 and n-6 fatty acids is tissue specific, and n-3 and n-6 fatty acid requirements should be based on the needs of the whole body.
Table 5.2 Hypotheses and summary of major findings for Chapter 3.

**Null hypothesis #1:** Hepatic gene expression for enzymes of fatty acid oxidation will not change from embryonic day 19 to postnatal day 10.

*Outcome:* Gene expression for enzymes of fatty acid oxidation showed a sharp increase at birth, with a plateau from 48-72 hours after birth to postnatal day 10.

**Null hypothesis #2:** Manipulation of the n-3 and n-6 content of the maternal diet will not lead to differences in growth or n-3 and n-6 fatty acids in the neonatal offspring liver.

*Outcome:* The amount of 18:3n-3 and 18:2n-6 in the maternal diet did not influence growth or 20:4n-6, but did influence 20:5n-3 and 22:6n-3 in three-day-old offspring livers.

**Null hypothesis #3:** Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not lead to differences in protein or gene expression for enzymes of fatty acid, glucose or amino acid metabolism in neonatal liver.

*Outcome:* Higher 18:3n-3 and 18:2n-6 in the maternal diet was associated with higher protein abundance for gluconeogenesis and serine metabolism, lower expression of a urea cycle enzyme, higher gene expression for enzymes of fatty acid oxidation and lower gene expression for enzymes of glycolysis and fatty acid synthesis in three-day-old livers.

**Null hypothesis #4:** Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter the concentration of triglyceride, phospholipid or cholesterol in neonatal liver.

*Outcome:* Higher 18:3n-3 and 18:2n-6 in the maternal diet led to higher cholesterol, but did not influence triglyceride or phospholipid concentrations in three-day-old livers.

**Null hypothesis #5:** Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter glucose, glycogen or amino acid concentrations in neonatal liver.

*Outcome:* Higher 18:3n-3 and 18:2n-6 in the maternal diet led to lower glycine, but did not alter serine, glucose or glycogen in three-day-old livers.

**Null hypothesis #6:** Manipulation of the n-3 and n-6 fatty acid content of the maternal diet will not alter markers of oxidative stress in neonatal livers.

*Outcome:* Higher 18:3n-3 and 18:2n-6 in the maternal diet led to higher glutathione and NADPH, but did not alter protein carbonyls in three-day-old livers.

**Conclusion:** Maternal dietary n-3 and n-6 fatty acids alter n-3 and n-6 fatty acid accumulation and gene expression and protein abundance of enzymes known to be rate limiting in metabolic pathways in three-day-old offspring livers. Higher n-3 fatty acids in the neonatal liver may be important in the early metabolic adaptation to postnatal feeding.
Table 5.3 Hypotheses and summary of major findings for Chapter 4.

**Null hypothesis #1:** Levels of MCFA in milk will not differ among rats fed diets differing in fat content and fatty acid composition.

*Outcome:* Milk MCFA levels were higher in rats fed a low fat, high carbohydrate diet compared to rats fed a high fat diet. At constant fat content, the 18 carbon chain unsaturated fatty acid composition of the diet did not alter milk MCFA, but feeding high 20:5n-3 and 22:6n-3 led to increased MCFA in milk.

**Null hypothesis #2:** Maternal plasma triglyceride and insulin concentrations will show no association to the levels of MCFA in milk.

*Outcome:* Maternal plasma triglyceride, but not insulin concentrations were inversely associated with the milk MCFA, suggesting the availability of maternal plasma fatty acid determines mammary gland fatty acid synthesis and secretion in milk.

**Null hypothesis #3:** The maternal fat and fatty acid composition will not alter the expression of genes relevant to fatty acid and glucose metabolism in the neonatal rat liver.

*Outcome:* Higher 20:5n-3 and 22:6n-3 in the maternal diet was associated with lower expression of genes for enzymes of fatty acid synthesis and glycolysis, and higher gene expression of the enzyme regulating ketogenesis in neonatal rat liver.

**Null hypothesis #4:** There will be no associations between MCFA levels and the levels of n-6, n-3, saturated or monounsaturated fatty acids in human milks.

*Outcome:* MCFA in human milk showed an inverse association with the 18 carbon unsaturated fatty acids. Because 18:1n-9, 18:2n-6 and 18:3n-3 are all consumed in vegetable oils, higher milk 18:1n-9, 18:2n-6 and 18:3n-3 may simply reflect higher fat intakes among lactating women with lower MCFA in their milk.

**Conclusion:** The availability of plasma fatty acids for the mammary gland, not the fatty acid composition of the maternal diet, determines MCFA secretion in milk. Regulation of mammary gland fatty acid synthesis likely differs from that in the liver, because 20:5n-3 and 22:6n-3 decrease hepatic, but not mammary gland fatty acid synthesis. Differences in gene expression in neonatal liver are best explained by differences in the milk supply and subsequent neonatal liver accumulation of long chain n-3 fatty acids, not 18:2n-6, 18:1n-9 or MCFA. The supply of n-3 fatty acids for the neonatal liver may be important in metabolic development.
5.2 Discussion

5.2.1 Integration of results

The goal of my PhD research was to begin to elucidate whether the early n-3 and n-6 fatty acid supply is relevant to the developing liver. In Chapter 2, I used the colostrum-deprived piglet and showed that the fatty acids in the milk diet impact n-3 and n-6 fatty acid accretion in the developing liver, but the physiological relevance was not addressed. In Chapter 3, I manipulated the maternal diet during pregnancy and showed that altering the liver n-3 fatty acids, but not 20:4n-6 was associated with altered protein abundance, gene expression and several metabolites in the neonatal rat liver; however, both the piglet diets and the rat maternal diets differed in medium chain fatty acids (MCFA), in addition to the n-6 and n-3 fatty acids. In Chapter 4, I separated the effects of n-6 and n-3 fatty acids, and resolved the question of potential confounding effects of MCFA, again using rats, and showed that the n-3 fatty acids, not the n-6 fatty acids or MCFA, are likely to be key mediators of the changes in gene expression.

5.2.2 Additional information revealed by integration of results from Chapters 3 and 4

By integrating the results of Chapters 3 and 4 additional insights are gained on the impact of maternal dietary fatty acid composition on offspring liver fatty acids, and the role of n-3 and n-6 fatty acids on gene expression in the neonatal liver. These are described below.

Dietary fatty acids and the effects on tissue fatty acids:

Four of the maternal rat diets from Chapter 3 and 4, collectively, had 20% energy from fat and <0.1% energy from 18:3n-3. These were the n-3 deficient diet in Chapter 3 and the High fat diets with 18:1n-9, 16:0, and 18:2n-6 in Chapter 4 (Table 5.4). Unexpectedly, the levels of 22:6n-3 were two-three fold higher in neonatal livers from the three groups of rats fed <0.1% energy 18:3n-3 in Chapter 4 than the n-3 deficient diet in Chapter 3 (Table 5.4). The remarkable difference in 22:6n-3 as well as 20:5n-3 in the neonatal livers at three days of age warrants
further discussion and several explanations are possible. First, the higher 22:6n-3 in neonatal livers of rats fed <0.1% energy 18:3n-3 in Chapter 4 compared to Chapter 3 may be explained by other fatty acids provided in the milks, specifically the MCFA, which was much higher in rats fed the n-3 deficient diet in Chapter 3 compared to Chapter 4. MCFA are absorbed bound to albumin and transported directly to the liver and, therefore, higher amounts of MCFA received in milk may dilute the apparent proportion of long chain n-3 fatty acids received by the neonatal liver. A study in which dietary n-3 fatty acids are kept constant, but MCFA intakes are varied, either through varying MCFA levels in milk by maternal diets high or low in carbohydrate, or through using oils rich in MCFA, such as coconut oil, would be useful to determine whether MCFA influence long chain n-3 fatty acid accretion in the liver.

