The relationship between long chain polyunsaturated fatty acids and body composition in infants

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Table of Contents

Abstract

Body composition in early life is a key factor that impacts human health outcomes throughout the lifespan. There are several published studies describing the role of maternal n-3 polyunsaturated fatty acid (PUFA) status in altering offspring body composition. However, less information is available for the relationship between infant n-3 PUFA and body composition. The present study aimed to test whether there is a relationship between newborn n-3 PUFA status and infant body composition. Seventy healthy mother-infant pairs from the Lakeshore General Hospital located in Greater Montreal were recruited. Infant body composition was assessed using dual-energy X-ray absorptiometry (DXA). Maternal and infant blood samples were taken, red blood cell (RBC) fractions collected and washed with saline then methylated to prepare fatty acid methyl esters with methanolic acetyl chloride for PUFA analysis using gas chromatography. Fatty acid status was expressed as % of total fatty acids (FA) in RBC. Infants were ranked according to RBC PUFA proportions using tertiles of: docosahexaenoic acid (DHA, 22:6 n-3), arachidonic acid (AA, 20:4 n-6), ∑n-3 PUFA, ∑n-6 PUFA and the ratio of n-6 PUFA: n-3 PUFA (n-6:n-3 ratio). The difference between the highest and lowest tertile was tested using MIXED model ANOVA. Correlation analysis and multiple regression models were performed to examine the relationships between the PUFA percentage and infant body composition. All infants (n=40 male; n=30 female) were of healthy weights and lengths for age (0.65±0.18 mo) and breastfed. On average, infants had 5.4±0.7% DHA, 14.7±1.3% AA, 6.4±0.9% ∑n-3 PUFA, 29.4±1.5% ∑n-6 PUFA of total FA and a n-6:n-3 ratio of 4.7±0.7 in RBC membranes. Infant RBC DHA and AA were negatively related to percentage of body fat (R^2=0.26, p=0.03 and R^2=0.27, p=0.02 respectively) in regression models adjusted for family income, maternal education, pregnancy weight gain, race, infant postnatal age, infant serum 25-hydroxyvitamin D concentration and sex. RBC AA was also positively associated with lean mass (g/kg) (R^2=0.31, p=0.01). Infants in the highest tertile of the n-6:n-3 ratio had a higher percentage of body fat compared to those in the lowest tertile (15.9±2.5% vs 13.3±2.6%, p=0.04). These results suggest that the neonatal PUFA percentage might partially explain the variation of infant fat stores obtained *in utero* and during early infancy. As such, balancing the ratio of n-6 PUFA and n-3 PUFA could be explored as a strategy to positively regulate body composition in infants. Further research is needed to replicate this study specifically in this age group.

Résumé

La composition corporelle pendant la petite enfance est un facteur déterminant de la santé tout au long de la vie. Plusieurs études ont permis de constater que le statut maternel en acides gras polyinsaturés (PUFA) influence la composition corporelle du nourrisson. Par contre, il existe peu de données sur la relation entre le statut en PUFA du nourrisson et la composition corporelle. L'objectif de cette thèse est d'examiner en détail si la composition en PUFA de la membrane des globules rouges (RBC) du nourrisson est reliée à la composition corporelle infantile. Soixante-dix paires mère-nourrisson en santé ont été recrutées à l'Hôpital général du Lakeshore, situé dans la région métropolitaine de Montréal. La composition corporelle des nourrissons a été mesurée par absorptiométrie à rayons X en double énergie (DXA). Des échantillons sanguins ont été prélevés sur les mères et les nourrissons, lavés dans une solution saline et méthylés au moyen d'une solution méthanolique de chlorure d'acétyle. Les esters méthyliques d'acides gras (FAME) résultant ont été analysés par chromatographie en phase gazeuse. Le statut en PUFA a été exprimé en % des acides gras totaux des RBC. Les nourrissons ont été classés par tertiles en fonction de la proportion de plusieurs types de PUFA, incluant l'acide docosahexaénoïque (DHA, 22:6 n-3), l'acide arachidonique (AA, 20:4 n-6), ∑n-3 PUFA, ∑n-6 PUFA et le ratio n-6 PUFA: n-3 PUFA. La différence entre le tertile le plus élevé et le tertile le plus faible pour chaque type a été déterminée par la procédure MIXED model ANOVA. Les relations entre les % de PUFA des RBC et les indices de composition corporelle des nourrissons ont été déterminées à l'aide d'analyses de corrélation et de modèles de régression multiple. Tous les nourrissons (n=40, garçons; n=40, filles) avec une taille et un poids normaux. En moyenne, la membrane des RBC des nourrissons était composée de 5.4±0.7% DHA, 14.7±1.3% AA, 6.4±0.9% ∑n-3 PUFA et 29.4±1.5% ∑n-6 PUFA des acides gras totaux. Une relation inverse a été observée entre la teneur en AA (R^2=0.26, p=0.03) et DHA (R^2=0.27 p=0.02) des RBC et le pourcentage de gras corporel des nourrissons par des modèles ajustés pour le revenu familial, le niveau d'éducation de la mère, le gain de poids pendant la grossesse, ainsi que le statut en vitamine D, la race, l'âge postnatal et le sexe du nourrisson. La teneur en AA des RBC était aussi associée positivement au pourcentage de masse maigre (R^2=0.31, p=0.01). Les nourrissons du tertile supérieur pour le ratio n-6: n-3 avaient un pourcentage de gras plus élevé en comparaison avec ceux du tertile inférieur (15.9±2.5% vs 13.3±2.6%, p=0.04). Ces résultats suggèrent que les variations dans les réserves adipeuses des nouveau-nés acquises *in utero* ou au début de la vie peuvent être expliquées en partie par le pourcentage de PUFA des RBC à la naissance. Des stratégies permettant d'équilibrer le ratio n-6: n-3 pourraient contribuer au maintien d'une composition corporelle optimale pendant la petite enfance. Des recherches supplémentaires sont requises pour reproduire cette étude avec ce groupe d'âge spécifiquement.

Author's Contribution

The data included in this thesis was obtained from a randomized controlled trial, which was conducted at the Mary Emily Clinical Nutrition Research Unit at McGill University.

Ye Yuan was the primary author of this thesis and contributed the major work of this project. She and Maryam Razaghi assisted in measuring the serum 25-hydroxyvitamin D concentration of infants using a Liaison auto analyzer to provide information regarding recruitment criteria. She attended study visits and assisted in anthropometric assessments. She was trained to analyze infant blood ionized calcium using radiometry, and to collect and store infant blood and urine samples. She helped to enter maternal 24-hour dietary recalls into Nutritionist pro. She also conducted red blood cell fatty acid direct methylation and analysis on gas chromatography. The statistical analysis was completed by Ye Yuan with the suggestions from Dr. Hope Weiler.

Kristina Mullahoo, Laura Glenn, Maryam Razaghi and Nathalie Gharibeh were involved in recruitment of mothers and infants. They administered 24-hour recalls and contributed to collecting and entering anthropometric, sociodemographic and DXA data. Kristina Mullahoo, Atheer Attar and Sharina Patel helped in analyzing maternal food frequency questionnaires.

Sherry Agellon and Paula Lavery were lab technicians in this study. They were involved in designing and performing blood collection, storage and analysis. Sherry Agellon trained Ye Yuan in analyzing RBC PUFA and measuring serum 25-hydroxyvitamin D.

Catherine A. Vanstone was the coordinator of this study and is a registered nurse. She was involved in designing and preparing the protocol and recruitment process for this study. She was responsible for collection of DXA and anthropometric data as well as maternal and infant blood in each study visit.

Dr. Hope Weiler was the principal investigator of the trial. She was responsible for designing, preparing, conducting and coordinating each aspect of the study. She reviewed and approved this thesis.

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List of tables

Literature review

Manuscript

List of figures

Literature review

Manuscript

List of abbreviations

AA	Arachidonic Acid
ADP	Air Displacement Plethysmography
AI	Adequate Intake
ALA	α-Linolenic Acid
AMDR	Acceptable Macronutrient Distribution Range
AMPK	5'-AMP-Activated Protein Kinase
BAT	Brown Adipose Tissue
BHT	Butylated Hydroxytoluene
BMI	Body Mass Index
BMC	Bone Mineral Content
BMD	Bone Mineral Density
C/EBPα	CCAAT/Enhancer Binding Protein α
CRP	C-Reactive Protein
COX	Cyclooxygenase
CPT-I	Carnitine Palmitoyl Transferase I
CV%	Coefficient of Variation
DHA	Docosahexaenoic Acid
DPA	Docosapentaenoic Acid
DRI	Dietary Reference Intake
DXA	Dual-energy X-ray Absorptiometry
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FAMEs	Fatty Acid Methyl Esters
FFQ	Food Frequency Questionnaire
FM	Fat Mass
GC	Gas Chromatograph
GPCRs	G-Protein–Coupled-Receptors
IGF-1	Insulin-like Growth Factor 1

IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
LA	Linoleic Acid
LM	Lean Mass
LTB-4	Leukotriene B-4
MUFA	Monounsaturated fatty acids
NF—κB	Nuclear Factor-κB
NO	Nitric Oxide
PC	Phosphatidycholine
PGE-2	Prostaglandin E-2
PPAR	Peroxisome Proliferator-Activated Receptor
PS	Phosphatidylserine
PTH	Parathyroid Hormone
PUFA	Polyunsaturated Fatty Acid
SFA	Saturated Fatty Acid
RBC	Red Blood Cell
SFT	Skin Fold Thickness
SNPs	Single Nucleotide Polymorphisms
TNF	Tumor Necrosis Factor
TX	Thromboxane
WAT	White Adipose Tissue
WC	Waist Circumference
UCP-3	Uncoupling Protein 3
USDA	The United States Department of Agriculture
VDR	Vitamin D Nuclear Hormone Receptor
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D

1. Introduction

Worldwide, obesity is a significant health issue that is increasing in prevalence in North America as well as most low and middle-income countries [1]. The prevalence of obesity among infants and through to adulthood remains high in the United States, as demonstrated in surveillance studies between 2003 to 2012 [2]. This is also true in Canada [3]. Many epidemiological and animal studies support the early origins of health and disease theory that small changes in the environment during development lead to phenotypic changes, which can affect an individual's response(s) to later environment(s) [4]. Due to the complications and numerous determinants of obesity, it is challenging to determine how closely it is related to early nutrient exposure(s) [5]. During the last decade, however, there is a growing body of fairly consistent evidence that demonstrates that childhood overweight and obesity exert profound adverse consequences on physical morbidity and premature mortality in adulthood [6]. During the fetal and postnatal periods, maternal nutrient support plays a paramount role in altering infant phenotype and health status, making this an important period for acquiring risk of subsequent obesity in later life [7]. Finding feasible and effective nutritional interventions for mothers and infants that can modify offspring body composition is critical in the achievement of human health [8].

Among all macronutrients and micronutrients, polyunsaturated fatty acids (PUFAs) are a promising nutrient series that may help relieve the burden of obesity and related chronic diseases. The n-3 and n-6 PUFA are the two major families of PUFA. While a balanced dietary intake of n-3 and n-6 PUFA are beneficial for human beings, a Western-style diet contains much more n-6 than n-3 PUFA [9]. Furthermore, some of the longer chain n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are studied and considered beneficial for infant neurodevelopment [10]. While the parent form of n-3 PUFA, α-linolenic acid (ALA), can only be obtained exogenously through food or supplements, the longer chain EPA and DHA can be synthesized from ALA [11]. This is true as well for the n-6 PUFA where linoleic acid (LA) is essential in the diet and the longer chain PUFA arachidonic acid (AA) is synthesized or obtained from dietary sources. Since an infant has limited capacity for synthesizing these longer chain PUFA, sufficient PUFA stores from maternal-fetal transfer *in utero* and postnatally from mother's milk is critical at this stage of life [12].

The effect of n-3 PUFA on immune, visual, cognitive, and motor function of infants is well studied [13]. A large double-blind multicenter study improved growth and developmental score in preterm infants using PUFA supplementation [14]. Less information is available on the effect of n-3 PUFA on infant growth and body composition. Animal studies provide relatively consistent results that indicate n-3 PUFAs can help maintain lean mass and prevent excessive fat storage through a variety of mechanisms [15]. These mechanisms include suppressing appetite, stimulating gene expression for β-oxidation, suppressing genes for supporting lipogenesis, regulating anti-catabolic and/or anabolic pathways in skeletal muscle and increasing brachial artery blood flow during exercise [11, 16-18]. While the results of animal studies are encouraging, the results of human studies are less consistent. The different body composition assessment tools used by researchers, the paucity of high quality human studies and inconsistent results from few studies to date make it hard to argue whether n-3 PUFAs exert a beneficial effect on human infant body composition or not [19, 20].

Thus, the primary objective of this thesis research was to test the relationship between newborn red blood cell (RBC) n-3 PUFA and body composition. It was hypothesized that infants with the highest levels of RBC n-3 PUFA would have higher lean mass (LM) and less fat mass (FM) compared to infants with the lowest levels of RBC n-3 PUFA.

The secondary objectives were to:

1) investigate the associations between both maternal RBC n-3 PUFA and infant body composition at birth. It was hypothesized that infants with the highest levels of maternal RBC n-3 PUFA would have more LM and less FM compared to infants with the lowest levels of RBC n-3 PUFA; and

2) examine the relationships between the n-6:n-3 ratio in infant RBC and infant body composition at birth. It was hypothesized that infants with the highest levels of n-6:n-3 ratio would have less LM and more FM compared to infants with the lowest levels of RBC n-3 PUFA.

2. Literature review

2.1 Fatty acids

Fatty acids are critical components of plasma and tissues, including cell membranes, skin, brain and adipose stores [21]. There are many categories of fatty acids of which two are pertinent to this thesis: n-6 and n-3 fatty acids, which are unsaturated fatty acids. Saturated fatty acids (SFAs), are without carbon-carbon double bonds, and remain solid at room temperature. The unsaturated fatty acids, which have one or more double bonds and are liquid at room temperature, can be subdivided into two classifications: monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) based on the number of double bonds in the fatty acid chain. Common types of MUFA are n-9, n-7 fatty acids, with one double bond compared to PUFAs that have at least two double bonds. According to the location of the last double bond relative to the terminal methyl group at the end of the PUFA molecule, there are two types of PUFAs [22]. The first double bond of n-3 PUFA is between the third and fourth carbon and the first double bond of n-6 PUFA is between the sixth and seventh carbon [11]. While each of the SFA, MUFA and PUFA have linkages to health, the n-3 and n-6 PUFA are the focus of this thesis.