The differences in 22:6n-3 in neonatal livers from rats fed the n-3 fatty acid deficient diets may also be explained by the duration of feeding the maternal diets. In Chapter 3, diets were fed from two weeks prior to mating and throughout gestation, whereas in Chapter 4 the maternal diets were fed beginning on day 10 of gestation. It is possible that the longer feeding time in Chapter 3 influenced the fatty acid composition of maternal adipose tissue, and hence the composition of fatty acids available for transfer to the fetus and to support lactation after delivery. This would explain the lower 22:6n-3 in milk from rats fed the n-3 deficient diet in Chapter 3 compared to Chapter 4. Whether the differences in the duration of maternal feeding with an n-3 fatty acid deficient diet explain the large differences in the neonatal liver fatty acids is not known. To resolve this, a study could be designed in which rats were fed either from two weeks prior to mating or from day 10 of gestation with identical diets and offspring liver fatty acids analyzed after birth.
Table 5.4 Comparison of diet, milk and neonatal liver fatty acids from the n-3 deficient diets in Chapters 3 and 4.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Chapter 3 n-3 deficient</th>
<th>Chapter 4 High fat-16:0</th>
<th>Chapter 4 High fat-18:1n-9</th>
<th>Chapter 4 High fat-18:2n-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3n-3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>1.4</td>
<td>2.1</td>
<td>2.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>14.6</td>
<td>0.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Σ Sat ≥16:0</td>
<td>2.2</td>
<td>9.9</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Σ Mono</td>
<td>1.7</td>
<td>7.7</td>
<td>15.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.10 ± &lt;0.01</td>
<td>0.29 ± 0.03</td>
<td>0.26 ± 0.02</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.09 ± &lt;0.01</td>
<td>0.03 ± &lt;0.01</td>
<td>0.02 ± &lt;0.01</td>
<td>0.05 ± &lt;0.01</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.12 ± &lt;0.01</td>
<td>0.09 ± 0.01</td>
<td>0.05 ± &lt;0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>0.40 ± 0.02</td>
<td>0.47 ± 0.05</td>
<td>0.39 ± 0.03</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>4.89 ± 0.14</td>
<td>7.62 ± 0.42</td>
<td>8.79 ± 0.47</td>
<td>26.9 ± 0.58</td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>50.4 ± 2.28</td>
<td>22.1 ± 1.72</td>
<td>16.8 ± 2.60</td>
<td>20.4 ± 1.22</td>
</tr>
<tr>
<td>Σ Sat ≥16:0</td>
<td>22.3 ± 0.14</td>
<td>17.9 ± 0.37</td>
<td>19.2 ± 0.63</td>
<td>17.9 ± 0.37</td>
</tr>
<tr>
<td>Σ Mono</td>
<td>22.0 ± 1.99</td>
<td>39.3 ± 1.16</td>
<td>54.8 ± 1.66</td>
<td>34.2 ± 0.61</td>
</tr>
<tr>
<td>Neonatal liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.99 ± 0.40</td>
<td>6.98 ± 1.11</td>
<td>8.05 ± 0.49</td>
<td>8.84 ± 0.66</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.04 ± 0.01</td>
<td>0.16 ± 0.05</td>
<td>0.17 ± 0.05</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>3.83 ± 0.47</td>
<td>8.45 ± 1.08</td>
<td>8.96 ± 0.54</td>
<td>10.1 ± 0.78</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>24.9 ± 1.07</td>
<td>26.6 ± 0.73</td>
<td>30.0 ± 0.37</td>
<td>38.2 ± 0.28</td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>6.09 ± 0.71</td>
<td>1.41 ± 0.11</td>
<td>1.13 ± 0.15</td>
<td>0.83 ± 0.10</td>
</tr>
<tr>
<td>Σ Sat ≥16:0</td>
<td>34.6 ± 0.70</td>
<td>35.8 ± 0.87</td>
<td>33.3 ± 0.29</td>
<td>33.1 ± 0.38</td>
</tr>
<tr>
<td>Σ Mono</td>
<td>30.6 ± 1.40</td>
<td>27.7 ± 2.17</td>
<td>26.6 ± 0.55</td>
<td>17.8 ± 0.84</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=6/group. Bold rows indicate data of specific interest to the preceding paragraphs.
In addition to the difference in 22:6n-3 between neonates born to dams fed the n-3 deficient diets in Chapters 3 and 4, a difference is also apparent between neonates born to rats fed the 18:3n-3 adequate diets, which include the n-3 adequate diet in Chapter 3 and the High fat n-3 diet in Chapter 4 (Table 5.5). Despite similar 18:3n-3 in the two maternal diets, milk and neonatal liver 22:6n-3 was higher in rats fed the n-3 adequate diet in Chapter 4 compared to Chapter 3 (Table 5.5). Notably, the 18:2n-6/18:3n-3 ratio of the two diets differed, with higher 18:2n-6 in the diet in Chapter 4 compared to Chapter 3. This is important as the results of Chapter 2 clearly illustrate that 18:2n-6 interferes with long chain n-3 fatty acid accumulation in infant liver. Notably, when we consider the precursor/product ratios as an indication of desaturation and elongation, the higher maternal dietary and milk 18:2n-6 in Chapter 3 is associated with significantly higher 18:3n-3/20:5n-3, 18:3n-3+20:5n-3+22:5n-3/22:6n-3, 18:2n-6/20:4n-6, and 18:2n-6+20:4n-6+22:4n-6/22:5n-6 ratios. Thus, it appears that the higher 18:2n-6 is associated with lower desaturation products, which is consistent with the lower long chain n-3 fatty acids in infant livers fed a diet with 10% compared to 1% energy from 18:2n-6 with similar 18:3n-3 in the piglet diets.
Table 5.5 Comparison of the diet, milk and neonatal liver fatty acids from the n-3 adequate diets in Chapters 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Chapter 3 n-3 adequate</th>
<th>Chapter 4 High fat-18:3n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet</strong></td>
<td>% energy</td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>3.9</td>
<td>2.7</td>
</tr>
<tr>
<td>18:2n-6/18:3n-3</td>
<td>2.6</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Milk</strong></td>
<td>g/100 g fatty acid</td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>9.55 ± 1.22</td>
<td>6.40 ± 0.37</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.49 ± 0.04</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>22:6n-3</td>
<td><strong>0.25 ± 0.01</strong></td>
<td><strong>0.52 ± 0.07</strong></td>
</tr>
<tr>
<td><strong>Neonatal Liver</strong></td>
<td>g/100 g fatty acid</td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>1.15 ± 0.13</td>
<td>0.74 ± 0.06</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>1.38 ± 0.17</td>
<td>1.07 ± 0.06</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>3.07 ± 0.33</td>
<td>2.37 ± 0.16</td>
</tr>
<tr>
<td>22:6n-3</td>
<td><strong>8.86 ± 1.41</strong></td>
<td><strong>11.8 ± 0.65</strong></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>9.02 ± 0.40</td>
<td>7.55 ± 0.21</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>8.60 ± 1.24</td>
<td>14.5 ± 0.36</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.09 ± 0.14</td>
<td>1.29 ± 0.10</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.22 ± 0.03</td>
<td>0.85 ± 0.16</td>
</tr>
<tr>
<td><strong>Precursor/product ratios</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3/20:5n-3</td>
<td><strong>0.88 ± 0.10</strong></td>
<td><strong>0.69 ± 0.04</strong></td>
</tr>
<tr>
<td>(18:3n-3 + 20:5n-3 +22:5n-3)/22:6n-3</td>
<td><strong>0.68 ± 0.07</strong></td>
<td><strong>0.35 ± 0.01</strong></td>
</tr>
<tr>
<td>18:2n-6/20:4n-6</td>
<td><strong>1.15 ± 0.15</strong></td>
<td><strong>0.52 ± 0.02</strong></td>
</tr>
<tr>
<td>(18:2n-6+20:4n-6+22:4n-6)/22:5n-6</td>
<td><strong>89.4 ± 11.9</strong></td>
<td><strong>32.9 ± 6.20</strong></td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=6/group. Bold rows indicate data of specific interest to the preceding paragraphs. Saturates and monounsaturates did not differ in the maternal diets and were 1.4% and 13.2% in the n-3 adequate diet and 1.7% and 13.6 % energy, respectively.
Whereas the preceding discussion has focused on fatty acids, it is important to note that triglycerides, not fatty acids were fed in my studies. To date, almost all published studies have focused on dietary fatty acids with very little consideration given to the oils and their triglyceride compositions. A desired average dietary fatty acid composition can be achieved by mixing together different oils; however, triglyceride fatty acid distributions may also be important. The n-3 fatty acid adequate diets in Chapters 3 and 4 were prepared using canola oil or a blend of flax seed oil and safflower oil, respectively. The prominent triglycerides in canola oil are (denoting the sn-1, sn-2 and sn-3 positions, with sn-2 representing the centre position) 18:1n-9/18:1n-9/18:1n-9, 18:2n-6/18:1n-9/18:1n-9 and 18:1n-9/18:1n-9/18:3n-3, but in flax seed oil they are 18:3n-3/18:3n-3/18:3n-3, 18:3/18:3/18:2n-6 and 18:3n-3/18:3n-3/18:1n-9, and in safflower oil they are 18:2n-6/18:2n-6/18:2n-6, 18:2n-6/18:2n-6/18:1n-9, and 18:2n-6/18:2n-6/16:0 (320). Accordingly, the rats in Chapter 3 were fed 18:3n-3 mainly on the triglyceride sn-3 position, but in Chapter 4 large amounts were fed on the sn-2 position, as well as the sn-1 position. Triglyceride digestion results in the release of free fatty acids from the sn-1 and sn-3 positions, which are absorbed along with the sn-2 monoglycerides and re-esterified. At the tissues, triglycerides are hydrolysed at the sn-1 and sn-3 positions, releasing free fatty acids to tissues, and the sn-2 monoglycerides are transported to the liver in chylomicron remnants (Figure 5.1). Thus, it is possible that the higher 22:6n-3 in milk in rats fed the flax-safflower oil in Chapter 4 than in the milk of rats fed canola oil in Chapter 3 (Table 5.5) was due to greater delivery of 18:3n-3 to the maternal liver for desaturation. This is a novel concept that should be further studied.
Figure 5.1 Schematic illustration of triglyceride digestion and absorption to illustrate that fatty acids at the sn-2 position of dietary triglycerides may have greater delivery to liver in remnant particles than fatty acids at the sn-1 or 3 positions. LPL: lipoprotein lipase.