Research has been conducted to look at the metabolism of PUFA in human beings because of their important role in human health. The n-3 and n-6 PUFA are known as essential fatty acids (EFA), which cannot be synthesized nor be converted from other fatty acids in humans and many other mammals. Furthermore, mammalian cells are not able to convert n-6 to n-3, as they lack the n-3 desaturase enzyme. In contrast, saturated fatty acids and MUFA can be synthesized in mammalian tissue from glucose or amino acid precursors. More importantly, the endogenous synthesis of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from their precursor α-linolenic acid (ALA); is limited with between 0.01% and 8% of ALA being elongated to EPA and less to DHA [23, 24]. For infants (either pre-term or term), they are able to make this conversion from their first month of life [25, 26]. Still, the amount of synthesis is limited. Infants who consume less breast milk which is rich in longer chain n-3 PUFAs, will be at higher risk for having lower EPA and DHA in tissues [26]. Hence, to ensure adequate tissue levels in infancy, EFAs and corresponding long chain PUFAs must be obtained through maternal-fetal transfer through the placenta or postnatally through maternal milk and eventually from introductory

foods later in infancy.

Owing to the important structural contribution of EFAs to cell membrane structure and cell signaling, EFAs are not treated only as a source of energy, but also as functional nutrients due to their biological effects. EFAs are known to stimulate growth, provide maintenance of skin and hair growth, regulate cholesterol metabolism, lipotropic activity, maintenance of reproductive performance, and other physiological and pharmacological effects. Additionally, these fatty acids have an important role in maintaining membrane integrity, immune response, an optimum level of unsaturation in tissue lipids and are components of structural lipids in many tissues, notably brain and retina [27]. More importantly, PUFA composition in cell membranes highly depends on dietary intake, certain enzyme activities, hormonal stimulation and their related genotype [28], highlighting the importance of obtaining sufficient EFA through diet.

2.2 The metabolism and utilization of n-3 and n-6 fatty acids

The essential n-3 and n-6 PUFAs, linoleic acid (LA) and ALA, are elongated and desaturated to long chain PUFA such as EPA and DHA mostly in the liver (Figure 2.1) [27]. LA (18:2 n-6) is converted by Δ-6 and Δ-5 desaturase and elongase enzymes to γ-linolenic acid (18:3 n-6) and dihomo-γ-linolenic acid (20:3 n-6) to form a key intermediate, and finally arachidonic acid (AA; 20:4 n-6).Then, most of AA is either further metabolized to docosapentaenoic acid (DPA; 22:5 n-6) or after incorporation into cell membranes used to make eicosanoids. As for ALA (18:3 n-3), it is converted to stearidonic acid (18:4 n-3) and eicosatetraenoic acid (20:4 n-3) and then EPA (20:5 n-3) and DHA (18:3 n-3), utilizing the desaturase and elongase enzymes. Since the same series of enzymes are used in the synthesis of n-3 and n-6 fatty acids, these two categories of PUFAs compete with each other during the metabolic process [22]. Hence, consuming greater amounts of one type may lead to reduced conversion of the other type. Consequently, the amount of AA (if high n-3 intakes) or EPA and DHA (if high n-6 intakes) synthesized may not be sufficient for tissue needs [29]. Besides being metabolized to different lipid mediators, these two groups of fatty acids also present in phospholipids to form the structure of cell membranes.

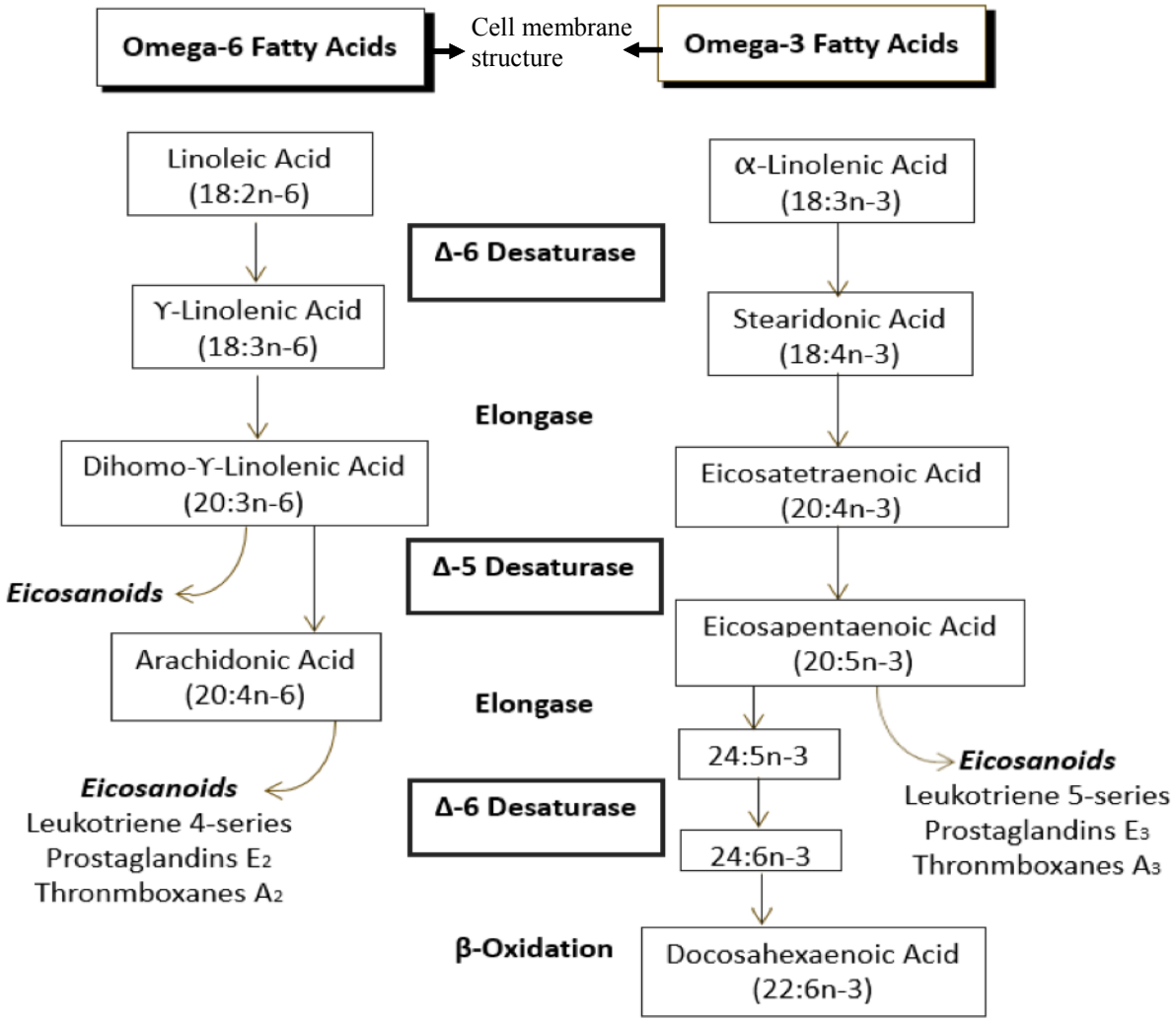


Figure 2.1. n-3 and n-6 fatty acids metabolism

The AA and EPA products of PUFA metabolism can be further metabolized to eicosanoids, including prostaglandin E (PGE)-2, leukotriene B (LTB)-2, thromboxane A (TXA)-4 series and PGE-3, LTB-5 and TXA-3 series respectively [22]. DHA can be converted to other autocoids such as resolvins, docosatrienes and neuroprotectins [30]. Although infants have the capacity to metabolize PUFA, the longer chain PUFA levels in blood are higher in infants fed formula rich in DHA and AA compared with those fed formula with only the EFA. This indicates that infants are unable to synthesize sufficient amounts of long chain PUFA to support optimal growth and development [12].

Due to the competition between the two EFAs, these bioactive eicosanoids produced from LA and ALA metabolism vary according to dietary PUFA intake. The products from AA metabolism are generally proinflammatory, proaggregatory, prothrombotic whereas products derived from DHA and EPA metabolism are less-inflammatory or inactive or even anti-inflammatory and inhibit platelet aggregation [22, 29]. A balance of these two classes of PUFAs should be warranted for homeostasis and normal development [31]. Thus, sufficient n-3 PUFA intake is critical in prevention of diseases and maintenance of body health.

2.3 Source and recommended intake of fatty acids

For pregnant women, and therefore as pertains to maternal-fetal transfer, the n-6 fatty acid LA can be obtained from vegetable oil, including corn, safflower and soybean oil. ALA can be obtained from plant-based foods, such as flaxseed and flaxseed oil, chia seed, perilla seed, walnuts, canola oil and rapeseed oil. Longer chain n-6 PUFA, AA is usually found in animal products and eggs, some fish and marine mammals in the diet of Greenland Inuit are also high in AA [23, 29]. In contrast, EPA and DHA are rich in oily fish (salmon, trout, halibut, herring and tuna) as well as oil that is extracted from the liver of fish species such as cod [32, 33]. For the fetus and infant, the major source of PUFAs is derived from the mother by maternal-fetal transfer and then through mother's milk, which is influenced by the nutritional status of pregnant and lactating mothers. More importantly, the secretion of fatty acids in mammary glands needs coordination of both endogenous and exogenous fatty acid supplies [34], making maternal dietary intake important in maintaining the PUFA concentration in breast milk. However, many pregnant and non-pregnant U.S and Canadian women, do not consume sufficient seafood, commonly having low n-3 PUFA intake during pregnancy and lactation [35, 36]. There are

bioenriched and enriched foods that contain dietary PUFAs, including eggs, yogurt and spreads that mainly contain fish derived and purified DHA and EPA. However, dietary recommendations should be based on the consumption of quality foods which naturally contain PUFAs [23], as the triglyceride structure of PUFAs in fortified food may not be the same as those in natural food due to autoxidation and purification processes [37]. Based on the prevalent low n-3 PUFA consumption among North American populations and diversity of food resources rich in PUFAs between regions and countries, efforts to establish dietary recommendations for n-3 PUFA have been made by different health organizations.

Given the beneficial effect of n-3 PUFAs, particularly DHA and EPA, and the failure to establish a Dietary Reference Intake (DRI) value for DHA and EPA by the Institute of Medicine (IOM; now called the National Academies of Science) report in 2002 [38], the issue of target n-3 PUFAs needs to be clarified with evidence-based recommendations. There is currently only an Adequate Intake (AI) value for ALA (0.5 g/day for infants from birth to 1 year, 1.6 g/day for adult men and 1.1 g/day for adult women) and LA (4.4 g/day for infant from birth to 6 months of age, 17 g/day for adult men and 12 g/day for adult women); these recommendations are based on median intakes in the U.S without any signs of nutrient deficiency [23]. Although there is no DRI for DHA and EPA, the National Academies of Science in the US recommend approximately 0.6-1.2% of energy of the Acceptable Macronutrient Distribution Range (AMDR) for n-3 PUFA intake [39]. The 2010 Dietary Guidelines for Americans recommends 8 oz/week (two 4-oz servings) of seafood, which equates to 250 mg/day of EPA and DHA, to prevent cardiovascular diseases [40]. Other health organizations recommend more than 500 mg/day for adults [41]. Specifically for pregnant women, the International Society for the Study of Fatty Acids and Lipids recommends 200 mg/day of DHA [40]; the European Food Safety Agency recommends 250 mg/day of EPA and DHA plus an additional 100-200 mg/day of DHA in consideration of neonatal brain development [42]. While Health Canada has not established a dietary recommendation for DHA, they did confirm that 3 grams of DHA plus EPA is safe for pregnant women. It also recommended 3% of energy from n-6 fatty acids and 0.5 % from n-3 fatty acids for general healthy individuals or 1% n-3 fatty acids for infants who do not receive a performed source of EPA and DHA [43].

A concern has been raised over excessive bleeding time resulting from high doses of n-3 PUFA. However, current research evidence shows no adverse effect on bleeding episodes or bleeding complications with 5 g/day of n-3 PUFA intake [44]. The n-3 PUFA supplementation during pregnancy has been reported to have no obvious adverse effect on preterm or term infants [45]. The safety of adding AA and DHA from algal and fungal oil to infant formula has also been confirmed by Health Canada, although its benefits are still inconclusive [46].

2.4 The importance of the n-6:n-3 fatty acid ratio

With the change of modern agriculture, a decrease of n-3 fatty acids has been found in many foods, including green leafy vegetables, animal meats, eggs and even fish [47]. For example, the n-6:n-3 ratio in egg yolks from free-range chickens is 1.3:1, while the United States Department of Agriculture (USDA) egg has a ratio of 19.9:1 and the ratio is reduced to 3-3.5:1 in DHA enriched eggs [9]. Consequently, a western style diet may be low in n-3 PUFA with a ratio of n-6:n-3 PUFA from 15:1 to 16.7:1, compared to the ratio 1:1 from diets of wild animals and ideally human beings [28]. Human milk from a group of healthy mothers in the US had a ratio of n-6:n-3 from 9.37:1 to 10.33:1 [35]. Furthermore, data from 65 studies demonstrated that lactating mothers in Canada, the Netherlands, Pakistan, rural South Africa and France had the lowest breast milk DHA concentration and the highest n-6:n-3 ratio compared with the value from other regions worldwide, where AA concentration varies less than DHA [48].

However, there is no confirmed optimal n-6:n-3 ratio for the average person. A ratio of 2-3:1 suppressed inflammation in patients with rheumatoid arthritis, and a ratio of 5:1 had a beneficial effect on patients with asthma, whereas a ratio of 10:1 had adverse consequences. These results imply the optimal ratio varies with consideration of different diseases [49]. Nonetheless, little information is available for health promotion and the exact requirements of EFA in humans have not been clearly identified, especially in infants. Jumpsen et al. observed small changes of n-6:n-3 ratio during neuronal and glial cell development in rats can have a significant effect in early development [50]. They stated that a n-6:n-3 ratio of 4:1 is optimal for the development of cerebellum, cortex and glial cells. A slight change of the n-6:n-3 ratio to 6:1 had an adverse effect on the development rate [50]. Health Canada currently recommends an n-6:n-3 ratio of 4:1 to 10:1, particularly for infants as well as pregnant and lactating women [51]. Addition of DHA

and AA in infant formula is not mandatory in Canada, but adequate LA and ALA needs to be ensured [46].

2.5 Genetic variation

Admittedly, there is variation in individual ability to endogenously synthesize long chain PUFA, both among infants and among mothers, including the synthesis in mammary glands and secretion of long chain PUFA in breast milk [52]. Moreover, the variant genotypes across racial and ethnic groups impact the result of n-3 and n-6 PUFA intake and supplementation. During the last few decades, researchers have put efforts into understanding the relationship(s) between diet-gene interactions and fat metabolism. Genome-wide association studies (GWAS) have identified many genetic polymorphisms altering molecular traits that increase risk of chronic diseases, such as diabetes and cancer. Based on this, Geiger and colleagues combined metabolites and single nucleotide polymorphisms (SNPs), and found it is a powerful way to identify SNPs that induce individual variation in complex lipid (glycerophospholipids) synthesis which affect lipoprotein and cellular phospholipid metabolism [53, 54]. Furthermore, the same group observed a strong association between SNP and two gene families, FADS1 and FADS2, that are involved in PUFA metabolism [54].