Dietary fatty acids and the effects on gene expression

Table 5.6 provides neonatal liver fatty acids for the groups used for gene expression studies. The expression of Acly, which encodes ATP citrate lyase involved in acetyl CoA export from the mitochondria as citrate, Scd1, which encodes stearoyl CoA (Δ 9) desaturase, and Pklr, which encodes pyruvate kinase, a regulatory enzyme of glycolysis, were decreased in neonatal livers with increased levels of long chain n-3 fatty acids in both Chapter 3 and 4. These results indicate that the n-3 fatty acid supply is a key regulator of these enzymes, with the reproducibility of the findings giving confidence to the interpretation. However, while Cpt1a and Shmt1 transcripts were higher in neonatal livers with higher n-3 fatty acids in Chapter 3, Chapter 4 showed no significant difference in Cpt1a or Shmt1 among neonatal livers with differing amounts of n-3 fatty acid. Several explanations are possible. First, the responses of biological systems are not linear. It is expected that induction/inhibition of gene expression by n-3 fatty acids will have a threshold above which no further change is seen. Cpt1a and Shmt1 expression were lower when the liver had 2.55 compared to 8.86 g 22:6n-3/100 g fatty acid, but not different among three groups when the liver 22:6n-3 ranged from 8.05 to 9.00 g/100 g fatty acid, or had very high 22:6n-3 of 28.0 g/100 g fatty acid. This may be interpreted to suggest that the
maximum stimulating effect of 22:6n-3 is achieved at 8-9% 22:6n-3 in the liver total fatty acids. Thus, it may be more appropriate to consider that deficiency of long chain n-3 fatty acids results in low activity of hepatic Cpt1a and Shmt1, rather than to say that n-3 fatty acids stimulate Cpt1a and Shmt1 expression. Another possible explanation is that differences in the other milk fatty acids explain the apparent differences in Cpt1a and Shmt1 expression. In Chapter 3, where differences in Cpt1a and Shmt1 were apparent, the milks also differed in MCFA and total unsaturated fatty acids (Table 5.6). It seems unlikely that MCFA were involved because hepatic Cpt1a and Shmt1 expression were not different in neonates fed milks with 33.5 compared to 18.2 g MCFA/100 g milk fatty acid in Chapter 4 (Figure 4.7). Possibly, Cpt1a and Shmt1 abundance were influenced by the sum of unsaturated fatty acid. The diets in Chapter 4 were prepared with safflower oils rich in either 18:1n-9 or 18:2n-6 and thus had different types, but not total amounts of unsaturated fatty acids (Table 5.6). The current study design does not enable separation of these differences.

In contrast to Cpt1a and Shmt1, differences in Hmgcs2 and Fasn were present in livers in Chapter 4 but not Chapter 3. Specifically, Hmgcs2 was higher and Fasn was lower in pups in the High fat-high long chain n-3 fatty acid compared to the low n-3 fatty acid groups in Chapter 4. This may reflect an effect of dietary rather than endogenously formed 20:5n-3 and 22:6n-3, or the much higher levels (by 10-fold) of 20:5n-3 and 22:6n-3 in milk when fish oil (Chapter 4) was fed than when only 18:3n-3 was fed (Chapter 3). In summary, both Chapters 3 and 4 showed that the composition of maternal dietary fatty acids impact gene expression in the neonatal liver. It is clear, however, that interpretation of effects of specific dietary fatty acids in in vivo studies is complex.
Table 5.6 Comparison of milk and neonatal liver fatty acids among diet groups for which gene expression was analyzed in Chapters 3 and 4.

<table>
<thead>
<tr>
<th></th>
<th>Chapter 3</th>
<th></th>
<th>Chapter 4</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-3 deficient</td>
<td>n-3 adequate</td>
<td>Low fat</td>
<td>High fat-18:1</td>
<td>High fat-18:2n-6</td>
<td>High fat-LC n-3</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.12 ±&lt;0.01</td>
<td>2.33 ±0.20</td>
<td>0.08 ±0.01</td>
<td>0.05 ±&lt;0.01</td>
<td>0.18 ±0.01</td>
<td>0.49 ±0.02</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.09 ±&lt;0.01</td>
<td>0.49 ±0.04</td>
<td>0.02 ±&lt;0.01</td>
<td>0.02 ±&lt;0.01</td>
<td>0.05 ±&lt;0.01</td>
<td>3.48 ±0.12</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.10 ±&lt;0.01</td>
<td>0.25 ±0.01</td>
<td>0.26 ±0.03</td>
<td>0.26 ±0.02</td>
<td>0.33 ±0.04</td>
<td>5.96 ±0.26</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>4.89 ±0.14</td>
<td>10.9 ±1.44</td>
<td>8.51 ±0.61</td>
<td>8.79 ±0.47</td>
<td>26.9 ±0.58</td>
<td>8.99 ±0.45</td>
</tr>
<tr>
<td>Σ Mono</td>
<td>22.0 ±1.99</td>
<td>48.2 ±3.48</td>
<td>32.4 ±2.87</td>
<td>54.8 ±1.66</td>
<td>34.2 ±0.61</td>
<td>21.9 ±1.07</td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>50.4 ±2.28</td>
<td>19.5 ±4.76</td>
<td>33.5 ±4.34</td>
<td>16.8 ±2.60</td>
<td>20.4 ±1.22</td>
<td>36.5 ±2.25</td>
</tr>
<tr>
<td>Σ Sat ≥16:0</td>
<td>22.3 ±0.14</td>
<td>17.9 ±1.20</td>
<td>25.2 ±1.32</td>
<td>19.2 ±0.63</td>
<td>17.9 ±0.37</td>
<td>19.2 ±0.52</td>
</tr>
<tr>
<td>Σ Unsat</td>
<td>27.3 ±2.13</td>
<td>62.6 ±5.01</td>
<td>44.2 ±1.77</td>
<td>63.0 ±1.95</td>
<td>61.8 ±1.04</td>
<td>44.2 ±1.77</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.04 ±0.01</td>
<td>1.15 ±0.13</td>
<td>0.13 ±0.03</td>
<td>0.05 ±0.01</td>
<td>0.11 ±0.03</td>
<td>0.25 ±0.02</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.04 ±0.01</td>
<td>1.38 ±0.17</td>
<td>0.24 ±0.04</td>
<td>0.17 ±0.05</td>
<td>0.18 ±0.05</td>
<td>4.75 ±0.30</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.99 ±0.40</td>
<td>8.86 ±1.41</td>
<td>9.00 ±0.57</td>
<td>8.05 ±0.49</td>
<td>8.84 ±0.66</td>
<td>28.0 ±0.49</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>24.9 ±1.07</td>
<td>20.4 ±1.32</td>
<td>32.1 ±0.44</td>
<td>30.0 ±0.37</td>
<td>38.2 ±0.28</td>
<td>16.0 ±0.38</td>
</tr>
<tr>
<td>Σ Mono</td>
<td>30.6 ±1.40</td>
<td>34.2 ±2.51</td>
<td>20.6 ±0.87</td>
<td>26.6 ±0.55</td>
<td>17.8 ±0.84</td>
<td>16.5 ±0.50</td>
</tr>
<tr>
<td>Σ MCFA</td>
<td>6.09 ±0.71</td>
<td>1.34 ±0.34</td>
<td>1.89 ±0.28</td>
<td>1.13 ±0.15</td>
<td>0.83 ±0.10</td>
<td>2.14 ±0.29</td>
</tr>
<tr>
<td>Σ Sat ≥16:0</td>
<td>34.6 ±0.70</td>
<td>29.3 ±1.74</td>
<td>35.0 ±0.45</td>
<td>33.3 ±0.29</td>
<td>33.1 ±0.38</td>
<td>25.4 ±0.42</td>
</tr>
<tr>
<td>Σ Unsat</td>
<td>59.4 ±0.36</td>
<td>69.3 ±1.53</td>
<td>63.1 ±0.57</td>
<td>65.5 ±0.37</td>
<td>66.1 ±0.34</td>
<td>72.4 ±0.43</td>
</tr>
</tbody>
</table>

Mono: monounsaturates; Sat: saturates; Unsat: unsaturates.
5.2.3 Specific contributions

This dissertation opens a new area addressing the importance of n-3 and n-6 fatty acid nutrition in early metabolic development in the liver. The contributions of this PhD research to the field of fatty acid nutrition are summarized into four major areas.

1) These studies are the first to show that the maternal dietary fatty acid composition at constant, moderate fat intakes influences offspring liver development. A growing field of interest relates to the effects of high dietary fat intakes during gestation and lactation on early markers of metabolism and subsequent metabolic programming in the offspring. In this regard, a number of studies have shown evidence of altered liver triglycerides, gene expression and developmental programming secondary to maternal high fat feeding in pregnancy (321-325). However, in most cases, the importance of the fatty acid composition, particularly the n-6 and n-3 fatty acids, has not been considered. Moreover, the addition of high fat to experimental diets in these types of studies also involved a reduction in the percent energy from protein, making it difficult to dissociate the effects of fat from those of an altered protein supply. The studies in this dissertation used diets with a constant proportion of energy, fat and protein, with constant vitamins and minerals per kcal, and provided fat at a moderate level to avoid potential confounding effects due to excess hepatic triglyceride accumulation. These studies clearly show that the fatty acid composition, at constant fat intakes, is relevant to early infant metabolic development.

2) These studies provide the first evidence that dietary fatty acid composition, specifically the n-3 fatty acids, influence liver gene expression in the developing liver. Although a large number of studies have shown that the n-3 and n-6 fatty acids regulate the expression of enzymes of fatty acid and glucose metabolism in adult liver cells both in vivo and in vitro (10), changes in gene expression may have different implications for the growing infant from that in the adult.
Notably, the infant consumes a high fat milk diet, at short inter-feed intervals, has higher energy needs per kg body weight, is in positive energy balance and is accumulating new tissues. Therefore, the effects of altered energy substrate metabolism must be considered in this context. In addition, while many of the gene targets linked to n-3 fatty acids in neonatal liver in the present studies have previously been shown to be regulated by n-3 fatty acids in adult liver, including Fasn, Acaca, Acox1, Cpt1a and Pklr (142, 143, 157, 290-295, 326, 327), several new targets of n-3 fatty acids were identified using 2D gel proteomics. These new targets include fructose-1,6-bisphosphatase, glycerol-3-phosphate dehydrogenase, serine hydroxymethyltransferase (SHMT1) and argininosuccinate synthase. Whether these proteins are also regulated by n-3 fatty acids in adult liver remains to be determined.