The two important enzymes in n-6 and n-3 PUFA metabolism, the delta-5 and delta-6 desaturases, are encoded by the FADS1 and FADS2 genes respectively [55, 56]. In 2006, Schaeffer et al. showed that common genetic variants of the FADS1 and FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in serum phospholipids (p values $<1.0 \times 10^{-13}$) and emphasized that the fatty acid composition of serum phospholipids is genetically controlled by the FADS1 and FADS2 gene cluster [57]. A study in Dutch women extended this finding, reporting that women who were homozygous for minor alleles in the FADS1/FADS2 gene cluster had lower DHA in plasma and human milk, suggesting genotype may also affect PUFA content of breast milk [58]. There are also interactions among genes, nutrients and diseases. Evidence exists showing that individuals may require different amounts of dietary LA, ALA, or AA, EPA and DHA, for both normal developmental needs and in the prevention and management of chronic diseases [59]. It is

recommended for researchers to take into consideration the FADS1 and FADS2 polymorphisms when exploring the requirement of n-3 and n-6 fatty acids consumption [52].

2.6 Opposite effect on inflammatory cytokine of n -3 and n-6 PUFAs

There are several factors that can affect health state and lead to many chronic diseases, including TXA-2, LTB-4, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor (TNF) and C-reactive protein (CRP). These factors are associated with diabetes, cancer, obesity, autoimmune disease, rheumatoid arthritis, asthma and depression, and can be increased by increasing n-6 PUFA intake and decreased by increasing n-3 intake, indicating the antagonistic effect between n-6 and n-3 PUFAs [28]. Unlike adults, infants are born with an immature immune system with reduced ability to produce cytokines. Then immune maturation occurs during the first months of life and is characterized as a Th1-polarization and an improvement in the capacity to produce cytokines such as IL-2 [60]. Several studies suggested the types of PUFAs during early infancy might affect the cytokine phenotype during development. Although its immunological effects on host defense are not conclusive, most data show that n-3 PUFA modulates inflammatory and immune responses [61]. These opposite effects from n-3 and n-6 PUFAs can be explained through several different mechanisms.

First, eicosanoids synthesized from AA and EPA have similar potency but act differently in inflammation processes. Prostaglandins PGI-2 and PGE-2, generated from AA have pro-inflammatory effects and prostaglandins PGI-3 and PGE-3, generated from EPA have anti-inflammatory effects [62]. PGE-2 is also suggested to have an anti-inflammatory effect [63]. During the initial phase of the inflammatory response, PGE-2 facilitates tissue influx of neutrophils, macrophages and mast cells from the bloodstream, which can cause swelling and edema. While in the later phase of inflammation, PGE-2 plays a role in inhibiting COX-2 enzymes and preventing synthesis of IL-2 and the expression of the IL-2 receptor in T cells [64]. TXB-2 produced from AA activates platelets and is a vasoconstrictor. These effects can be inhibited by prostaglandins formed from EPA [65]. Moreover, LTB-4 from n-6 PUFA increases vascular permeability and the production of inflammatory cytokines. On the other hand, LTB-5 from n-3 PUFA prevents the LTB-4 biosynthesis [22]. In obese patients, the chronic low-grade inflammation occurs with the increased level of plasma inflammatory cytokines, such as IL-6, CRP and TNF. These inflammatory cytokines cause insulin resistance or interfere with insulin

signaling, which result in increased free fatty acid circulation and ectopic fat accumulation [66].

Besides being involved in cytokine metabolism, the eicosanoids interact with G-protein–coupled-receptors (GPCRs). Studies have shown that PGE-2 binds to four types of GPCRs (the EP1, EP2, EP3 and EP4-GPRCs) that play important roles in various physiological and pathophysiological responses, including pain sensation regulation, peripheral circulation during acute inflammation and inducing hyperemia and swelling [67]. Meanwhile, GPCRs are also evolved in important pathways that regulate glucose homeostasis and adipogenesis [68]. The potential of GPCRs as new therapeutic targets for treating obesity and type 2 diabetes has been realized and is being explored [69]. Similar to PGE-2, LTB-4 interacts with two types of cell receptors, BLT1 and BLT2, which regulate production of LTB-4, thus mediate its inflammatory process [70].

As important components in cell membranes, n-3 and n-6 fatty acids influence cell membrane fluidity. This results from the fact that the PUFA acyl chain is extremely flexible due to the variety of double bonds in the structure [71]. Evidence indicates that DHA can modulate phosphatidylserine (PS) levels *in vitro* and *in vivo*, which is crucial in cell survival. DPA which is used to replace DHA in neuronal cells during n-3 PUFA deficiency proved to be less effective in PS accumulation and prevention of apoptosis [72]. The abnormal function of erythrocytes, including alterations in insulin receptors and Na^+-K^+ ATPase activity is commonly seen in human obesity. Whereas the fatty acid composition is responsible for this change that affect the enzymatic activities [73]. Additionally, T-cell cytokine, IL-22, plays a key role in stimulating IL-1β release, an inflammatory pathway in adipose tissue [74]. While T-cell rafts and soluble membrane phospholipid and fatty acyl composition can also be modulated by dietary n-3 fatty acids [75], and therefore suppress T-cell activation [75, 76].

Additionally, PUFAs exert effects through the regulation of gene expression. The major effect is through the interaction with nuclear receptors, which control the genes in relation to lipid metabolism and inflammatory signaling [77]. As gene regulation is an important pathway in which PUFAs level is associated with body composition, the mechanism is outlined in detail in later sections of this review.

2.7 PUFAs for infants

PUFAs play important roles during pregnancy, providing precursors to synthesize eicosanoids and components of cell membrane phospholipids [78]. Moreover, maternal PUFA status is closely related the fatty acid level of infants at birth. Data show that the postnatal changes in infant DHA and AA status are negatively related to the corresponding status at birth ($p< 0.01$), these changes were not significantly affected by the types of milk consumed by the infant [79]. During pregnancy, the ratio between placental and maternal plasma DHA is highest compared to other fatty acids [80], this is ascribed to placental preference to transfer DHA compared to other fatty acids [81] resulting in higher DHA concentration in cord blood than that in maternal blood [82]. This implies that a supplement of DHA might be needed to meet the requirement for mothers and the DHA supply to the fetus [78]. Although estrogen stimulates DHA synthesis in women [83], sufficient n-3 PUFA status is still difficult to reach in most pregnant women both with or without supplementation, based on the their regular low intake of dietary n-3 PUFA [84]. The effect of prenatal n-3 PUFA on infant functional outcomes should be investigated thoroughly, so that future recommendations for PUFA supplementation for pregnancy and lactation could be made accurately.

Clinical studies indicate the positive effects of long chain PUFA on infant cognitive and neural development, although some of these benefits are transitory [85, 86]. However, many dietary recommendations are made for preventing and reducing chronic disease, specifically cardiovascular diseases rather than health promotion for healthy people [23]. Health Canada suggests a n-6:n-3 ratio of 4:1 to 10:1; this ratio however, was considered optimal for brain and retina development [87] and was not set based on other tissue needs during development. Besides neural benefits, PUFAs also play a potentially significant role in the modulation of developmental processes affecting short- and long-term health outcomes related to growth, body composition, immune and allergic responses, and the prevalence of nutrition-related chronic diseases [88]. Nutrients influence gene expression, and many chronic diseases are programmed because of undernutrition during the fetal or infant period [49], these changes *in utero* can lead to the programming of endocrine and metabolic systems, so that the changes can be permanent or predisposed [89]. Maternal dietary intake of PUFAs influence the PUFAs available to transfer to the fetus [90] and an adverse maternal fatty acid profile can limit fetal growth [91]. Moreover,

the PUFA level in postpartum milk, the major source of PUFAs for infants during early infancy, highly depends on maternal stores, diet and synthesis by the mammary glands [92]. To improve the PUFA level in breast milk, prenatal and postnatal supplementation are recommended [93]. The health benefit of n-3 PUFA and the scarcity n-3 PUFA level in mothers and infants implies the necessity for mothers and infants to obtain sufficient PUFAs.

2.8 Adipose tissue development in early life

The first detectable adipose in a fetus is observed between the 14th and 16th week of gestation. Then, fat lobules appear followed by lobules increasing in size and adipocytes appearing in the main fat depot areas by the 23rd week of gestation [94]. The adipose tissue in newborns can be divided into two different physiological and functional types: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT has more blood vessels and heat producing mitochondria for dissipation of energy through heat production. BAT is abundant in small mammals and newborns and decreases with age. Conversely, WAT stores energy as triglycerides [95, 96], and may increase over time.

Humans start storing fat before birth and are born with enough fat to ensure a sufficient energy supply during the transition to postnatal life [95]. Before parturition, fetuses tend to mobilize fat themselves before being disconnected from the maternal nutrient flow through the placenta. During the first year of postnatal life, fat cells increase in size (hypertrophy) which is considered as a characteristic of fat accumulation [97]. A few study results also support the hypothesis that adipose cell proliferation is a major contributor to adipose tissue growth in infancy based on the hyperactive DNA synthesis activity in precursor cells [98]. As the early onset of obesity is partially a result of the increase in adipocyte number, the capacity of the cell proliferation and differentiation in precursor cells might be highest in early life (infancy) and contribute to obesity later in life [99]. Based on the results of rat studies, adipose tissue develops rapidly in some but not all depots. Although the capability for precursor cells to proliferate and differentiate to adipocytes may vary between different depots, the adipocyte cell replication rate is highest in early life [100, 101]. Therefore, infancy is a crucial and sensitive period to control fat tissue expansion while ensuring healthy growth.

2.9 The methods of measurement of infant body composition

Infants undergo a rapid growth period after birth, during which their body composition changes dramatically. Body mass index (BMI) is as a proxy for nutrition status, yet cannot differentiate each compartment of body composition [102]. There are many techniques available for differentiating and measuring the components of human body composition. Each method has assumptions, disparate advantages and limitations (Table 2.1) [103]. Most of these techniques are difficult to apply to infant measurements and are of limited reference value for measuring infant body composition. Further, there is a lack of validation and cross-calibration studies for several of the methods. Because of these reasons, four methods tend to be acceptable as suitable measurements for infant body composition, which will be reviewed here [104].

Anthropometric measurements, such as skinfold thickness or circumference of the body, provide better estimation of infant fat mass (FM) and fat free mass than weight or length. However, their accuracy and precision in infants could be attenuated by many potential errors, including the variability of the caliper used and operator error. The formulas for estimating FM and lean mass (LM) have not been well examined in infants [104]. The association between skinfold results and body fat also varies between different populations regarding gender, maturity and ethnicity [105]. Another technique, bioelectrical impedance analysis is based on assessing the electrical resistance of the body through a small current. Various age specific equations have been proposed for calculating total body water to obtain fat free mass [105]. It is a simple quick procedure. However, changes in room and body temperature, infant position, movement, crying and urination can affect the accuracy and conductivity of the instrument, which can invalidate results [104].

Densitometry is a technique assessing total body density using either hydrostatic weighing (HW) or air displacement plethysmography (ADP) for differentiating FM and fat free mass by their specific densities [106]. Recently developed devices for measuring infant and child body composition are called PeaPod (birth to 6 mo) and BodPod (2-6 years of age) respectively equipped with ADP, which make the procedure simple, quick and practical [106]. Still, more research of the specific density of fat and fat free mass in infants, accounting for their maturity, ethnicity and gender, should be explored to validate this technique in the context of infancy [103].

Table 2.1. Summary of techniques for measuring body composition

Technique	Assumptions made	Reference	Advantages/disadvantages
Skinfold-raw	Constant skin protein content	Y	For: simple measure of regional fat Against: no information on lean mass
Skinfold-equations (Jackson and Pollock)	Constant fat storage underneath skin	N	For: simple and quick Against: poor accuracy in individuals and groups
Body mass index	Var weight= Var fat	Y	For: simple and quick Against: measures nutritional status not body composition
Body circumference	Girth of sites for assuming body proportion and size	Y	For: simple, quick, robust measure of abdominal fat (waist circumference) Against: not so accurate as measure of internal visceral fat
BIA	Constant body water and H_{ffm}	N	For: simple and quick Against: poor accuracy in individuals and groups
DXA	Constant attenuation of FFM and FM	N	For: accurate for limb lean and fat Against: radiation exposure; whole body bias size, sex, fatness
Densitometry	Constant D_{ffm} and D_{fm}	N	For: acceptable two-component technique Against: effects of disease on lean mass reduce accuracy
Isotope dilution	Constant H_{ffm}	N	For: only technique acceptable in all age groups Against: delayed results; inaccurate if disease affects H_{ffm}
MRI	Voxel volume in slices for assuming constant body components	N	For: accurate for regional AT Against: expensive, limited availability, measures AT not fat
TOBEC	Constant H_{ffm}	N	For: acceptable two-component technique Against: rarely available, accuracy unknown
TBK	Constant K in cell mass	N	For: measures functional component of body composition Against: rarely available, poor accuracy for fatness
Multicomponent models*	Constant D_{prot} and D_{min},	N	For: most accurate approach, all measurements acceptable Against: expensive, specialist research approach

BIA: bioelectrical impedance analysis; DXA: dual-energy X-ray absorptiometry; MRI: magnetic response imaging; TOBEC: total body electrical conductivity; TBK: whole body potassium scanning; AT, adipose tissue; D_{fm}, density of fat; D_{ffm}, density of fat-free mass; D_{min}, density of mineral; D_{prot}, density of protein; FM, fat mass; FFM, fat-free mass; H_{ffm}, hydration of fat-free mass; K, potassium; Var, variability

*based on body fat, water and hydration, density, and mineralization of fat free mass

Information was obtained and modified from [103].

Lastly, dual-energy x-ray absorptiometry (DXA) is also one of the most widely used advanced technologies in pediatric applications; it provides whole body and regional assessments of bone mass, FM and LM [103]. The photon absorption level in bone and lean soft tissue is measured and distinguished at two different energy levels. Then fat can be extrapolated by subtracting bone and lean from weight, so that the assumption of each compartment could be made [107]. DXA has been validated in studies comparing its results with chemical analysis on carcasses of pigs [105]. DXA is also reported to provide accurate and reliable assessment of neonatal body composition in comparison with magnetic resonance imaging and anthropometric measurements [108]. However, there are several commercial DXA scanners and software from various companies, and not all of these have been validated for measuring infant body composition [104]. Challenges in interpreting the results provided by different instruments and software should be addressed by more cross-validation studies.

2.10 Body composition during infancy

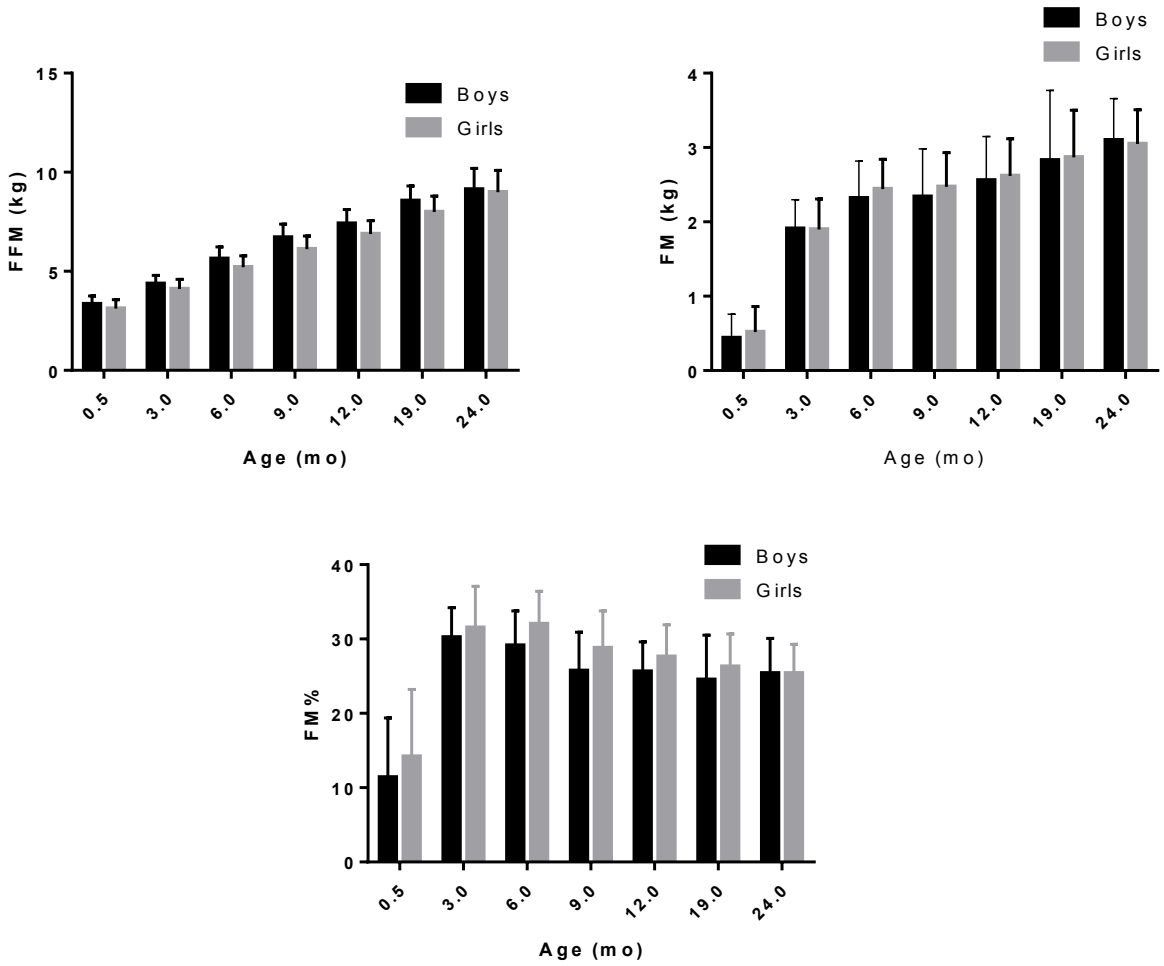
A typical weight loss has been observed in healthy newborns during the first three days after birth, which is attributed to reductions in body water and body solids, where greater FM is lost [109]. The finding of several studies also indicates that preadipocyte differentiation and self-renewal might be highest during the first year of life in infants [110]. The percentage of FM (FM%) increases after birth until 6-9 months of age, followed by a decrease for 5-6 years. After that, the proportion of fat will increase again, which is characterized as "adiposity rebound" [111]. A recent updated reference for the body composition of healthy infants in the US from 0.5 months to 24 months is summarized in Figure 2.2 [112]. If the adiposity rebound occurs before 5-6 years of age, the risk of obesity is increased [111].

Obesity is a condition when excessive body fat is accumulated. Due to the relative complication and limited accessibility of FM measurements, body weight percentile (>95th) and BMI are widely used to define overweight and obesity [111]. In adults, a BMI of 25-30 kg/m^2 suggests the individual is overweight. While a BMI greater than 30 kg/m^2 indicates obesity in individuals and risk of related negative effects [113]. In children, BMI percentiles are preferred as these are age specific. Ellis et al. reported that BMI does not always correspond to FM% and proposed that healthy girls and healthy boys should have a FM% that ranges from 17% to 32% and from 10% to 25% respectively [114].

2.11 PUFA and body composition

In addition to the well-documented benefits of n-3 PUFA intake on reducing cardiovascular diseases and improving cognitive function, dietary fat provides energy while contributing to energy stores and thus is reflected in body composition. Studies have shown that dietary SFA

Figure 2.2. Fat free and fat mass estimated from a multicomponent model* in boys and girls, ages 0.5 to 24 mo.



Values are mean ± standard deviation
*Measurements based on total body water, total body potassium and bone mineral content
Data source was obtained from [112].

and MUFA can be considered as predictors of human adiposity [115]. N-6 PUFA and n-3 PUFA play opposing functional roles in affecting the differentiation of adipose precursor cells in adipocytes through adipogenic mechanisms. *In vitro*, peroxisome proliferator-activated receptor (PPAR)δ, a member of the PPAR family, and C/EBPβ and C/EBPδ, two members of CCAAT/enhancer binding protein α (C/EBPα), upregulate the expression of PPARγ which induces adipogenesis [116]. PUFAs act as adipogenic hormones in this process, participating in the regulation of expression of lipid-related genes and adipose cell differentiation [117]. One of the main adipogenic components in serum is derived from AA which is a precursor of PGI-2 (prostacyclin), and is synthesized and released from preadipocytes. The prostacyclin upregulates the gene expression of C/EBPβ and C/EBPδ as well as PPARγ expression [118].

In contrast to n-6 PUFA, n-3 fatty acids have the potential to decrease body fat through multiple mechanisms [119, 120]. Adult appetite can be affected by n-3 PUFA consumption. A large weight loss study (n=278) showed that the extent of weight loss from energy restriction could be enhanced by consuming fish or fish oil in adults (20 – 40 y). The validated visual analogue scale (VAS), an assessment for hunger sensation, indicates that n-3 PUFA is associated with greater satiety immediately after the test dinner and after 120 min. The author concluded that consuming n-3 PUFA from fish and fish oil modulates postprandial satiety in overweight and obese volunteers during weight loss [121]. Therefore, it might be feasible to improve weight loss and body composition by following a n-3 PUFA enriched diet, as it reduces food intake. However, the proportion of FM loss resulting from this intervention needs to be verified. Although adipose tissue mass and distribution might be programmed during fetal and infant life, the effect of infant weight gain and later body composition appears to differ between high-income and low-middle-income countries [122]. Hence, whether n-3 PUFA can alter infant body composition and contribute to healthy growth and body composition later in life by suppressing appetite or altering adipogenesis needs more studies to clarify.

Gene expression by n-3 PUFA may also affect fat deposition. Mitochondrial carnitine palmitoyl transferase I (CPT-I) enzyme catalyze coenzyme A facilitates fatty acids transport into the mitochondria for β-oxidation in liver, cardiac muscle and skeletal muscle cells, to control the main point of β-oxidation [11]. The CPT-I gene expression in mitochondrial is regulated upstream by PPARs and by 5'-AMP-activated protein kinase (AMPK), and then is activated by

EPA in both adipose tissue and skeletal muscle. An earlier study showed that dietary n-3 PUFA intake increases CPT-1 acitivity in rat heart and skeletal muscle, which explained the stimulation effect of EPA for AMPK [16]. Additionally, an increased expression of uncoupling protein 3 (UCP-3) mRNA in skeletal muscle, and the expression of peroxisomal acyl-CoA oxidase gene in skeletal muscle, liver and heart have been shown in rats supplied with n-3 PUFA (40% of energy fat in diet), which results in decreased efficiency of mitochondrial oxidative phosphorylation, thus increasing energy production and decreasing fat formation [123]. Mori et al. also found that rats fed with a fish oil diet had significantly higher expression of genes regulating CPT 1a, cytochrome P450 4A10 and malic enzyme. Hence, the lipid metabolism-related enzyme activity: fatty acid β-oxidation, ω-oxidation, and malic enzyme activities in the small intestine of mice with 8% fish oil diet was increase by 1.2-, 1.6-, and 1.7-fold those in mice fed a diet that offers same amount of energy (30% triacylglycerol) [124]. A down regulation of genes for supporting lipogenesis and encoding stearoyl-CoA desaturase mRNA (a key enzyme in the lipogenic pathway) in epididymal fat was observed in mice fed with EPA/DHA concentrated diet (6% EPA, 51% DHA of total lipids (wt/wt)) [125]. Altogether, the effect of n-3 PUFA on gene expression lead to increased fat oxidation in organs rather than fat storage [18].

Animal studies reveal the potential of n-3 PUFA in the maintenance of lean body mass through changing the activity of anti-catabolic and/or anabolic pathways in skeletal muscle [18]. Evidence has shown that EPA is likely to be involved in a lipoxygenase metabolite as it deactivates the transcription of nuclear factor-κB (NF-κB) followed by suppression of activation of the ubiquitin-proteaseome pathway, a key pathway in muscle proteolysis during energy restriction [126]. N-3 PUFA is also claimed to increase whole body protein synthesis via promoting insulin sensitivity and activating insulin signaling to the Akt-mTOR-S6K1 pathway [127]. The increased metabolic rate caused by promotion of lean body mass can contribute to the reduction in FM. Nonetheless, this potential of n-3 PUFA has not been proven in humans.

Finally, Walser et al. states the potential of DHA and EPA in enhancing brachial artery blood flow during exercise [17]. Nutrient disposal is influenced by skeletal muscle blood flow and vasodilator function, which are often impaired in obese people [128, 129]. Improving these can increase nutrient delivery to muscle so that more nutrients are utilized for energy production instead of fat storage [18].

N-3 fatty acids also have effects on bone mineral content (BMC). This is suggested by studies that found that n-3 PUFA improve bone formation by reducing cytokine production. Three cytokines, IL-1, IL-6 and TNF, are regarded as inflammatory cytokines that regulate osteoclast progenitor differentiation, stimulates the early stages of osteoclastogenesis, increases bone resorption and inhibits bone formation [130]. N-3 PUFA consumption decreases the production of these cytokines and other stimuli including lipopolysaccharide, AA, proteolysis-inducing factor [131] with the net effect being reduced NF-κB activation and modulates receptor activator of NF-κB ligand (RANKL) signaling [132]. Another suppression of cyclooxygenase (COX)-2-dependent prostaglandin (PG) synthesis induced by cytokine reduction is crucial for osteoclastogenesis and bone resorption [133]. Nitric oxide (NO) is involved in bone resorption as well. An increase in the production of constitutive NO is associated with a decreased number and diminished activity of osteoclastic cells and therefore decreased bone resorption both *in vitro* and *in vivo* studies [134]. Activation of inducible nitric oxide synthase (iNOS) pathway and an increase in the concentration of NO during inflammation has been linked to enhanced osteoclastic activity and reduced osteoblastic activity as seen in an animal model of inflammation-induced osteoporosis [135]. N-3 PUFA can elevate the production of constitutive NO while suppressing the production of inducible NO by inhibiting the actions of inflammatory cytokines [133]. In contrast, n-6 PUFAs are able to increase the production of pro-inflammatory cytokines (TNF, IL-6) and NO posing deleterious effects on bone resorption, which can occur in both physiological and pathological processes [136].

2.12 Animal and human studies

The benefits of n-3 PUFA supply during prenatal and postnatal period on infant adiposity have been demonstrated in several studies, which strongly predict health status in adulthood [20]. Dietary n-3 PUFA in the neonatal period also has an influence on bone mass, BMC and related disease in adulthood [137]. This evidence implies the importance of controlling body composition at early stages of life. Therefore, the role of various PUFA in body composition should be clarified.

2.12.1 Animal studies

As in the sections reviewed previously, several animal studies provided the proof that adiposity can be reduced with n-3 PUFA consumption from a mechanistic perspective. Nonetheless, when it comes to how maternal n-3 PUFA supplementation affects infant body composition, there is not enough data to make a solid conclusion [15]. For experimental studies investigating this topic, the rat is a commonly used model. N-3 PUFA supplementation or placebo is provided through specific oils within a basic rodent diet, although the dosage of supplement varies considerably.

To mimic maternal-fetal-infant transfer, Korotkova et al. chose to provide the intervention group linseed oil (high n-3 diet) and the comparison group either sunflower oil (high n-6 diet) or soybean oil (n-6:n-3 diet) during last ten days of gestation and throughout lactation. Body weight and inguinal fat pad weight of pups were lower in the intervention group compared to the soybean and sunflower oil fed groups at 3 weeks of age. Adipocyte size was also lower in the intervention group compared to the soybean group [138]. Similarly, Massiera et al. supplied pregnant mice a diet rich in ALA, a standard diet or one made with corn oil. The offspring of the mice kept receiving the same diet as fed to their mothers, and the outcome measurements were done at 8 weeks of age. Similar to the previous study, the total FM, epididymal fat pad weight and adipocyte size of pups were lower in both the ALA group and the standard diet group [118]. As the authors did not explain the PUFA composition of the standard diet, it is not clear whether the decrease in FM results from greater intakes of n-3 PUFA or not.

In addition to altering the essential PUFA content and ratios, some researchers chose to add semi-purified EPA and DHA in n-3 PUFA diet. In order to examine the longer term effect of maternal n-3 PUFA supplementation, Wyrwoll et al. fed the rats and their offspring a n-3 PUFA enriched diet (high in EPA and DHA) or a standard diet, and measured the adiposity indicators until the pups were 6 months of age [139]. Comparing the outcomes between the n-3 PUFA group and the control group, the epididymal fat pad weight was reduced in the n-3 PUFA group. But surprisingly, there was no difference in FM assessed by DXA between the two groups. Ibrahim et al. mated the rats and supplied the dams and pups with a diet rich in LA or ALA or a mixture of LA, ALA and n-3 long chain PUFA (fish oil) respectively. At 105 days of age, they

found there was no effect of n-3 PUFA supplementation on adipocyte lipolysis, gene expression or glucose tolerance of the offspring [140]. Altogether, more robust animal data are needed to conclude whether there is an effect of maternal n-3 PUFA supplementation on FM in the offspring. In addition, the relationships among the various PUFAs and the ideal amounts in the diet are not clear as no dose-response studies regarding n-3 PUFA intake and alterations in body composition have been reported to the best of this author's knowledge.

2.12.2 Human adult studies

Several human studies have been conducted to investigate the association between fatty acid levels and body composition during the last two decades. However, the evidence about this topic is not clear, because different investigators conducted research on this topic adopting different measurement tools and have reported contradictory results.

PUFA status during the gestational period has an effect on mothers themselves. A longitudinal dietary intervention study during pregnancy found fish intake during the first and second trimester correlates to the increase of EPA and DHA, however, it did not make any difference to body composition. In contrast, meat intake in early pregnancy increases fat-free mass in the mothers [141].