3) **These studies provide the first evidence that the n-3 fatty acids regulate enzymes involved in amino acid and protein metabolism.** To the best of my knowledge, there are no published reports to show that the composition of dietary fatty acids regulates enzymes involved in amino acid metabolism at any stage of the lifespan. A role for n-3 fatty acids in contributing to amino acid metabolism is supported by studies that reported differences in amino acids in infant plasma and piglet liver after feeding with supplemental 22:6n-3. Piglets fed formula with 22:6n-3 had lower liver glycine, taurine and β-alanine, and higher asparagine than piglets fed formula without 22:6n-3 (328). These results are consistent with the findings of higher SHMT1 and lower glycine in neonatal rat livers with higher 22:6n-3 in Chapter 3. Plasma threonine was also reported to be lower in infants fed formula with 22:6n-3 and 20:4n-6 compared to formula without 22:6n-3 and 20:4n-6, and plasma phospholipid 22:6n-3 was inversely associated with plasma levels of alanine, histidine, methionine, serine and threonine (329). Whether the lower free amino acids in these studies and in rat livers with higher n-3 fatty acids in Chapter 3 reflect altered amino acid uptake by tissues, with increased incorporation of amino acid into proteins is intriguing. This possibility may be answered by examining effects of n-3 fatty acids on lean
tissue accretion and growth. One recent study addressed whether n-3 fatty acids influence muscle protein synthesis in elderly subjects (300). Supplementation with 20:5n-3 and 22:6n-3 was associated with greater relative incorporation of labeled phenylalanine into muscle proteins, suggesting higher protein synthesis, when compared to a corn oil placebo. However, to date, data on the effect of n-3 and n-6 fatty acids on infant body composition is limited and conflicting (199, 201, 202, 330, 331).

The association between long chain n-3 fatty acids and protein metabolism is further intriguing as 20:5n-3 and 22:6n-3 are naturally present in the human diet only in animal tissues, specifically the phospholipids of muscles and organs and triglycerides of fatty fish, all of which are also rich sources of protein. Because they are usually consumed with dietary protein, it seems logical that the long chain n-3 fatty acids may regulate protein and amino acid metabolism. The role of dietary 20:5n-3 and 22:6n-3 in protein synthesis and amino acid metabolism, and potential implications of adding 20:5n-3 and 22:6n-3 to foods low in protein requires further attention.

4) The studies with human milk provide a clear example of the complexity of human studies due to the unavoidable associations between nutrients. As described in Chapter 4, analysis of human milk fatty acids showed an inverse association between 18 carbon chain unsaturated fatty acids (18:1n-9, 18:2n-6 and 18:3n-3) and medium chain fatty acids (MCFA), which could be interpreted as inhibition of mammary gland MCFA synthesis and secretion by maternal dietary unsaturated fatty acids. However, the use of a rat model to enable design of diets with constant fat but varying in specific fatty acids, showed that the dietary content of 18:1n-9, 18:2n-6 and 18:3n-3 does not influence MCFA secretion in milk. Rather the results point to a role for the proportion of dietary energy from fat and carbohydrate, and the maternal plasma triglyceride pool as key factors regulating MCFA secretion in milk. An important
limitation of human studies is that foods, not nutrients, are consumed and therefore it is difficult to interpret which components of the diet explain statistically significant associations between dietary and biochemical variables.

5.2.4 General comments

A number of important messages arise from this research on the role of n-3 fatty acids in developing liver.

1) Dietary recommendations need to consider the entire lifespan and address the unique metabolic requirements of young infants. Metabolic regulation in the infant is different from other stages of the lifespan. The infant consumes a high fat milk diet and feeds more frequently than an adult, without apparent excess fat accumulation in the liver. The infant is growing and requires macronutrients and amino acids to support tissue growth. The infant is still developing, which means that many metabolic pathways, for example fatty acid oxidation and gluconeogenesis, are low at birth and increase postnatally. Current dietary recommendations for infants under 6 months of age are based on the average intake of nutrients from human milk, and for infants 7-12 months of age, based on the average intake of nutrients from human milk plus complementary foods. This is problematic because the nutrient content of human milk is highly variable and dependent on the maternal diet, which may not be ideal. Because of the unique metabolism and macronutrient needs of the infant, dietary recommendations should address the specific needs for infant growth and development.

2) Recommendations for intakes of n-6 and n-3 fatty acids should not be considered independently. The 2002 Dietary Reference Intakes from the National Academy of Sciences gave an acceptable macronutrient distribution range for the n-3 fatty acids of 0.6-1.2% energy and for the n-6 fatty acids of 5-10% energy (19). However, the 2002 Dietary Reference Intakes did not address that high n-6 fatty acid intakes influence the needs for dietary n-3 fatty acids. For
example, a diet could have 10% energy 18:2n-6 and 0.6% energy 18:3n-3 and meet the acceptable macronutrient distribution range for both the n-6 and n-3 fatty acids. The studies described in Chapter 2 showed that intakes of 18:2n-6 at about 10% dietary energy resulted in apparent “n-3 fatty acid deficiency” in tissues, despite provision of 18:3n-3 at about 1.2% dietary energy, an amount that was adequate with a low 18:2n-6 diet. Future studies should not overlook the inter-dependence of the n-3 and n-6 fatty acids.

3) The regulation of fatty acid, carbohydrate and protein metabolism are integrated and should not be studied in isolation. While it is well understood that metabolic pathways must be coordinately regulated for energy needs, it is less well appreciated that fatty acids, glucose and amino acids have additional functional roles beyond that of energy requirements. For example, fatty acids, in addition to providing sources of energy, are important components of cell membranes, precursors to signaling molecules, and regulators of gene and protein expression. When fat is abundant in the diet, fatty acids can be oxidized for energy in the citric acid cycle, but this requires a source of oxaloacetate. The anapleurotic reactions that regenerate oxaloacetate require glucose or amino acids, therefore, even when fat is the source of carbons for acetyl CoA, glucose and/or amino acids are still essential for oxidative metabolism. In addition, fatty acid oxidation requires nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) as electron acceptors, both of which contain amino groups. Fatty acid synthesis itself also requires NADPH, which can be generated from NADP in the pentose phosphate pathway, for which glucose is required. Glucose, in addition to the roles listed above, is also required for synthesis of non-essential amino acids. Amino acids are needed not only for protein synthesis, but also to provide amino acids or nitrogen for numerous other compounds including, for example, sphingolipids, phospholipid head groups, neurotransmitters, purines, pyrimidines, polyamines, nitric oxide and peptides such as carnitine and glutathione. From the perspective of the infant, it is clear that the infant needs to adapt not only to use fatty acids as a primary source
of energy, but also to ensure the supply of glucose carbons and amino acids and nitrogen for other essential functions. The findings of this dissertation suggest that the n-3 fatty acids are important for the coordinated metabolic adaptation to the high fat and lower protein milk diet at birth.

4) The response to changes in fatty acid flux cannot be extrapolated across organs with very different metabolic functions. Metabolic regulation and the impact of dietary fatty acids differs across organs. Many current recommendations for fatty acids in growing infants focus on 22:6n-3 and 20:4n-6 for the brain and retina. However, Chapter 2 showed that while addition of 22:6n-3 and 20:4n-6 to an infant milk formula high in 18:2n-6 enabled accumulation of 22:6n-3 in the brain, the effects on liver fatty acids were very different. Presumably, with an adequate and appropriate diet, the essential functions of all organs are met. Before proceeding to guidelines, we need to understand effects of n-3 and n-6 fatty acids across organs and cells, including the immune system, and determine optimal intakes for the whole body.

5.3 Strengths and limitations

The most important strength of this research is that it addressed the effects of altered fatty acids in the liver during development, a novel topic about which little was or still is known. Recognizing that early development is complex, metabolism may differ from adults, and that not all of the effects of fatty acids are understood, I used a combination of both proteomic and targeted molecular biology approaches. Many of these methodologies are still emerging as research tools in the field of nutrition; thus, an important part of this research was developing methodologies not yet established in our laboratory, including 2D gel proteomics. As data were gathered and new questions emerged, additional methods and collaborations were established, for example the analysis of glycine and serine by HPLC. This strategy allowed a conceptual
approach to how fatty acids may impact multiple pathways, rather than detailing increased or decreased expression of every enzyme within a given pathway. An important outcome was that in addition to pathways of fatty acid oxidation and synthesis, this dissertation raises questions related to the pentose phosphate pathway and synthesis of non-essential amino acids. It is unlikely that this would have been revealed with more traditional approaches. A limitation of this research is that the relevance is unknown, since no functional effects were assessed and no kinetic measures of metabolite flux were addressed. However, these studies and my results now set the stage for studies on metabolic flux using stable isotope tracers and measures of enzyme activity. Clearly many more studies can follow.