As for the studies conduct in non-pregnant human adults, adults with BMI greater than 25 kg/m^2 and defined cardiovascular risk factors are reported to have less body fat after 12 weeks of fish oil consumption [142]. Some investigators researched the effect of PUFA in groups of people with metabolic illness. For example, Warner et al. investigated the effect of fish oil consumption with or without aerobic exercise on FM% of participants with hyperlipidemia. Since only the group with both exercise and fish oil consumption showed a decrease in FM%, it is unclear how much of this change is attributed to fish oil consumption or if it was due to synergistic interactions [143]. In another study, Izabella et al. studied the effect of fish oil supplements on females with type 2 diabetes by offering different doses of n-3 PUFA (1.5 g/d, 2.5 g/d, control) for 30 days. A significant body mass and waist circumference reduction was observed in the intervention group with the lower dosage (1.5 g/d fish oil) instead of the higher dose of n-3 PUFA (1.5 g/d fish oil) [144].

2.12.3 Human infant studies

Similarly, there are studies on the effect of PUFA status on infant body composition (Table 2.2). Some previous studies evaluated the effect of maternal PUFA status on infant growth. A randomized controlled trial that recruited 802 Mexican infants whose mothers had been supplemented 400 mg/d DHA or placebo during later pregnancy (from week 18-22 to delivery) found that only the crown-heel length of newborns of primigravid mothers was slightly greater than those in control group at 18 months of age [145]. However, there was no difference in height, weight, BMI and corresponding z scores between the intervention group and the control group when the children were 60 months old [146]. It is also important to note that the participants in this study are highly susceptible to suffer from other nutrient deficiencies, which may confound the study results. In addition, quality of growth was not reported.

The effect of PUFAs has also been studied in the context of multiple nutrient supplements (minerals, vitamins and probiotics) in fish oil supplements for pregnant women. Interestingly, infant BMI was lower in the n-3 PUFA supplement group than the control group at 1.7 years of age [147]. Besides the studies using BMI to assess infant body composition, the research group added BMI z-score and skin-fold thickness in a follow up study when the children turned 6 years of age. No difference between groups in adiposity measurements was observed, except the BMI z-score in the intervention group was significantly lower at 21 months (0.978 vs 1.030) but not at 6 years (1.033 vs 1.020), where their height was negatively correlated to the increase in maternal red blood cell DHA status (height z score was not explored). A delayed increase of BMI z-score compared with the WHO (World Health Organization) growth standards was also observed in the intervention group, which occurred after 21 months. This could be a sign of a favorable BMI pattern caused by DHA supplementation (200 mg/day) during pregnancy [148].

There are also several studies focusing on the effect of PUFA in breast milk on infant body composition. A randomized clinical trial with 208 pregnant women supplemented with 1200 mg n-3 PUFAs per day and guidance to reduce n-6 PUFA intake from the 15th week in pregnancy to 4 months of lactation versus following their regular diet successfully reduced the n-6:n-3 PUFA ratio. With the measurement of skinfold thickness (SFT) and ultrasonography, the investigators reported that the intervention of n-3 PUFA appears to have had no effect on FM of infants [149].

Table 2.2: Randomized clinical trials investigating relationship between n-3 PUFA and infant body composition

Authors and countries	Population	Intervention and control	Outcome (s)	Results
Ramakrishnan et al. **Gonzalez-Casanova, I., et al.** **[145, 146]** **Mexico** (n=1094)	Pregnant women	N-3 LCPUFA group: algal oil (400 mg DHA per day) Control group: olive oil (18-22 Wk GA to birth)	BMI z-score	No difference between the groups at birth and at 60 mo of age
Bergmann, R.L., et al. **[147, 148]** **German**y (n=144)	Pregnant/ lactating women	LCPUFA group: fish oil (200 mg DHA and 60 mg EPA per day) plus basic supplement containing vitamins and minerals along probiotics; Control group: basic supplement containing vitamins and minerals with or without probiotics (21st Wk GA to third mo of lactation)	1.7 yr: BMI 6 yr: BMI z-score, SFT	1.7 yr: covariate-adjusted BMI was lower in the n-3 LCPUFA group compared with the control group 6 yr: no difference between the groups in any adiposity measure
Much et al. **[147, 149]** Germany (n=208)	Pregnant/ lactating women	LCPUFA group: (1,200 mg n-3 LCPUFAs per day (1,020 mg DHA and 180 mg EPA) + instructions to normalize their AA intake to 90 mg/d) Control group: healthy diet according to the current guidelines in Germany (15th Wk GA to fourth mo of lactation)	SFT	No difference between the groups in SFT Breast milk n-3 LCPUFAs appear to stimulate fat mass growth over the first y of life,
Helland et al. **[150, 151]** Norway (n=590)	Pregnant/ lactating women	N-3 LCPUFA group: cod liver oil (1183 mg DHA and 803 mg EPA per day); Control group: corn oil (17-19th Wk GA to third mo of lactation)	BMI	No difference between the groups during first y of age
Lauritzen et al. **[152, 153]** Denmark (n=175)	Lactating women with low fish intake	LCPUFA group: fish oil (790 mg DHA and 620 mg EPA per day); Control group: olive oil; Reference group: high fish intake (first four mo of lactation)	BMI, WC, SFT	2.5 yr: adiposity measures were higher in the n-3 LCPUFA group compared with the control group 7 yr: no difference in any adiposity measure between the groups
Groh-Wargo et al. **[154]** Cleveland, Ohio (n=60)	Preterm infants	DHA + AA supplementation until 1 yr corrected age	Body weight, lean mass, and fat mass, BMC	Infants who were fed DHA+AA-supplemented formulas had significantly greater lean body mass and significantly less fat mass at 1 yr of age

LCPUFA: long chain PUFA, SFT: skin fold thickness, BMI: body mass index, WC: waist circumference, Wk: week, GA: gestation, mo: month, yr: year

However, breastmilk n-3 PUFA level is positively related to FM, whereas the n-6 PUFA level is inversely associated with weight, BMI and LM of infants at 4 months of age [155]. A subsequent paper reported the extended effect of the intervention at 1 year of age and concluded that the change in the n-6:n-3 PUFA ratio did not alter the FM of children in spite of the positive effect of PUFAs on birth weight and length. Unlike the negative association between n-3 PUFA and BMI observed in adult studies, an inverse association between n-6 PUFA and BMI was found [156]. It thus appears that developmental stage may dictate the response of tissue compartments and growth to PUFA intake. Whether this is related to LM or FM requires further studies.

Some studies failed to detect any differences in infant weight, length and body composition from lactating mothers who were supplemented with much higher amounts DHA (1183 mg) and EPA (803 mg) per day than those in other studies [150]. Additionally, no difference in BMI was reported among 7 year old children whose mothers took 10 mL of cod liver oil or corn oil during pregnancy [151]. Because corn oil has been suggested to alter lipoprotein metabolism and increase postprandial energy expenditure, a concern of this trial was that using corn oil as the control group might be inappropriate [157]. However, Lauritzen found that although the difference is not detectable in 9 month old infants, a significant increase of BMI (0.6 kg/m^2; $p= 0.022$) and waist circumference was observed at 2.5 years in children whose mothers received the fish oil (4.5 g/d) or olive oil during the first four months of lactation [152]. Surprisingly, no relation between n-3 PUFA supplementation and BMI, BMI z-score or skinfold thickness was found, when the children were 7 years old [153].

Some researchers studied fatty acid supplementation on infants after their birth rather than giving the intervention to the mother. A study involving sixty preterm infants supplemented with DHA and AA showed significant increases in lean body mass ($p< 0.05$) and a significant decrease of FM ($p< 0.05$) compared to the control group. No difference was observed in BMC and bone mineral density (BMD) [154].

2.12.4 Cohort studies

Besides clinical trials, cohort studies are worth reviewing and provide guidance about research directions for further studies. In the Copenhagen Prospective study on childhood asthma birth

cohort, the fatty acid profile of 281 mother's breast milk samples and the FM of their children at 6-9 years of age measured by DXA were collected. The significant inverse association between breast milk DHA and BMI from 2-7 years of age was obtained. However, the negative relation between DHA level and FM are only observed between 6-9 years of age. The effect of breast milk n-6:n-3 PUFA ratio on body composition at each time point was not found [110]. A similar result was obtained in the project Viva cohort. The maternal plasma EPA+DHA concentration was not associated with child adiposity at three years of age. The ratio of n-6:n-3 PUFAs was positively associated with SFT and risk of obesity which is determined as BMI $\geq 95^{th}$ percentile for age and sex [158], although BMI is not an accurate indicator of body composition. A most recent report from the Generation R study in Netherlands examined 4830 mothers' plasma PUFA concentration in the second trimester and the BMI and FM% of their children at 6 years of age using DXA. They found lower maternal n-3 PUFA and higher n-6 PUFA concentration is associated with higher total body FM% and abdominal fat in childhood [159]. This study lost 46% of participants before measurement of the children, which leads to possible attrition bias. The plasma PUFA levels were measured only once during pregnancy and might not truly reflect the long term PUFA content the fetus was exposed to during the prenatal period. Still, the large sample size and many confounding variables that they accounted for in the analysis made these observations convincing.

However, not all cohort studies observed the relation between maternal n-3 PUFA consumption and offspring body composition. A cohort study in Denmark assessed the relation of 965 pregnant women's n-3 fatty acid intake during the second trimester and the indicators of the risk factors of cardiovascular diseases among the 20-year-old offspring. There was no association between n-3 PUFA consumption during pregnancy and the BMI and waist circumference of the offspring, nor with other cardiometabolic risk factors including glucose metabolism, adipose tissue-derived hormones and lipid profile [160]. Similarly, another observational study from the United Kingdom was conducted in 293 women, measuring maternal plasma PUFA concentration at the 34^{th} gestational week and their offspring's body composition at the age of 4 years and 6 years by using DXA. Opposite to the Danish cohort's finding, a positive association between plasma n-6 fatty acid concentration of the mothers and FM but not LM at both 4 years and 6 years in the children was observed. However, the association between maternal n-3 PUFA

concentration and the FM of infants either at 4 years or 6 years was not detected. Besides, n-3 fatty acid concentration in mothers was correlated with higher offspring LM, however, was confounded by the positive relation between n-3 PUFA and offspring height. This observation can be a possible explanation of the limited changes in BMI after n-3 PUFA consumption from previous studies results [161]. It is worthy to note that, instead of only measuring the PUFA intake, maternal plasma PUFA concentration was obtained to determine the effectiveness of supplement absorption, which makes the observed direct relation between maternal PUFA concentration and offspring's body composition more reliable. Further studies also should consider how maternal PUFA status can be modified by how much PUFA is transferred to the fetus and the correlates of dietary n-6:n-3 ratio to adiposity measures.

2.12.5 Systematic reviews on results and methodology

Although there are not enough existing studies and robust evidence supporting the relationship between PUFA and body composition, some tentative systematic reviews have been conducted, providing extensive knowledge of existing studies and suggestions for future research. A review of trials looking at the effect of n-3 fatty acid supplementation on body weight change found four out of five trials in which adult participants have no change in body weight after supplementation. But two out of six trials reported an increase of birth weight and the rest showed no effects in terms of fatty acid supplementation during pregnancy [19]. It was not possible to examine body composition at birth, since no data was available.

As for the systematic review of PUFA supplementation during pregnancy and/or lactation on infant body composition, one paper reported on only 3 trials on pregnant or lactating women that met the inclusion criteria for further analysis, reporting positive, negative and neutral effects of n-3 PUFA on offspring FM in 2010. The supplement dose of PUFA varied between these three trials, ranging from 0.2 to 1.18 g DHA/d and 0.62 to 0.80 g EPA/d. The direct measurement of body composition using DXA was encouraged by the reviewers, as no trial used this technique before. Meanwhile, there is no evidence that a dose response is implicated in BMI from the three trials, as the lowest dose reduced the BMI of infants, the highest dose exerted no effect on BMI, while the intermediate dose increased the BMI [157]. This might result from the range they observed, which seems too narrow. Stratakis et al. analyzed the research results of recent clinical

trials examining the changes in adiposity in infants as a result of maternal PUFA supplementation. The review included six studies related to this topic. Similar to the review mentioned above, the paucity of data for body composition assessed using DXA measurements is implied. Improving on previous qualitative reviews, this review provides a comprehensive analysis of the study results from the six trials with well-established methods. Unfortunately, current information provides insufficient evidence to support that n-3 fatty acid supplementation during pregnancy and lactation has positive effects on child adiposity [20].

2.13 PUFA and infant bone health

The majority of research studies to date have examined the association between PUFA level and body composition by simply focusing on the effects on adiposity without reporting the changes in BMC. Still, there are studies looking at the effect of PUFA on infant bone health. Rats with n-3 PUFA deficiency are highly likely to have osteoporosis. But with the treatment of adequate n-3 PUFA after 49 days of low n-3 PUFA intake from birth, the ratio of n-6:n-3 PUFA in bone compartments can be restored and the bone loss in n-3 deficient rats can be reversed [162]. Similarly, a combination of AA and EPA or DHA increases bone mass in infant animals, including piglets [163] and chicks [164]. Additionally, Yin et al. reported a sex difference in the effect of n-3 fatty acid on bone mass in Guinea pig pups. They found the effect of either maternal AA + DHA or maternal DHA supplementation on bone outcomes is positive in female pups, but negative in male pups [165].

Most human studies have focused on the potential effect of n-3 PUFA on seniors who are vulnerable to osteoporosis. An observational study in Japan reported the positive relation between fish and shell fish consumption from dietary patterns of Japanese premenopausal women and their BMD [166]. Another study conducted by Amy et al., which is known as the first controlled feeding study in humans investigating the effect of plant-derived n-3 PUFA on bone turnover, indicated the protective effect of n-3 PUFA on bone health. This effect might be due to decreased bone resorption according to the evidence that the bone turnover marker serum N-telopeptides decreased significantly with a high ALA diet [167]. In a review on this topic, most human data from observational studies on elderly people indicate a positive association between n-3 PUFA and femoral BMD, although a number of trials failed to observe a significant

improvement in BMD of participants who received n-3 PUFA supplementation [168]. Furthermore, Eriksson et al. examined the relationship with serum phospholipid PUFA and BMD in 85 healthy 8-year-old children. No relation was found between BMD and EPA, DHA. However, AA was positively associated with whole body BMD, while LA was negatively correlated with whole body BMD and lumbar spine BMD [169].

In terms of the effect of maternal fatty acids status on offspring's bone health, a study recruited 727 mother and child pairs followed by maternal fatty acid measurement in plasma phosphatidycholine (PC) at 34-week gestation and offspring BMD assessment. A positive association between maternal EPA and whole body BMD as well as lumbar spine BMD were observed in the 4 year old offspring [170]. Another study that measured thirty mothers' RBC as well as cord red blood cell PUFA concentration and the BMC of their infants reported that both maternal RBC and cord RBC AA were positively related to whole body BMC in newborns. Interestingly, maternal RBC DHA is inversely associated with lumbar spine and femur BMC. The paper underlined the importance of n-6 and n-3 PUFA balance in maintaining and supporting bone mass accretion in infants [171].

2.14 Interaction between PUFA and vitamin D

The philosophy of nutrition has moved away from considering single nutrients and to considering nutrient-nutrient interactions. In the area of PUFA and body composition, vitamin D has been suggested as a potential factor to consider. Some of the evidence for this comes from adult literature where low vitamin D status is hypothesized to cause excess parathyroid hormone (PTH) secretion and calcium influx into adipocytes, which leads to weight gain [172]. 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), a product of 25-hydroxyvitamin D (25(OH)D), which is related to elevated serum PTH, might inhibit adipogenesis through several molecular mechanisms [173]. Particularly in adipose tissue, $1,25(OH)_2D$ also regulates the expression of leptin, a satiety hormone, which balances energy storage and expenditure [174, 175]. Hence, the interaction between these two nutrients might affect the actual result of PUFA or vitamin D status in infant body composition, although researchers have not clarified the effect of this interaction yet.

First, there are some studies the indicate the interference in nutrient absorption and activation between PUFAs and vitamin D. Niramitmahapanya et al. analyzed the relationship between types of dietary fat and plasma 25(OH)D in participants with vitamin D supplementation. They found the changes of plasma 25(OH)D in response to 2 years of vitamin D supplementation is positively associated with MUFA, but negatively associated with PUFA intake assessed by a food frequency questionnaire [176]. This finding suggests that PUFA might reduce the effect of vitamin D on body composition. The result of another study did not agree with this finding. Healthy women or men (n=50) were supplied with a single dose of vitamin D supplement along with a meal containing no fat or 30 % of energy as fat with a high (4:1) or low (1:4) MUFA/PUFA ratio. The mean peak plasma vitamin D level was higher in those consuming the fat containing meal compared with those who had the fat free meal. The vitamin D absorption was not affected by the ratio of MUFA/PUFA [177]. However, these studies did not investigate the types of PUFA that affect vitamin D absorption. Whereas specifically considering the interference between n-3 PUFA and vitamin D, Itariu et al. found n-3 PUFA supplementation did not change the vitamin D status in obese patients. But the inverse association between vitamin D concentration and inflammatory biomarkers (IL-6 and CRP) was lost after n-3 PUFA treatment [178]. Moreover, 25(OH)D can be activated to ($1,25(OH)_2D$) by the enzyme 1α-hydroxylase in the kidney. Meanwhile, n-3 PUFA have anti-inflammatory effect and might regulate enzyme activities in uremic condition. A study of a combination of n-3 PUFA and vitamin D supplement in hemodialysis patients showed a tendency for increases in serum $1,25(OH)_2D$ concentration among those receiving the combined intervention compared with those had vitamin D supplementation alone [179].

There is not much information about the influence of such nutrient interaction on body composition. It is reported that infants with higher vitamin D status had higher LM and lower FM which is evident at 1 y of age [180]. In older female mice, low vitamin D exacerbated the adiposity result from a high fat diet. But vitamin D supplementation did not relieve the high dietary fat induced adiposity [181]. Indeed, further research is needed to elucidate whether the types of fatty acid in the diet can alter the level of adiposity.

Still, the mechanistic roles of n-3 PUFA and vitamin D suggest their combined effects on body composition. Vitamin D is the precursor of calcitriol, a steroid hormone. It can bind to vitamin D

nuclear hormone receptor (VDR) to regulate target gene expression [182]. Excessive expression of VDR in mouse adipocytes suppress fatty acid β-oxidation and lipolysis [183]. Whereas the physiological result of this effect on adipose tissue has not been clarified. Besides, inflammation has been realized as a significant contributor to obesity-associated complications [66]. It is previously reviewed that n-3 PUFA can reduce inflammatory biomarkers. Similarly, vitamin D is negatively associated with BMI and inflammatory biomarkers. It is reasonable to assume that n-3 PUFA and vitamin D have same tendency of effect on inflammation in adipose tissue. Some data also suggests the anti-inflammatory effect of n-3 PUFA still works even in vitamin D deficiency [178]. Besides, $1,25(OH)_2D$ also limits differentiation of preadipocyte cells *in vitro* by suppressing C/EBPα and PPARγ up-regulation. Likewise, n-3 PUFA down-regulate these two genes [173]. Moreover, vitamin D and n-3 PUFA have common effects on energy metabolism through enhancing uncoupling proteins [184], up-regulating β-oxidation [185] and muscle promotion and maintenance [186, 187]. Further studies regarding how these similar effects from vitamin D and n-3 PUFA cooperate with each other would be worthwhile.

2.15 Rationale for the present study

Excessive FM deposition should be controlled at an early stage of life, since individual FM and fat distribution may have adverse effects on health and increase the risk of serious diseases during later life. It is advocated for future investigators to conduct studies with a focus on these early age groups for preventing them from developing childhood obesity [8]. Clearly, the relationship between n-3 PUFA status and infant body composition is understudied. Some researchers proposed that intervention studies are needed to determine the effect of n-3 PUFAs on infant body composition. The inconsistent results from previous studies probably are due to the different measurement tools used to assess body composition and various patterns of n-3 PUFAs supplementation that different researchers adopted. Therefore, observational studies investigating association between n-3 PUFAs level and body composition by using DXA could contribute to the body of knowledge. After that, the findings from observational studies could be confirmed by subsequent intervention trials. Besides, dose-response studies should be explored in order to set a target n-3 PUFA level for further intervention studies. The role of the n-6:n-3 ratio in infant body composition should be examined as well. Furthermore, previous studies examine the PUFAs level by measuring maternal PUFA intake and the PUFA concentration in

maternal plasma or breast milk. However, maternal PUFA status may not fully reflect the actual infant PUFA status. Studying the PUFA biomarkers in infant blood and their relationships to infant body composition could provide more direct information on the role of n-3 PUFA in infant adiposity.

3. Manuscript

The relationship between long chain polyunsaturated fatty acids and body composition in infants

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3.1 Abstract

Background: It has been suggested that maternal n-3 polyunsaturated fatty acid (PUFA) status plays a role in altering infant body composition. However, the infant's own n-3 PUFA status may better relate to body composition.

Objective: The present study aimed to test whether there is a relationship between newborn n-3 PUFA status and infant body composition.

Methods: Healthy mother-infant pairs (n=70) from the Greater Montreal region were studied within 1 month post-partum. Infant red blood cells (RBC) were directly methylated to measure PUFA profiles using gas chromatography and expressed as % of total fatty acids (FA). Infants were ranked according to RBC PUFA using tertiles of docosahexaenoic acid (DHA, 22:6 n-3), arachidonic acid (AA, 20:4 n-6), ∑n-3 PUFA, ∑n-6 PUFA and the ratio of n-6 PUFA: n-3 PUFA (n-6:n-3 ratio). Infant body composition was measured using dual-energy X-ray absorptiometry (DXA). The difference in body composition variables between the highest and lowest tertiles was tested using MIXED model ANOVA. Correlation analysis and multiple regression models were conducted to examine the relationship between the PUFA percentage as well as n-6:n-3 ratio and infant body composition.

Results: All infants (n=40 male; n=30 female) were of healthy weights and lengths for age. On average, infants had 5.4±0.7% DHA, 14.7±1.3% AA, 6.4±0.9% ∑n-3 PUFA, 29.4±1.5% ∑n-6 PUFA of total FA and a n-6:n-3 ratio of 4.7±0.7 in RBC membranes. Infant RBC DHA and AA percentages were negatively related to percentage of body fat (R^2=0.26, p= 0.03 and R^2=0.27, p= 0.02 respectively) in regression models, after adjusting for family income, maternal education, pregnancy weight gain, race, infant postnatal age, infant serum 25-hydroxyvitamin D concentration and sex. RBC AA was also positively associated with lean mass (g/kg) (R^2=0.31, p= 0.01). Infants in the highest tertile of the n-6:n-3 ratio had higher percentage of body fat compared to those in lowest tertile (15.9±2.5% vs 13.3±2.6%, p= 0.04).

Conclusion: This observational study suggests that neonatal PUFA status might help to explain the variability in infant body composition during early infancy. Balancing the ratio of n-6 PUFA and n-3 PUFA or providing longer-chain PUFA such as DHA and AA could be explored as a strategy to prevent excessive fat accretion in infancy.

3.2 Introduction

The global epidemic of obesity is a major public health issue with high relevance to morbidity and mortality [2, 5]. It is now known that obesity in childhood increases the risk of cardiovascular diseases and the corresponding rate of death as an adult [188]. Findings from many studies have shown that the fetal and infantile nutritional environment influence body composition in childhood as well as adulthood [189]. In particular, the relationship between breastfeeding and childhood quality of growth has been reviewed, revealing that the duration of breastfeeding and breast milk quality are important factors that contribute to optimal infant growth and development [190]. Moreover, a recent meta-analysis concluded that both exclusivity and duration of breastfeeding is a protective factor against childhood obesity [191].

Despite the confounding factors that may affect the relationship between breastfeeding and lower risk of obesity, the micronutrients and macronutrients in breast milk are considered as a mechanistic explanation that supports healthy growth and regulates patterns of infant weight gain [122, 191-193]. Among these nutrients, polyunsaturated fatty acids (PUFAs), especially the subgroup n-3 PUFAs, are essential for early infant development. Studies have shown that breastfeeding is associated with higher n-3 PUFA concentration, which benefits infant immune function, visual acuity, cognitive and motor function [13].

In addition, dietary lipid composition during early life significantly affects tissue differentiation, organ function and body composition [194]. Several lines of evidence have suggested that n-3 PUFA plays a critical role in altering infant body composition. Data from animal studies suggests that an increased n-6:n-3 ratio in infant formula may promote adipocyte differentiation, growth and inflammation [99]. Furthermore, inflammation can independently affect the development of obesity during early life, and is also strongly linked to obesity-related diseases, such as diabetes, heart disease in adulthood [195]. Offspring born to rats fed a n-3 PUFA enriched diet during pregnancy had either lower whole body fat mass or epididymal fat pad weight compared to a control diet from 8 week to 6 months of age [118, 139, 196]. However, in a similar study using n-3 PUFA supplementation on mated female rats, these results in the male offspring were not confirmed [140]. The contradictory evidence highlights the necessity of

further research on the relationship between maternal n-3 PUFA transfer to the fetus and subsequent body composition of the infant.

A few observational studies analyzed the relationship between fetal/infant n-3 PUFA exposure during pregnancy or lactation and their postnatal body composition up to 7 years of age [110, 158-161]. Body composition was measured using a variety of techniques including body mass index (BMI), skin fold thickness and dual-energy X-ray absorptiometry (DXA). Only a few studies reported an inverse association between maternal n-3 PUFA status and infant adiposity indicators from 2-7 years of age [110, 158, 159]. For trials in pregnant or lactating women, the amount of DHA tested varied from 400 to 1200 mg per day. The results of these studies are controversial, reporting no difference, increased or decreased adiposity in infants born to the mothers supplied with n-3 PUFA compared with the respective control group when the infants were 6 months to 7 years of age [146, 148, 152, 154]. Only the studies from Lauritzen et al. and Groh-Wargo et al. suggest higher maternal n-3 PUFA status is related to lower fat in offspring [152, 154]. Altogether, the evidence for the effect of n-3 PUFA on infant adiposity remains unclear.

During pregnancy and early infancy, maternal PUFA supply is an important determinant of infant blood PUFA profile and the only source of n-3 and n-6 PUFA for infant growth [197]. Although maternal dietary intake of PUFA, or PUFA concentration in milk and maternal plasma were chosen in previous studies as an indicator of infant PUFA exposure, a biomarker in infant blood could better reflect infant PUFA status. However, few studies have looked at the association between the PUFA profiles in red blood cell (RBC) membranes of infants in relation to body composition assessments. Therefore, we conducted an observational study with 70 mothers and their newborn infants to investigate the association between maternal or newborn RBC n-3 PUFA proportions and infant body composition using a 3-compartment model. We also examined whether variability in maternal demographic factors altered these associations.

3.3 Participants and methods

3.3.1 Study population:

The data and samples used in this analysis were obtained at the baseline visit of an ongoing trial. Participants were mothers and their neonates recruited from the Lakeshore General Hospital located in the Greater Montreal area as part of a study regarding vitamin D status (NCT02563015). All screened infants were healthy and term-born with an appropriate weight for their gestational age and born to a healthy mother. The eligible participants recruited in the trial were born to mothers not taking medications known to affect vitamin D metabolism and infant health and who intended to breastfeed for at least 3 months. Infant and maternal serum 25-hydroxyvitamin D (25(OH)D) were measured on a dedicated auto-analyzer (Liaison Diasorin Inc.). Infants, who had low vitamin D status (<50 nmol/L of 25(OH)D) were not excluded on the basis of maternal pregnancy BMI, but relevant weight gain and obstetrical history were recorded. While the rest of the infants with higher vitamin D status were recruited only if their mothers had healthy weights (pre-pregnancy BMI 18.5-24.9 kg/m^2) and weight gain. Exclusion criteria included maternal smoking in pregnancy, diabetes, preeclampsia, celiac disease, inflammatory bowel disease and use of medications that impact vitamin D/mineral metabolism.

3.3.2 Postnatal Assessments:

Eligible mother and infant pairs were contacted and scheduled for a family visit at the McGill University Mary Emily Clinical Nutrition Research Unit between April 2016 and May 2017. During the baseline visit, mothers provided written informed consent and completed a series of questionnaires including: supplement use, breastfeeding, general health and sun exposure; 24-hour recall of their food intake, as well as, a food frequency questionnaire regarding mothers' usual intake during pregnancy. Anthropometric measurements of mothers including height and weight were taken. For infants, their weight, length, head and arm circumferences were recorded. DXA scans were performed of the lumbar spine (LS1-4) and whole body. Information on pregnancy history and infant birth data were obtained from the hospital medical records.

3.3.3.1 Infant body composition assessment:

Infant weight was measured using an electronic scale with a dynamic weighing program (Mettler-Toledo Inc., Switzerland). Length (0.1 cm) was measured using an infantometer (O'Learly Length Boards, Ellard Instrumentation Ltd., US). Head circumference was measured (0.1 cm) using a non-stretchable tape (Perspective Enterprises, US). Weight-for-age, height-for-age, and BMI-for-age Z-sores were calculated using WHO growth standards and software (WHO AnthroPlus, Switzerland).

A fan-beam DXA (APEX version 13.3:3, Hologic 4500A Discovery Series, Bedford, MA) was used to assess infant body composition (LS1-4 and whole body). Infants were scanned wearing only a diaper and a light sleeper with no metal or plastic components using infant whole body software. The whole body scan provides lean mass (LM; g and g/kg), fat mass (FM; g and %), and bone mineral content (BMC; g). The lumbar spine (LS1-4) scan provided for BMC and bone mineral density (BMD; g/cm^2).