While it would be ideal to do the research described in this dissertation in humans, this is neither practical nor possible. Animal models were used to address the effects of the neonatal diet on tissue lipids, accessing tissues that are unattainable in human infants. The animal model allowed control of the nutrient composition of diets in addition to housing and other environmental conditions. The experimental diets were designed to be identical in micronutrients and protein relative to dietary energy, and only differed in the type, or in the case of the low fat diet in Chapter 4, the amount of fatty acid provided. The diets were also designed to be relevant to current maternal and infant diets. In the studies with neonatal piglets two of the formulas had fatty acid compositions modeled exactly after marketed infant formulas and in the studies with pregnant and lactating rats, the fat in the maternal diets did not exceed 20% energy, giving fat and fatty acids within the nutritional range.

While the animal studies were appropriate to address the study objectives, caution must be taken when translating the findings to humans, because of the species differences between pigs, rodents and humans. While pigs, rodents and humans must all transition from in utero nutrition to a higher fat postnatal milk diet, the growth and the development of adipose and lean
tissues differ between species. In the human infant the third trimester of gestation represents a period of adipose tissue expansion and triglyceride accumulation, such that infant has fat stores representing about 16% of body weight at birth (332). In contrast, the rat is much less well developed at birth and has very little adipose tissue (333). In addition, both the rat and piglet develop much more rapidly after birth than humans, and the rapid postnatal growth is supported by a higher protein content of rat and pig milk compared to human milk (174). Finally, metabolism also differs across species, for example, the major circulating lipoprotein in rodents is HDL rather than LDL as in humans (333). The physiological and metabolic differences between rodents and humans must be considered before extrapolating the findings of the present studies to humans, as the impact of diet in animals may over or under estimate the effects in humans.

Further limitations of the present study relate to the study designs of Chapters 3 and 4. First, the study designs did not enable separation of the effects of the in utero or postnatal supply of n-3 fatty acids, and because dietary manipulation was achieved through the maternal diet in gestation and lactation, it is also possible that differences in gene and protein expression occurred secondary to change in the maternal fatty acid metabolism. Analysis of neonatal livers from Chapter 3 from birth to 10 days postnatal revealed that differences in 22:6n-3 were apparent in livers at birth (less than 12 hours after parturition), reflecting differences in 22:6n-3 transfer in gestation, whereas differences in the n-6 fatty acids were only apparent in the postnatal period (Table A3.3, A3.4). It is therefore likely that the observed changes in gene and protein expression are explained by a combination of placental fatty acid transfer and the postnatal milk diet. However, while the study could have been designed to separate effects of the fatty acid supply in gestation from that in lactation, it would then be difficult to translate the relevance to a particular time point in human development (i.e. the postnatal rodent adipose tissue development may more closely reflect human infant adipose accretion in utero). In
addition, dietary patterns of women are unlikely to change considerably between gestation and lactation, therefore the model used in Chapters 3 and 4 is appropriate to consider the effects of the maternal dietary fatty acids for infant liver development. Second, while I chose to study the neonates at day three postnatal, based on the ontogenic expression of genes for enzymes of fatty acid oxidation, it is possible that some other age would have been more appropriate to assess changes due to the early fatty acid supply. As such, the long-term significance is not known. Finally, while the preceding section outlined the complications of using different oils due to the different positions of fatty acids on the triglyceride molecule, it is also possible that the oils differed in other fat-soluble nutrients, for example Vitamin E. However, because of the known roles of fatty acids in regulation of metabolism and gene expression, it is reasonable to expect that the differences in gene and protein expression between groups are explained by differences in the fatty acid content of the diets provided.

5.4 Future directions

The research described in this thesis has opened the door for many new avenues of research. An important next step would be to determine if the changes in gene and protein expression correspond to changes in metabolic activity or nutrient substrate flux. This could be accomplished through the use of stable isotopically labeled substrates. For example, $^{13}$C glucose could be provided to animals and the label measured in respired CO$_2$, non-essential amino acids or pentose phosphate pathway metabolites. This would show if the n-3 fatty acids decrease glycolysis and increase the flux of glucose carbons to non-essential amino acids, such as serine and glycine. Alternatively or in addition, the activity of the enzymes shown to differ in abundance could be measured ex vivo and this would resolve whether enzymes are also altered post-translationally. Another important future direction would be to determine if n-3 fatty acids
influence the metabolic adaptation to feeding at birth. This might be best tested by assessing the ability to maintain plasma glucose in response to a stressor, or to mount a ketogenic response.

Future studies should assess whether the early changes in gene and protein expression in response to dietary fatty acids persist at older ages or perhaps increase or disappear, and consider whether early changes in metabolic pathways impact long-term health. In addition, because the rat studies did not distinguish between in utero effects or effects of the postnatal milk diet, a cross-over study design, in which rats born to dams fed a high n-3 fatty acid diet through gestation are cross-fostered to dams fed a low n-3 fatty acid diet during suckling, and the reverse would enable separation of the specific effects of the in utero and postnatal fatty acid supply.

The studies in Chapter 3 and 4 focused on manipulation of 18:3n-3, 18:2n-6 and long chain n-3 fatty acids in the maternal diet, but there are several other dietary treatments that would be interesting to study. Future studies could use different doses of the long chain n-3 and n-6 fatty acids, an altered ratio of n-3/n-6 fatty acids, or address the effects of diets providing the same fatty acid composition but with different fat sources and thus different triglyceride structures. Based on the pig study, where addition of 22:6n-3 to the diet increased 22:6n-3 in tissue lipids but did not support the increase in 20:5n-3 seen when the diet had low 18:2n-6, it would seem important to understand if 20:5n-3 plays important roles in early development of the liver or other organs, different from 22:6n-3. This has important implications for infant formula development and also for women taking 22:6n-3 supplements.

The levels of MCFA in milk vary over four-fold, yet the experimental studies in this dissertation found no evidence that the amount of MCFA in rat milk alters gene expression in the offspring liver. Potential effects on pathways not investigated in this dissertation should be considered in future studies. In addition, future studies could address whether dietary MCFA, at constant n-3 and n-6 fatty acid intakes, alters n-3 or n-6 fatty acid accretion in tissues.
Another important direction that could be taken is to elucidate molecular mechanisms through which n-3 or n-6 fatty acids regulate gene expression. For example, gel-shift or chromatin immunoprecipitation assays could be used to determine which transcription factors are involved in the regulation of gene expression. In addition, while some of the mechanisms for n-3 and n-6 fatty acid regulation of gene expression have been elucidated using adult animal livers or cells, the potency of the different n-3 and n-6 fatty acids as regulators of gene expression is still unclear. Studies using cell cultures would also enable specific manipulation of each fatty acid, without complication due to changing the total fat supply.

5.5 Significance

Early nutrition is important for infant growth and development with potential lasting effects (334, 335). Maternal intakes of n-3 and n-6 fatty acids vary widely leading to a wide variability in n-3 and n-6 fatty acid transfer across the placenta and secretion in milk. Understanding implications of variability in the perinatal supply of n-3 and n-6 fatty acids is complex, as the functions of n-3 and n-6 fatty acids are wide ranging, vary among tissues and are still poorly understood. The present dissertation has shown that the n-3 and n-6 fatty acid supply influences metabolic development in the neonatal liver. These studies provide scientific evidence that maternal and infant fatty acid nutrition is important in development, with effects extending beyond the brain. Dietary guidance that focuses only on the brain may overlook important needs or even adverse effects of the diet in other developing organs. This work provides a foundation for many new research directions, which should continue to address the functions of n-3 fatty acids across different organs and systems and consider the unique nutrition and metabolic environment in early infancy.
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APPENDIX 1 Supplemental methods

A1.1 Procedure for lipid extraction. Modified from Folch et al., 1957 (336).

1. Homogenize samples in 18 volumes of saline. Use 0.25 mL of the combined sample + saline homogenate for extraction.

2. Add 2 mL saline, vortex briefly.

3. Add 3 mL methanol, vortex briefly.

4. Add 6 mL chloroform, vortex briefly.

Note: the volume of sample used can be increased but the proportions of chloroform: methanol: saline + sample must be 6:3:2.25 v/v/v.

5. Centrifuge tubes at 2000 revolutions per minute (rpm) for 5 minutes at 4°C.

6. Remove lower (organic) layer.

7. Rinse remaining aqueous layer with 6 mL chloroform. Centrifuge at 2000 rpm for 5 minutes at 4°C. Remove the organic layer and combine with the first collection.

8. Dry the organic solvents under nitrogen.

A1.2 Procedure for methylating fatty acids (337).

1. Add internal standard(s), usually an odd chain saturated fatty acid of chain length 9 to 19, such as 17:0.

2. Add reagents to samples as per Table A1.1, vortex and cap tubes. Incubate tubes at 105°C for the amount of time provided in Table A1.1.
Table A1.1 Reagents and methylation times for fatty acid methylation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>BF3</th>
<th>Methanol</th>
<th>Benzene</th>
<th>Methylation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Phospholipid (TPL)</td>
<td>1.0 mL</td>
<td>--</td>
<td>--</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Free Fatty Acids (FFA)</td>
<td>0.5 mL</td>
<td>--</td>
<td>--</td>
<td>15 minutes</td>
</tr>
<tr>
<td>PE, PC, PS</td>
<td>1.0 mL</td>
<td>--</td>
<td>--</td>
<td>10 minutes</td>
</tr>
<tr>
<td>PI</td>
<td>1.0 mL</td>
<td>--</td>
<td>--</td>
<td>20 minutes</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>1.0 mL</td>
<td>--</td>
<td>--</td>
<td>90 minutes</td>
</tr>
<tr>
<td>Triglycerides (TG)</td>
<td>0.25</td>
<td>0.55</td>
<td>0.20</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Cholesterol Esters (CE)</td>
<td>0.35</td>
<td>0.35</td>
<td>0.30</td>
<td>45 minutes</td>
</tr>
</tbody>
</table>

3. Cool samples to room temperature.

4. To extract the methyl esters add 3 mL saline and 6 mL pentane to the test tube. Vortex for 15 seconds.

5. Centrifuge the samples at 2500 rpm for 10 minutes at room temperature, then take off the top (pentane) layer and transfer to a clean test tube.