3.3.3.2 Dietary assessment:

Mothers completed a 24-hour dietary intake assessment with a dietitian. The dietitian recorded the types, brands, quantity of food that mothers consumed and the time of consumption to provide for nutrient intake assessments. Mothers were also asked to complete a semi-quantitative food frequency questionnaire (FFQ) [198] which has been validated for use in pregnant women in English and French. In the FFQ, the frequency and portion of the intake of 147 food items during pregnancy and corresponding daily portion size were recorded [199]. The 24-hour dietary intakes were analyzed using the computer software program Nutritionist Pro™ (Axxya Systems LLC, Stafford, TX, US) and the Canadian Nutrient File 2010b. FFQs were entered in an Excel template and analyzed for nutrient intakes according to the on-line version of Canadian Nutrient File 2015. The 24-hour dietary intake assessment and FFQ enabled further assessments of n-3 PUFA-rich food, including fish, fish oil and seafood intake.

3.3.4 RBC PUFA analysis

Non-fasting capillary blood (1 ml) was collected via heel lance from infants and 5 ml venous blood was collected from mothers. Maternal and infant blood samples were separated into plasma and cell fractions using a refrigerated centrifuge (6°C) at 4000X*g* for 20 minutes. The plasma and buffy coat layers were removed and the remaining RBC was then washed twice by adding an equal volume of isotonic saline into the tube, mixed by inversion and centrifuged for 5 minutes at 1500X*g*. After removing the saline following the second wash, an equal volume of saline with 0.01% butylated hydroxytoluene (BHT) (wt/vol) was added in the tube and inverted several times to resuspend the RBC. The RBC sample was then transferred to a clean tube and flushed with nitrogen gas to prevent oxidation. All RBC samples were stored at -80°C before proceeding to fatty acid analysis.

The lipid extraction method was a modified method of RBC direct methylation [200]. Prior to methylation, an internal standard mixture was prepared by adding 4 ml of C21:0 (1 mg/ml) in toluene, 8 ml of C19:1 (1 mg/ml) in hexane with 0.005% BHT and 188 ml of hexane + 0.005% BHT(wt/vol) in a clean glass bottle. A reference mixture was made at a concentration of 10 µg/ml methyl nonadecanoate C19:0 in hexane +0.005% BHT (wt/vol). Once the sample had thawed, 125 µl RBC was combined with the internal standard mixture (0.5 ml), methanol (3 ml) and acetyl chloride (0.3 ml) for fatty acid methyl esters (FAME) preparation, followed by one hour 70°C incubation. After cooling to room temperature, 3 ml sodium chloride and 1 ml reference standard mixture were added. The tube was vortexed for 5 seconds and centrifuged at 1500X*g* for 5 minutes. Then the top hexane layer was transferred to a 2 ml gas chromatograph (GC) vial. Another 0.5 ml hexane was added to the remaining mixture, vortexed and centrifuged again at 1500X*g* for 5 minutes. After the hexane layer was added, the sample was concentrated under nitrogen and transferred to a GC vial insert for subsequent GC analysis.

FAMEs were separated using a silica column (Varian Factor Four vf23ms, 60 meter by 0.25 mm capillary column, Varian-Chrompack, HP-88 112-8867), fitted in a Varian CP-3800 GC (Varian, Inc., Walnut Creek, CA, USA) with a flame-ionization detector. Each sample had 0.5 µl injected at a split ratio of 1:4. Hydrogen was used as the carrier gas and nitrogen was the make-up gas. The injector port temperature and the detector temperature was 270°C and 280°C, respectively.

The oven temperature started at 60°C for 1 min and then increased to 120°C at 20°C/min, from 121-180°C at 10°C/min, 181-200°C at 2°C/min and 221-220°C 5°C/min. The standard 461 (cat # GLC-461, Nu-Chek Prep, Inc. Elysian, MN) was used to identify individual PUFA peaks. Three pooled samples were methylated along with infant and maternal samples in each methylation batch. Coefficients of variation (CV%) of the percentage of total fatty acids for each PUFA peak from the three pooled samples were calculated. The CV% for the AA, eicosapentaenoic acid (EPA) and DHA in pooled samples are 4.2%, 6.9% and 9.8% respectively. Intra-essay CV% for the AA, EPA and DHA in pooled samples are 3.1%, 4.3% and 4.7%. Recovery was calculated according to the peak area of the internal standard (C19:1, C21:0) and the reference standard (C19:0), which was averagely 127.6% and 101% for C19:1 and C21:0 respectively in infant samples. Infant and maternal RBC PUFA were expressed as a % of total fatty acids.

3.3.5 Statistical analysis:

All data analyses were processed using the statistical software SAS (University Edition, SAS Institute Inc., Cary, North Carolina 27513, USA.). Normality and homogeneity were tested using the Shapiro-Wilk normality test and Levene's test respectively. For data that were not normally distributed (i.e., maternal PUFA intakes during pregnancy), Spearman correlation coefficients were calculated to examine their relationships to infant RBC PUFA% as well as body composition variables. Additionally, correlations between maternal RBC PUFAs and infant PUFA% were evaluated using Pearson tests. Mother's DHA was transformed using square root before correlation analysis. Spearman correlations were calculated for mother's ∑n-3 PUFA and ∑n-6 PUFA, since they were not normally distributed even after transformation. Tests were conducted with and without outliers, defined as greater than 3 standard deviation from the mean.

Infants were ranked according to RBC PUFA using tertiles, including DHA, AA, ∑n-3 PUFA, ∑n-6 PUFA and the ratio of n-6 PUFA: n-3 PUFA (n-6:n-3 ratio). Differences between the highest and lowest tertile were tested using MIXED model ANOVA. Random variables explored in the model included: maternal age at delivery, family income, maternal education, pre-pregnancy BMI, pregnancy weight gain, race, infant actual age infant serum 25(OH)D concentration, and sex. To determine the relationship between fatty acid proportions (%) in maternal or infant RBCs and infant body composition, Pearson correlation coefficients were

calculated in addition to multiple linear regression model (F-test, analysis of covariance) after confirming the normality of the data set. A p value < 0.05 was considered as statistically significant. Bonferroni adjustment of the p value for multiple comparisons was applied. The analysis was conducted for ∑n-3 PUFA, ∑n-6 PUFA, DHA, AA and n-6:n-3 ratio. The omega-3 index (DHA+EPA) was not included in this report, as the infants had very low EPA (mostly around 1-2%) that did not impact the interpretation of the results;.

3.4 Results

3.4.1 Participant characteristics

The general characteristics of mothers and infants are summarized in Table 3.1. In brief, for the 70 infants included in this study, the mean age of the mothers at the time of delivery was 31.6±4.8 y, 84% of mothers completed a college or university program, and 57% identified as white. Pre-pregnancy BMI was 24.5±5.0 kg/m^2 according to self-report, 57% of the infants came from families with annual household incomes greater than $70,000 Canadian.

The infants in this study (57% male) were born at 39.7±1.0 wk gestational age with a birth weight of 3.4±0.4 kg and weight for age and sex z-score of 0.17±0.83. At the postnatal assessment, infants were 0.65±0.18 mo of age, had a weight for age and sex z-score of -0.16±0.74, length z-score of -0.04±0.90 and BMI z-score of -0.20±0.76. There were no significant differences between sexes for whole body BMC, LS1-4 BMD, FM and LM (Table 3.2). Maternal and infant fatty acid profiles are presented in Supplemental table 3.1.

3.4.2 The relationships between maternal PUFA intake and infant PUFA and body composition

In sum, 58 maternal FFQs were obtained, and showed that maternal α-linolenic acid (ALA) and n-6 PUFA intake (Supplemental table 3.2) was inversely related to offspring RBC DHA, ∑n-3 PUFA, and positively related to infant n-6:n-3 ratio ($p< 0.05$). There was also an inverse association between maternal linoleic acid (LA) intake and infant DHA ($p= 0.02$). Moreover, maternal ALA intake positively associated with FM% ($p= 0.04$). Maternal ∑n-3 PUFA intake was not significantly related with infant PUFA proportions in RBC, but inversely related with LS

BMC (p= 0.04) and positively related with FM% (p= 0.047). There were no other significant relationships of maternal PUFA intake to infant AA, DHA, ∑n-3 PUFA, ∑n-6 PUFA, n-6:n-3 ratio in RBC (Supplemental table 3.2) nor to infant body composition variables (Supplemental table 3.3).

3.4.3 The relation between maternal and infant RBC PUFA

Maternal RBC DHA was positively correlated with infant RBC DHA and ∑n-3 PUFA; and inversely correlated with infant ∑n-6 PUFA and n-6:n-3 ratio ($p< 0.05$). Similarly, maternal ∑n-3 PUFA was positively correlated with infant DHA and ∑n-3 PUFA; and inversely correlated with infant ∑n-6 PUFA and n-6:n-3 ratio ($p< 0.05$) (Supplemental figure 3.1). In contrast, mother's ∑n-6 PUFA negatively associated with infant DHA and ∑n-3 PUFA; and positively associated with infant ∑n-6 PUFA and n-6:n-3 ratio ($p< 0.05$). Likewise, maternal n-6:n-3 ratio inversely associated with infant DHA and ∑n-3 PUFA; and positively associated with infant ∑n-6 PUFA and n-6:n-3 ratio (Supplemental figure 3.4). However, no significant associations between maternal AA and infant PUFA groups were observed (Table 3.3).

3.4.4 The relation between infant body composition and infant RBC PUFA

Maternal n-3 and n-6 PUFAs were not related to any of the parameters of infant growth or body composition (Supplemental table 3.3). Infant RBC DHA positively associated with LM relative to total body weight (g/kg) ($r=0.25$, $p= 0.04$); inversely correlated with FM ($r = -0.26$, $p = -0.03$) and FM% ($r= -0.30$, $p= 0.01$) (Figure 3.1). Similarly, ∑n-3 PUFA was inversely associated with FM% ($r= -0.26$, $p= 0.03$) and there was a positive association between n-6:n-3 ratio and FM% ($r= 0.25$, $p= 0.04$) (Figure 3.2). Infant RBC AA was positively associated with LM% ($r= 0.37$, $p= 0.002$); inversely associated with FM ($r= -0.32$, $p= 0.008$) and FM% ($r= -0.31$, $p= 0.009$). No other relationships between infant PUFA and body composition variables were observed (Supplemental table 3.5). There were no significant outliers that affected the observed relationships.

In the regression model adjusting for infant postnatal age and pregnancy weight gain, infant DHA was observed as a significant contributor to lower whole body BMC ($p= 0.05$), lower BMC/weight ($p= 0.028$), lower FM ($p= 0.04$) and lower FM% ($p= 0.03$) (Table 3.4). Infant

serum 25(OH)D concentration and sex, maternal demographic variables did not significantly contribute to the models. After adjusting for infant postnatal age and maternal pregnancy weight gain, higher AA remained associated with lower BMI (p= 0.04), lower FM% (p= 0.03), and higher LM (g/kg) (p= 0.01) (Table 3.5). ∑n-3 PUFAs was associated with lower BMC/weight (Supplemental table 3.6). ∑n-6 PUFAs was not related to infant body composition variables (Supplemental table 3.7). Potential confounding factors including family income, infant serum 25(OH)D concentration, gestational age and sex, maternal education, race, and maternal pre-pregnancy BMI were not significant as covariates in these models. The observed associations remained significant after removing non-significant variables from the regression model.

In mixed model ANOVA analyses, infants in the highest tertile of DHA (p= 0.04) and ∑n-3 PUFA (p= 0.03) had lower FM% than those in the lowest tertile. Infants in the highest tertile of ∑n-3 PUFA tended to have higher LM (g/kg) than those in lowest tertile (p= 0.0505). Infants in the highest tertile of AA had higher LM (g/kg) (p= 0.02) and lower FM% (p= 0.04) than infants in the lowest tertile. For infants in highest tertile of n-6:n-3 ratio, a higher FM% and a tendency of lower LM(g/kg) was observed compared to those in the lowest tertile (p= 0.04, 0.06 respectively) (Table 3.6). No difference was observed between the highest and lowest tertiles for any of the bone measurements (Supplemental table 3.8). The random effects included maternal demographic variables, infant serum 25(OH)D concentration, infant actual age and sex. Including maternal pre-pregnancy BMI did not significantly improve the model.

3.5 Discussion

This study tested for novel relationships between newborn infant RBC PUFA profile and body composition, which have not been well documented in the literature. The first important finding of this study is that infant DHA and AA were inversely associated with FM%, even after adjusting for family demographics, maternal and infantile characteristics. Conversely, a higher n-6:n-3 ratio was associated with higher FM%. Lastly, it is observed that infant DHA and AA were positively related to infant LM (g/kg), while ∑n-3 PUFA and ∑n-6 PUFA are not related to infant LM (g/kg). Interestingly, maternal PUFAs were not related to any of the infant body composition parameters, attesting to the importance of measuring PUFA status of the infant.

3.5.1 Interpretation of findings

Early weight gain and changes in body composition during infancy are affected by nutrient intakes and can be important factors in the development of adiposity in later life. Previously, researchers have investigated the relationships between maternal PUFA status and offspring body composition. However, there are very few studies that explored the association between infant PUFA status and body composition. In the present study, only maternal n-3 PUFA and ALA intake during pregnancy positively related with infant FM%, which was opposite to the inverse relationship between infant DHA and FM%. This is within reason, since the maternal intake could not accurately reflect the endogenous synthesis and metabolism of DHA, which is promoted by estrogen and insulin during pregnancy and affects infant n-3 PUFA status [201]. Moreover, relationships between maternal RBC n-3 PUFA or n-6 PUFA and infant body composition were not found. This is reasonable since maternal RBC PUFA after delivery may not sufficiently be reflected in offspring PUFA status [202].

Maternal lower n-6 PUFA and higher n-3 PUFA concentration in plasma during pregnancy is observed to be associated with lower childhood BMI at 2 years of age or later [158, 203]. Less information is available for these relationships during infancy. Low plasma n-3 PUFA in cord blood was reported to be related to higher skinfold thickness at birth and positive changes of infant BMI z-score during the first 6 months of life [204]. A study in 208 pregnant women in Germany showed that higher maternal plasma AA percentage were associated with lower BMI at

1 year of age (BMI z-score was not accounted for) [149]. Another observational study in The Netherlands reported that n-3 and n-6 PUFA (% wt/wt) in breastmilk did not significantly relate to infant BMI during first year of life [205]. Although there was no significant association between maternal PUFA status and BMI in the present study in newborn infants, we observed an inverse association between infant AA and their BMI, but not with BMI z-score, which is partially in line with the finding of the study in Germany by Much et al. [149].