6. Re-extract the lower layer by adding another 3 mL of pentane to the original test tube. Vortex. Centrifuge for 10 minutes at 2500 rpm. Remove the top layer and combine with the first extraction.

7. Add 3 mL of saline to the pentane layers. Vortex. Centrifuge the samples at 2500 rpm for 10 minutes, then remove the top (pentane) layer with a 9” Pasteur pipette, and transfer to a clean 13 X 100 test tube.

8. Evaporate the pentane under nitrogen in the nitrogen evaporator.

9. Rinse the walls of the test tubes with 200-300 µL hexane to concentrate the sample at the bottom of the tube. Dry under nitrogen.

10. Resuspend the fatty acid methyl esters in an appropriate volume of hexane and transfer to GLC autosample vials (one vial per sample).
A1.3 Procedure for 2D gel separation of proteins in liver samples

Sample preparation and immobilized pH gradient (IPG) strip rehydration

1. Homogenize ~50 mg liver in 500 µL of 40 mM Tris.

2. Determine protein concentration.

3. Calculate volume of homogenate needed to obtain 500 µg total protein and add to a new tube. Add 1.5X digestion buffer (4.2 g urea, 1.52 g thiourea, 0.4 g CHAPS, water to 7.5 mL) to a total volume of 100 µL (x 3 for triplicate analysis). Dissolve the protein by pipeting up and down. Centrifuge at 10 000 g for 5 minutes and transfer the supernatant to a new tube.

4. Reduce and alkylate proteins using the BioRad Reduction Alkylation Kit (163-2090)

5. Clean up samples using the BioRad Clean Up Kit (163-2130)

6. Dissolve pellets in 250 µL rehydration buffer (1.26 g urea, 0.46 g thiourea, 0.12 g CHAPS, 30 µl 100 X Bio-Rad ampholytes (pH 3-10), bromphenol blue (6 µL of a 1% solution), water to 3 mL). Pipeting up and down and vortexing will help to redissolve pellets. If pellets do not dissolve, incubate for a few minutes. Briefly centrifuge. ** All protein should dissolve. Any undissolved material will interfere with isoelectric focusing and should not be applied to the IPG strips. Combine triplicate tubes.

7. Rehydrate IPG strips (BioRad, pH 3-10). Prop up a disposable IPG strip tray using the cover or paper, so that the tray is on a slight incline. Pipet 225 µL of sample evenly into each lane of the tray. (225 µL is used as there will be some loss of sample during rehydration). Peel plastic backing off of IPG strips. Place strip gel side down in sample. Lay tray flat and cover with a slightly damp paper towel. After 15-30 minutes check strips by moving the ends with forceps. Strips should slide easily. If strips stick to the tray, there will be a dry spot in the gel. Lift strip and reapply. Cover the tray with a damp paper towel, cover with the lid and then seal using plastic wrap. Rehydrate overnight
Isoelectric Focusing

8. Clean isoelectric focusing tray with 1% Triton X-100 and a cotton swab. Allow to dry completely. Place wicks on each electrode and add 6 µL water to each wick. (Wicks absorb excess electrolytes and prevent overheating.) Fill a 100 mL graduated cylinder with water. Rinse the IPG strips by dipping in the water. Blot excess water off by touching the end of the strip on to paper towel. Place IPG strips in isoelectric focusing machine, gel side down, ensuring that the gel is in contact with the electrodes and the ± orientation of gel is correct. Cover the strips with mineral oil. Focus with a linear voltage ramp to 8000 V for 35000 V-hour.

2nd Dimension polyacrylamide gel electrophoresis


Prepare equilibration solution (36 g urea, 20 mL 10% sodium dodecyl sulfate solution [SDS], 25 mL of 5 M Tris-Cl pH 8.8, 20 mL glycerol, water to 100 mL) and split into 2 50 mL portions. Add 1 g dithiothreitol (DTT) to one part. Add 1.25 g iodoacetamide to the other part. Place focused strips into a clean disposable tray, gel side up. Add equilibration solution with dithiothreitol to each lane, enough to cover the strip. Incubate for 10 minutes on a rotating plate. Transfer strips to a new disposable tray. Add equilibration solution with iodoacetamide to each lane and incubate 10 minutes with rotation. Transfer strips to a new disposable tray. Cover with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) until ready to load onto the polyacrylamide gels.

10. Load strips onto precast gels (BioRad Criterion Tris-HCl Gel 345-0101). Load a protein molecular weight marker into the end well (~10 µL). Using a plastic transfer pipet, pour heated agarose (0.5% agarose + bromphenol blue) onto the top of the gel. Dip an equilibrated IPG strips into running buffer, blot excess then load the strip onto the gel, pushing down on the edges until the IPG strip is flat on the gel. Place gels into electrophoresis tank and fill the
upper chambers with running buffer.

11. Run gels at 200 V constant until blue dye front is near the bottom of the gel, about 1 hour.
12. Rinse and stain gels. Remove gels from cassettes. Place gel in a tray, cover with water and rinse 3 times for 5 minutes each on a rotating plate. Discard water and add BioSafe Coomassie (BioRad) to cover the gel. Incubate for at least 1 hour. Discard coomassie and destain the gels overnight in water.

**Scanning gels and spot matching with PDQuest Software**

13. Scan gels and compare spots using PDQuest Software (BioRad).

**A1.4 Procedure for analysis of gene expression by real time PCR.**

1. Extract RNA. Use frozen tissues that have not been thawed. It is important to keep tissues frozen until homogenized as RNA degrades very quickly. Extract RNA from tissues using the RNeasy Kit (Qiagen) following the manufacturer’s protocol. Perform the optional DNase step to degrade potentially contaminating genomic DNA. Use 50 µL water to elute RNA from liver tissue. After isolating RNA, keep it on ice as it is still susceptible to degradation.
2. Determine the concentration of RNA in 2 µL sample using a nanospec. The absorbance at 260 nm must be within the linear range of the nanospec (0.1 and 1) for the concentration of RNA to be determined accurately. If the absorbance at 260 nm is higher than 1, take an aliquot of the RNA and dilute it until the absorbance 260 is between 0.1 and 1. The ratio of 260/280 should be between 1.8 and 2. Ratios outside of this range indicate the presence of impurities in the sample (i.e. proteins). The concentration of RNA is calculated using the absorbance at 260 nm.
3. Determine RNA quality by gel electrophoresis. Aliquot about 3 µL of RNA to run on an agarose gel. Add 0.4 g agarose and 40 mL Tris base, acetic acid, ethylenediaminetetraacetic acid (EDTA) [TAE, Invitrogen] to an Erlenmeyer flask. Microwave for about 30 seconds or
until the agarose is completely dissolved. Swirl to mix and allow to cool slightly. Set up electrophoresis casting gel apparatus and combs (BioRad). Add 4 µL Sybr Green to the agarose solution and swirl to mix. Pour agarose into the gel apparatus. Remove any bubbles using a pipet tip. Allow to set for about 15-20 minutes. Remove gel and base tray and place into gel dock. Remove combs from gel and fill gel dock with 1X TAE until the gel is covered. Combine RNA samples (~3 µL) with Orange Loading Dye (~2 µL). Load samples into the gel wells. Run for about 45 minutes at 75 V or until the orange dye front is halfway down the gel. Remove the gel & base from the gel dock and visualize the bands using the Chemigenius (Perkin Elmer), using the transilluminator with EtBr/UV filter. Degraded RNA will appear as a smear on the gel, intact RNA will show two distinct ribosomal RNA bands on the gel.

4. Synthesize cDNA using the High Capacity Reverse Transcriptase Kit (Applied Biosystems). Use 1 µg RNA in an 80 µL reaction. Make a reverse transcription master mix, multiply by the number of reactions + 2 to account for losses during pipeting:

- 8.0 µL reverse transcription buffer
- 3.2 µL dNTP
- 4.0 µL reverse transcriptase
- 8.0 µL random primers
- 16.8 µL Rnase-free water
- 40.0 µL

Add 1 µg RNA to a PCR tube then add Rnase-free water up to 40 µL. Add 40 µL reverse transcription mix to each tube. Place the tubes in a PCR cycler for 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 seconds and hold at 4°C. When the run is complete, transfer cDNA product to 1.5 mL Eppendorf tubes. For realtime PCR, make a 1:50 dilution of the cDNA: 2 µL cDNA + 98 µL water. Store cDNA at -20°C.
5. Fill in the blank 96-well template with the sample names as shown in the sample plate below. Half of the plate is used for the endogenous control detector and the other half is used for the target gene. Each sample should be analysed in duplicate wells. Ensure that each plate has a calibrator sample and a non-template control. The calibrator is made by combining 10 µL of each of the control group samples. This sample is given an RQ value of 1 and all other values are expressed relative to the calibrator. The non-template control is used to ensure that the PCR mix and primers are not contaminated with PCR product. It will contain the same PCR mix and primers as the other wells, but without any template (cDNA). A reading from the non-template control wells indicates that the PCR mix or primers have been contaminated with PCR product.

6. Prepare a master mix for the target primer and endogenous control using the following recipe:

- 10 µL Taqman gene expression master mix (Applied Biosystems)
- 1 µL Target gene primer or endogenous control
- 4 µL water

Multiply by the number of samples that will be analysed + 2 to account for losses in pipetting.

7. Pipet 15 µL of endogenous control master mix into each well for Columns 1-6. Pipet 15 µL of the target gene master mix into each well for Columns 7-12.

8. Add 5 µL of cDNA (diluted 1:50) to each well following the prepared template.

9. Cover the plate with adhesive film and seal.

10. Spin the plate to ensure that all solution is at the bottom of the wells.

11. Load the plate into the realtime PCR cycler.

12. Run the plate using the 7500 Realtime PCR System (Applied Biosystems).

13. Analyze the data using the 7500 System software.
Figure A1.1 Sample 96-well plate layout for real time PCR.

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S: sample; CAL: calibrator; NTC: non-template control.

A1.5 Procedure for determining glycogen in liver samples.

1. Homogenize 15 mg liver in 1 mL of ice cold 0.3 M perchloric acid.

2. glycogen → glucose

   Prepare amyloglucosidase (AG) reagent, 10 mg/mL in 50 mM sodium acetate with 0.02% bovine serum albumin, pH 5.5. For each sample prepare an amyloglucosidase tube (AG) and a no enzyme (NE) control tube. Add 50 µL of sample to each tube. To the AG tube add 500 µL AG reagent, to the NE tube at 500 µL 50 mM sodium acetate with 0.02% bovine serum albumin, pH 5.5. Vortex and incubate at room temperature for 2 hours with shaking.

3. glucose → glucose-6-phosphate → 6-phospho-gluconate

   Centrifuge the samples at 10000 g for 10 minutes at room temperature.

   Transfer 400 µL of the supernatant into new tube.

   Prepare 3-5 standards with a range of 0 – 15 µg glucose.
Add 500 µL of glucose hexokinase assay reagent (Sigma) to each tube.

Incubate for 15 minutes at room temperature.

Read sample absorbance at 340 nm.

4. Calculate glucose in samples from the glucose standard curve.

\[
\text{Glycogen} = [\text{glucose in AG}] - [\text{glucose in NE}]
\]

A1.6 Preparation of liver samples for amino acid analysis.

1. Homogenize liver (100 mg) in 0.5 mL 4% sulphosalicyclic acid

2. Add \(^{13}\)C-labeled glycine (11.2 µg/100 mg liver) and serine (4.2 µg/100 mg liver) (Cambridge Chemical).

3. Centrifuge at 13500 g for 15 minutes, save pellet for protein assay

4. Filter supernatant using a Millipore centrifugal filter unit 3K (Millipore Cat UFC500 496).

   Add up to 0.5 mL to the filter, spin at 14000 g for 1 minute. Repeat with remaining sample and spin for 30 minutes.

5. Store at -80°C until analysis.

A1.7 Procedure for methylating fatty acid in milk samples (317, 336).

1. Thaw the milk samples in cold water.

2. Add 500 µg of tridecanoic acid (13:0) internal standard, and 500 µg of heptadecanoic acid (17:0) internal standard and dry the solvent under nitrogen.

3. Add 100 µL of milk sample to the test tube


5. Slowly add 200 µL of acetyl chloride.

6. Cap the tubes tightly. Vortex samples and incubate at 100°C for 60 minutes, vortexing briefly every 15 minutes.
7. Cool samples at 4°C.

8. Slowly add 5 mL of 6% potassium carbonate solution.

9. Cap the tubes tightly and shake for 3-5 minutes.

10. Carefully open the tubes and then add 4 mL of pentane. Vortex.

11. Centrifuge at 2000 g for 10 minutes.

12. Remove the top layer (pentane) with a Pasteur pipette, and transfer to a new test tube.

13. Add another 4 mL of pentane to the original sample, vortex, centrifuge and add the pentane layer to the first collection.

14. Dry the pentane under nitrogen gas being careful not to leave the samples for extended periods on the evaporator.

15. Resuspend the methyl esters in about 750 μL hexane and transfer directly to a GLC autovial.
## APPENDIX 2 Fatty acid composition of experimental diets

**Table A2.1** Complete fatty acid composition of diets fed in Chapter 2.

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<th>Supplemented</th>
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The formulas contained 60 g of fat and 989 kcal/L.
**Table A2.2** Complete fatty acid composition of diets fed in Chapter 3.

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The diets had 20% energy from fat and 4.03 kcal/g.
**Table A2.3** Complete fatty acid composition of diets fed in Chapter 4.

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<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</tr>
<tr>
<td>18:3n-3</td>
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<td>0.02</td>
<td>0.01</td>
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</tr>
<tr>
<td>20:4n-6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.07</td>
<td>1.08</td>
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<td></td>
</tr>
<tr>
<td>22:0</td>
<td>0.25</td>
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</tr>
<tr>
<td>22:1</td>
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<td>0.02</td>
<td>&lt;0.01</td>
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<td>0.02</td>
<td>0.06</td>
<td>0.56</td>
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<tr>
<td>20:5n-3</td>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>22:4n-6</td>
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<td>&lt;0.01</td>
<td>0.07</td>
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<tr>
<td>22:5n-3</td>
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<td>&lt;0.01</td>
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<td></td>
</tr>
<tr>
<td>22:6n-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.67</td>
<td>9.94</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:0</td>
<td>0.14</td>
<td>0.21</td>
<td>&lt;0.01</td>
<td>0.14</td>
<td>0.17</td>
<td>0.20</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24:1</td>
<td>0.10</td>
<td>0.24</td>
<td>&lt;0.01</td>
<td>0.10</td>
<td>0.20</td>
<td>0.23</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Low fat diet had 4% energy from fat and 3.70 kcal/g and the High fat diets had 20% energy from fat and 4.03 kcal/g.
### Table A3.1 Fatty acid composition of milk from rats fed diets differing in fat and fatty acid composition.

<table>
<thead>
<tr>
<th></th>
<th>Low fat</th>
<th>High fat-n-3 deficient</th>
<th>High fat-n-3 adequate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Σ medium</strong>¹</td>
<td>39.2 (36.9-41.6)</td>
<td>50.4 (46.0-53.7)</td>
<td>19.5 (12.7-28.7)*</td>
</tr>
<tr>
<td>16:0</td>
<td>20.8 (20.1-21.3)</td>
<td>18.8 (18.4-19.2)</td>
<td>14.8 (13.1-16.9)*</td>
</tr>
<tr>
<td>18:0</td>
<td>3.2 (3.1-3.3)</td>
<td>3.3 (3.1-3.6)</td>
<td>2.8 (2.72-2.98)*</td>
</tr>
<tr>
<td><strong>Σ saturates</strong></td>
<td>24.2 (23.4-24.7)</td>
<td>22.3 (22.1-22.6)</td>
<td>17.9 (16.1-20.2)*</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>3.6 (3.1-3.9)</td>
<td>2.2 (1.7-3.2)</td>
<td>2.1 (1.6-2.9)</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>2.4 (2.0-2.6)</td>
<td>1.8 (1.5-2.2)</td>
<td>2.3 (2.1-2.5)</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>22.8 (20.8-24.3)</td>
<td>17.0 (15.0-19.3)</td>
<td>41.8 (36.1-47.3)*</td>
</tr>
<tr>
<td><strong>Σ mono</strong></td>
<td>30.0 (26.9-32.1)</td>
<td>22.0 (19.2-25.8)</td>
<td>48.2 (41.8-53.7)*</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>5.1 (4.7-5.3)</td>
<td>3.9 (3.7-4.3)</td>
<td>9.5 (7.6-11.8)*</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.63 (0.51-0.72)</td>
<td>0.52 (0.46-0.62)</td>
<td>0.69 (0.57-0.86)</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>0.03 (0.03-0.04)</td>
<td>0.03 (0.02-0.03)</td>
<td>0.06 (0.03-0.09)</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>0.04 (0.03-0.05)</td>
<td>&lt;0.01²</td>
<td>0.01 (&lt;0.01-0.04)</td>
</tr>
<tr>
<td><strong>Σ n-6</strong></td>
<td>6.3 (5.9-6.5)</td>
<td>4.9 (4.6-5.1)</td>
<td>10.9 (8.8-13.6)*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.10 (0.09-0.11)</td>
<td>0.12 (0.12-0.13)</td>
<td>2.3 (2.0-2.7)*</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.07 (0.06-0.07)</td>
<td>0.08 (0.08-0.09)</td>
<td>0.49 (0.42-0.55)*</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.07 (0.06-0.08)</td>
<td>0.10 (0.09-0.10)</td>
<td>0.36 (0.32-0.43)*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>0.13 (0.12-0.14)</td>
<td>0.09 (0.08-0.12)</td>
<td>0.25 (0.23-0.27)*</td>
</tr>
<tr>
<td><strong>Σ n-3</strong></td>
<td>0.36 (0.34-0.39)</td>
<td>0.40 (0.37-0.43)</td>
<td>3.5 (3.1-3.9)*</td>
</tr>
</tbody>
</table>

Values are means with range in parentheses for milk from n=3 rats per diet group. Differences were determined using unpaired t-tests with preplanned comparisons between the Low fat and High fat-n-3 deficient group and between the High fat n-3 deficient and n-3 adequate groups. There were no significant differences between the Low fat and High fat-n-3 deficient groups. * Different from High fat-n-3 adequate, P<0.05. ¹Fatty acids with chain lengths of 14 carbons or less. ²Where a single value is given, all values were the same.
Table A3.2 Birth weight and three-day-old weight and liver triglyceride, phospholipid and phospholipid fatty acids in offspring of rats fed diets differing in fat content and composition during gestation.

<table>
<thead>
<tr>
<th></th>
<th>Low Fat</th>
<th>High Fat n-3 deficient</th>
<th>High Fat n-3 adequate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size, n</td>
<td>15 ± 0.49</td>
<td>15 ± 1.0</td>
<td>14 ± 0.67</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>6.8 ± 0.21</td>
<td>6.7 ± 0.42</td>
<td>6.9 ± 0.17</td>
</tr>
<tr>
<td>Weight at 3 days, g</td>
<td>8.9 ± 0.31</td>
<td>8.1 ± 0.49</td>
<td>9.0 ± 0.20</td>
</tr>
<tr>
<td>Liver TG, µg/mg protein</td>
<td>239 ± 52.3</td>
<td>229 ± 34.9</td>
<td>269 ± 45.6</td>
</tr>
<tr>
<td>Liver PL, µg/mg protein</td>
<td>163 ± 7.81</td>
<td>156 ± 2.67</td>
<td>173 ± 6.86</td>
</tr>
<tr>
<td>PE fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>1.49 ± 0.20</td>
<td>1.54 ± 0.14</td>
<td>1.99 ± 0.11</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>23.5 ± 0.80</td>
<td>22.2 ± 0.99</td>
<td>20.2 ± 0.63</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>2.46 ± 0.25</td>
<td>2.98 ± 0.41</td>
<td>0.84 ± 0.07*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>11.3 ± 1.71</td>
<td>14.3 ± 0.97</td>
<td>0.06 ± 0.03*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.18 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>0.64 ± 0.10*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>12.0 ± 1.74</td>
<td>10.7 ± 1.52</td>
<td>24.9 ± 0.92*</td>
</tr>
<tr>
<td>PC fatty acids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>4.91 ± 0.58</td>
<td>5.27 ± 0.44</td>
<td>6.29 ± 0.43</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>20.1 ± 1.25</td>
<td>20.5 ± 1.09</td>
<td>18.9 ± 0.38</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.11 ± 0.18</td>
<td>1.27 ± 0.25</td>
<td>0.23 ± 0.04*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>4.74 ± 0.80</td>
<td>5.60 ± 0.43</td>
<td>0.03 ± 0.02*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.58 ± 0.08*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>5.97 ± 0.96</td>
<td>5.05 ± 0.73</td>
<td>13.0 ± 0.69*</td>
</tr>
</tbody>
</table>

Values are means ± SEM for n = 6-12 litters/diet group. TG, triglyceride; PL, phospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine. Data were analyzed using unpaired t-test with preplanned comparisons between the Low fat and High fat n-3 deficient and between the High fat n-3 deficient and n-3 adequate groups. There were no significant differences between the Low fat and High fat n-3 fatty acid deficient groups (P>0.05).*Different from the High fat n-3 deficient group (P<0.05).
Table A3.3 Liver fatty acids from day 0 to day 10 postnatal of rats born to dams fed diets with adequate or deficient n-3 fatty acid.

<table>
<thead>
<tr>
<th>g/100g fatty acid</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-3 def</td>
<td>n-3 adeq</td>
<td>n-3 def</td>
<td>n-3 adeq</td>
</tr>
<tr>
<td>12:0</td>
<td>1.15 ± 0.79</td>
<td>0.36 ± 0.64</td>
<td>1.48 ± 0.64</td>
<td>0.20 ± 0.09*</td>
</tr>
<tr>
<td>14:0</td>
<td>3.17 ± 0.91</td>
<td>1.52 ± 1.21</td>
<td>3.12 ± 0.97</td>
<td>0.99 ± 0.21*</td>
</tr>
<tr>
<td>16:0</td>
<td>29.2 ± 3.55</td>
<td>27.5 ± 2.58</td>
<td>28.9 ± 2.02</td>
<td>24.6 ± 2.58*</td>
</tr>
<tr>
<td>18:0</td>
<td>12.0 ± 3.44</td>
<td>13.3 ± 5.43</td>
<td>10.9 ± 5.86</td>
<td>6.52 ± 1.96</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>3.61 ± 1.35</td>
<td>2.13 ± 0.54</td>
<td>2.25 ± 0.64</td>
<td>1.92 ± 0.48</td>
</tr>
<tr>
<td>18:1n-7</td>
<td>5.13 ± 0.33</td>
<td>3.76 ± 0.49*</td>
<td>3.97 ± 0.68</td>
<td>2.72 ± 0.45*</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>23.1 ± 1.86</td>
<td>23.0 ± 4.19</td>
<td>21.2 ± 4.85</td>
<td>31.5 ± 3.82*</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>4.81 ± 0.47</td>
<td>6.40 ± 1.75</td>
<td>4.12 ± 0.82</td>
<td>7.70 ± 0.92*</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>6.68 ± 1.15</td>
<td>8.21 ± 1.35</td>
<td>9.87 ± 3.13</td>
<td>7.04 ± 1.86</td>
</tr>
<tr>
<td>22:4n-6</td>
<td>1.18 ± 0.62</td>
<td>1.16 ± 1.28</td>
<td>2.26 ± 0.95</td>
<td>0.96 ± 0.12*</td>
</tr>
<tr>
<td>22:5n-6</td>
<td>2.30 ± 0.64</td>
<td>0.95 ± 1.38</td>
<td>3.73 ± 1.68</td>
<td>0.20 ± 0.04*</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.04 ± 0.01</td>
<td>0.26 ± 0.15*</td>
<td>0.03 ± 0.03</td>
<td>0.61 ± 0.45*</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>0.05 ± 0.02</td>
<td>0.57 ± 0.43</td>
<td>0.08 ± 0.07</td>
<td>1.95 ± 0.47*</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>0.20 ± 1.95</td>
<td>0.65 ± 0.36</td>
<td>0.44 ± 0.35</td>
<td>1.95 ± 0.45*</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>2.0 ± 0.86</td>
<td>5.47 ± 2.42*</td>
<td>2.60 ± 1.01</td>
<td>5.82 ± 2.22*</td>
</tr>
</tbody>
</table>

Values are means ± SD of 1 pup/litter for n=4-6 litters per diet group. Maternal diets are provided in Section 3.3. Differences were determined by unpaired t-tests.* Significantly different from n-3 deficient, P<0.05.
Table A3.4 Liver lipids from day 0 to day 10 postnatal of rats born to dams fed diets with adequate or deficient n-3 fatty acid.

<table>
<thead>
<tr>
<th>μg lipid/mg protein</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n-3 def</td>
<td>n-3 adequate</td>
<td>n-3 def</td>
<td>n-3 adequate</td>
</tr>
<tr>
<td>TG</td>
<td>53.7 ± 43.6</td>
<td>40.0 ± 36.4</td>
<td>102 ± 52.1</td>
<td>212 ± 121</td>
</tr>
<tr>
<td>CE</td>
<td>4.94 ± 0.78</td>
<td>4.33 ± 2.29</td>
<td>5.76 ± 2.70</td>
<td>7.24 ± 2.46</td>
</tr>
<tr>
<td>FC</td>
<td>10.7 ± 0.76</td>
<td>9.63 ± 0.34*</td>
<td>9.62 ± 0.89</td>
<td>9.79 ± 1.47</td>
</tr>
<tr>
<td>CL</td>
<td>4.62 ± 0.88</td>
<td>5.65 ± 0.78</td>
<td>5.35 ± 1.05</td>
<td>4.83 ± 0.60</td>
</tr>
<tr>
<td>PE</td>
<td>33.3 ± 2.25</td>
<td>27.5 ± 6.48</td>
<td>29.9 ± 2.75</td>
<td>32.0 ± 5.64</td>
</tr>
<tr>
<td>PC</td>
<td>52.6 ± 2.03</td>
<td>48.7 ± 7.04</td>
<td>49.0 ± 4.57</td>
<td>53.9 ± 7.95</td>
</tr>
<tr>
<td>PS</td>
<td>6.99 ± 1.45</td>
<td>7.72 ± 2.94</td>
<td>5.58 ± 0.22</td>
<td>5.31 ± 0.55</td>
</tr>
<tr>
<td>PI</td>
<td>10.1 ± 0.37</td>
<td>9.37 ± 0.68</td>
<td>9.28 ± 0.73</td>
<td>10.3 ± 1.82</td>
</tr>
<tr>
<td>SPH</td>
<td>7.40 ± 1.17</td>
<td>8.04 ± 2.64</td>
<td>6.12 ± 0.61</td>
<td>6.09 ± 0.88</td>
</tr>
<tr>
<td>LPC</td>
<td>11.5 ± 3.42</td>
<td>13.1 ± 4.74</td>
<td>8.58 ± 0.71</td>
<td>10.1 ± 4.88</td>
</tr>
<tr>
<td>Total PL</td>
<td>127 ± 7.87</td>
<td>121 ± 8.17</td>
<td>114 ± 9.55</td>
<td>123 ± 16.8</td>
</tr>
</tbody>
</table>

Values are means ± SD of 1 pup/litter for n=4-6 litters per diet group. Maternal diets are provided in Section 3.3 Differences were determined by unpaired t-tests.* Significantly different from n-3 deficient, P<0.05. TG, triglyceride; CE, cholesterol ester; FC, free cholesterol; CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SPH; sphingomyelin; LPC, lysophosphatidylcholine; PL, phospholipid.