Although BMI is strongly correlated with body composition variables, it is not an appropriate measurement of infant body composition and provides limited information regarding quantity and distribution of each body composition compartment [104, 206, 207]. Nevertheless, detailed body composition components were assessed in only a few studies examining the relationship between maternal PUFA supply and offspring body composition. A US pregnancy cohort of 1250 mothers and their children showed that higher DHA and EPA in both maternal diets and cord blood were associated with lower subcutaneous FM, which was expressed as the sum of the children's subscapular and triceps skinfold thicknesses at 3 years of age [158]. Another cohort study, the Southampton Women's Survey (SWS), in the United Kingdom included 293 mother-child pairs studied for maternal plasma PUFA concentration assessment and offspring body composition measurement using DXA. An positive association between n-6:n-3 ratio and infant FM was reported at age of 4 years, but not at age of 6 years [161]. Findings from the present study showed no relationship between infant body composition variables and maternal RBC n-3 or n-6 PUFA. However, we observed that higher infant DHA and lower n-6:n-3 ratio were related to lower FM%, which is consistent with previous findings.

A positive association between maternal n-6 PUFA status and offspring FM was also reported in the US pregnancy cohort and SWS study [158, 161]. Additionally, a Dutch prospective cohort study also reported higher maternal plasma n-6 PUFA concentration in the second trimester is associated with a higher FM% in the children assessed by DXA at the age of 6 years [159]. However, in our study with its cross-sectional nature, infant AA but not n-6 PUFA was observed as a significant contributor to lower FM%, whereas the n-6:n-3 ratio was positively related with FM%. There could be a few explanations for these inconsistencies. Firstly, the positive association between maternal n-6 PUFA status and infant fat mass reported in previous studies were observed after 3 years of age, which makes it difficult to compare it to the corresponding

relationship we observed in newborns. The relationship between infant n-6 PUFA or particularly AA and infant adiposity might differ between early and later postnatal life. Secondly, infant RBC AA concentration may not be comparable with the maternal AA concentration during pregnancy. Also, the fatty acid profiles of the participants differ between studies, the mean percentage of infant ∑n-6 PUFA and ∑n-3 PUFA were 29.4% and 6.4% respectively in this study, and lower than those values in maternal plasma and cord blood from the Dutch and the US pregnancy cohort respectively.

The observed inverse relationship between DHA and FM% is supported by the previous findings in animal studies from mechanistic perspectives. Researchers found that n-3 PUFAs inhibit preadipocyte differentiation and maturation by suppressing cyclooxygenase-dependent prostaglandin synthesis [208]. Interestingly, n-3 PUFA supplementation reduced fat deposition through activating peroxisome proliferator-activated receptor (PPAR), stimulating the expression of mitochondrial uncoupling proteins [16], increasing fatty acid oxidation [123] and lipoprotein lipase activity [209]. Conversely, n-6 PUFAs promote prostacyclin synthesis and stimulate preadipocyte differentiation and maturation [208]. But we observed an inverse association between AA and FM%. Hence, the positive association between n-6:n-3 ratio and FM% could reflect the beneficial effect of n-3 PUFA on infant adiposity. This also suggests that the equilibrium between n-6 PUFA and n-3 PUFA could be a more effective contributor in regulating infant FM development. Moreover, this may also explain why previous clinical trials reported contradictory results. Many trials did not control for or investigate infant AA status. Hence, the infant n-6:n-3 ratios based on the essential fatty acids alone may not be the best biomarker to test for relationships to body composition. Comprehensively examining the PUFA profile and n-6:n-3 ratio in infant blood after n-3 PUFA supplementation in pregnant women and their relationship to offspring body composition might be helpful to provide more evidence to confirm our findings.

The observed inverse relationship between infant DHA and BMC/weight in the present study was supported by a few previous studies, reporting that higher maternal n-3 PUFA intake is associated with reduced femur BMC and whole body BMC of offspring in rats and human infants respectively [171, 210]. However, the mechanistic explanation of this association is not certain, since there is not enough data confirming linking this relationship to bone formation and

resorption biomarkers, such as procollagen type 1 N-terminal propeptide, urinary pyridinolines, deoxipyridinolines, etc [211]. These relationships could be examined in future studies.

3.5.2 Strengths and limitations of methodology

A unique strength of our study is that we included infant RBC PUFA analysis, which might better reflect the concentration of PUFAs that infants were exposed to, rather than data on maternal PUFA intake and the PUFA level in maternal plasma or breast milk. Maternal–fetal circulation of PUFAs through the placenta involves complicated mechanisms including passive and facilitated diffusions of non-esterified fatty acids, which provides the major source of PUFAs in infant RBC before birth [161]. During later pregnancy, PUFAs are preferentially transferred from mothers to fetus, and PUFA percentage is usually higher in cord than maternal plasma [212]. A selective DHA and AA transfer through the placenta is also demonstrated [213]. Therefore, maternal PUFA profile may not adequately predict the PUFA status in infants [202]. More importantly, we conducted infant body composition measurements using DXA, which is advocated in previous systematic reviews [20]. The DXA technique we used is a validated body composition measurement, particularly for infants [214]. Movement artifacts were also minimized by scanning infants while sleeping. Although the infants in our study were from a vitamin D supplementation trial, we collected their blood samples and body composition information prior to the trial vitamin D supplementation, which eliminated any potential bias caused by the variation in infant vitamin D intake and tested for random effects of vitamin D status. Moreover, we used a detailed questionnaire to assess maternal and demographic information, which enable us to comprehensively consider potential confounders in data analysis.

Nonetheless, the observational design of this study does not enable us to explore the causality of the observed associations. Infant PUFA level might reflect other nutrient intake in maternal diet, lifestyle factors and genotype variation. Still, several sociodemographic and maternal factors have been adjusted in the analysis. Second, we did not measure infant bioactive factors, such as leptin, insulin-like growth factor 1, growth hormones, which regulate metabolism and energy intake, affecting infant weight gain and body composition [193]. Third, we only collected infant body composition and blood samples once after birth. It is unsure whether the infant PUFA

concentration we measured can fully reflect their PUFA content during the perinatal period of early adipose tissue development, although the PUFA levels in infant RBC reflects longer dietary exposure than those in plasma [159].

In conclusion, this study demonstrates that there are relationships between PUFA profile and body composition in neonates. We found that both infant AA and DHA were inversely associated with adiposity and positively associated with LM (g/kg), while higher infant n-6:n-3 ratio was associated with higher FM%. Moreover, infant DHA is inversely related to BMC/weight. Additional observational studies and clinical trials are necessary to confirm these results and explore long term effect as well as causality during this critical period of life.

Table 3.1: Participant characteristics according to sex

	Boys (n=40)	Girls (n=30)	Combined (n=70)
Infants at birth			
Gestational age (mo)	39.7±0.9	39.7±1.0	39.7±1.0
Birth weight (kg)	3.5±0.4	3.3±0.3	3.4±0.4
Birth weight z-score	0.15±0.91	0.19±0.73	0.17±0.83
APGAR score at 1 min	8.7±1.6	9.0±0.7	8.8±1.3
APGAR score at 5 min	9.4±0.9	9.6±0.7	9.5±0.8
Serum 25(OH)D (nmol/L)	40.5±20.6	38.1±19.0	39.4±19.8
Infants at postnatal visit			
Age at visit (mo)	0.66±0.18	0.64±0.17	0.65±0.18
Weight (kg)	3.9±0.5	3.8±0.4	3.9±0.5
Weight z-score	-0.24±0.81	-0.05±0.63	-0.16±0.74
Length (cm)	53.1±2.1	52.3±1.8	52.8±2.0
Length z-score	-0.07±0.92	0.01±0.86	-0.04±0.90
BMI (kg/m^2)	13.8±1.3	13.7±0.8	13.8±1.1
BMI z-score	-0.28±0.87	-0.09±0.58	-0.20±0.76
Head circumference (cm)	36.6±1.2	36.1±2.5	36.4±1.9
Head circumference z-score	0.14±0.9	0.38±2.1	0.24±1.5
Mothers			
Ethnicity, white	23(58%)	17(57%)	40(57%)
Education (≥college)	33(83%)	26(87%)	59(84%)
Annual family income (≥$70,000)[δ]	22(55%)	18(60%)	40(57%)
Age at delivery (y)	32.0±5.1	31.0±4.2	31.6±4.8
PWG (kg)	13.7±0.8	13.1±5.6	13.3±5.7
Pre-pregnancy BMI (kg/m^2)	24.3±4.3	24.8±5.9	24.5±5.0
BMI at visit (kg/m^2)	27.4±4.6	27.4±4.6	27.6±5.3

Values are mean±SD or n (%).

[δ] in Canadian dollars. PWG: pregnancy weight gain

Table 3.2: Infant bone and body composition

	Baseline (n=70)		
	Boys (n=40)	Girls (n=30)	Combined
LS 1-4			
LS1-4 BMC (g)	2.26±0.39	2.29±0.45	2.27±0.42
BMD (g/cm^2)	0.234±0.035	0.245±0.055	0.238±0.045
Whole body			
BMC (g)	95.6±13.9	90.8±13.1	93.5±13.6
BMC minus head (g)	52.4±7.2	50.2±8.2	51.5±7.7
BMC (g/kg weight)	24.4±2.9	24.3±3.4	24.4±3.12
FM (g)	761.4±291.8	745.1±183.1	754.4±249.6
FM (%)	17.6±5.2	18.3±3.3	17.9±4.5
LM (g)	3378.0±376.4	3208.5±274.0	3305.3±344.6
LM (g/kg)	864.0±61.6	857.5±39.6	861.2±53.1

Values are mean±SD.

LS: lumbar spine, FM: fat mass, LM: lean mass, BMD: bone mineral density, BMC: bone mineral content.

Table 3.3: Relationships between infant RBC and maternal RBC fatty acid (FA) (% of total fatty acids)

	Infant RBC FA (% of total FA)				
Maternal RBC FA (% of total FA)	DHA	∑n-3	AA	∑n-6	n-6:n-3 ratio
DHA (%)[γ]	0.52***	0.57***	-0.12	-0.33**	-0.60***
∑n-3(%)[#]	0.58***	0.64***	-0.06	-0.27*	-0.65***
AA (%)	0.09	0.05	0.19	0.11	-0.05
∑n-6(%)[#]	-0.32**	-0.38**	0.16	0.38**	0.44**
n-6:n-3 ratio	-0.57***	-0.64***	0.08	0.33**	0.67***

n=69, the PUFA values from 1 maternal RBC were excluded from analysis as extreme outliers

*p<0.05 **p<0.01 ***p<0.0001

[γ] Mother's DHA was transformed using square root

[#] Maternal ∑n-3 PUFA and ∑n-6 PUFA were transformed using the inverse of values before correlation analysis (the coefficient was transformed back to reflect original units)

Table 3.4: Relationship between infant RBC DHA (% of total fatty acids) and infant body composition

Variables	Regression coefficients	p value	95% confidence interval
BMC/weight (n=69) [δ]	Intercept=33.79		
DHA (%)	-1.12	**0.028**	-2.11 to -0.13
Mothers' race [a]	-0.64	0.41	-2.20 to 0.92
Family income [b]	0.27	0.66	-0.93 to 1.47
Mothers' education	-0.04	0.91	-0.70 to 0.78
Age at visit (mo)	-7.26	0.0006	-11.26 to -3.26
Mothers' PWG (kg)	0.18	0.004	0.06 to 0.31
Serum 25(OH)D (nmol/L)	-0.01	0.58	-0.05 to 0.03
Sex [d]	-0.69	0.34	-2.13 to 0.74
R^2=0.31			
LM (g/kg) (69) [δ]	Intercept=832.76		
DHA (%)	14.88	0.09	-2.53 to 32.29
Mothers' race [a]	1.71	0.90	-25.71 to 29.12
Family income [b]	12.18	0.25	-8.96 to 33.32
Mothers' education [c]	2.82	0.67	-10.25 to 15.89
Age at visit (mo)	-80.50	0.03	-150.84 to -10.16
Mothers' PWG (kg)	-2.75	0.01	-4.88 to -0.61
Serum 25(OH)D (nmol/L)	0.09	0.79	-0.58 to 0.76
Sex [d]	-1.36	0.91	-26.55 to 23.83
R^2=0.26			
FM (%) (69) [δ]	Intercept=23.96		
DHA (%)	-1.63	**0.03**	-3.11 to -0.15
Mothers' race [a]	0.24	0.84	-2.09 to 2.57
Family income [b]	-1.12	0.22	-2.91 to 0.68
Mothers' education [c]	-0.03	0.96	-1.14 to 1.08
Age at visit (mo)	5.20	0.09	-0.79 to 11.16
Mothers' PWG (kg)	0.21	0.03	0.03 to 0.39
Serum 25(OH)D (nmol/L)	-0.03	0.24	-0.09 to 0.02
Sex [d]	0.06	0.96	-2.20 to 2.08
R^2=0.26			

[δ]n=69, 1 maternal variable missing for mom pregnancy weight gain
PWG: pregnancy weight gain, 25(OH)D serum 25-hydroxyvitamin D,
[a]1=white 2=non-white.
[b] 1= < $70,000 2= ≥ 70,000 3=do not know/refuse to answer
[c] 2=high school 3=vocational school or apprenticeship training 4=CEGEP (pre-university) 5=university
[d] 1= male 2= female.

Table 3.5: Relationships between infant RBC AA (% of total fatty acids) and infant body composition parameters

Variables	Regression coefficients	p value	95% confidence interval
BMC/weight (n=69)[δ]	Intercept=32.00		
AA(%)	-0.27	0.39	-0.92 to 0.36
Mothers' race [a]	-0.80	0.32	-2.41 to 0.81
Family income [b]	0.20	0.76	-1.07 to 1.46
Mothers' education [c]	-0.04	0.91	-0.81 to 0.73
Age at visit (mo)	-7.55	0.002	-12.13 to -2.99
Mothers' PWG (kg)	0.18	0.008	0.05 to 0.30
Serum 25(OH)D (nmol/L)	-0.01	0.49	-0.05 to 0.03
Sex [d]	-0.12	0.86	-1.55 to 1.31
R^2=0.26			
LM (g/kg) (69) [δ]	Intercept=678.61		
AA(%)	13.75	**0.01**	3.22 to 24.28
Mothers' race [a]	0.46	0.97	-26.10 to 27.03
Family income [b]	16.90	0.11	-3.98to 37.78
Mothers' education [c]	4.72	0.46	-7.96 to 17.40
Age at visit (mo)	-45.01	0.24	-120.34 to 30.32
Mothers' PWG(kg)	-2.27	0.04	-4.37 to -0.16
Serum 25(OH)D (nmol/L)	0.17	0.60	-0.48 to 0.82
Sex [d]	-12.26	0.30	-35.84 to 11.32
R^2=0.31			
FM (%) (69) [δ]	Intercept=32.97		
AA (%)	-1.06	**0.02**	-1.98 to -0.14
Mothers' race [a]	0.23	0.84	-2.09 to 2.55
Family income [b]	-1.47	0.11	-3.29 to 0.36
Mothers' education [c]	-0.20	0.72	-1.31 to 0.90
Age at visit (mo)	2.69	0.42	-3.88 to 9.27
Mothers' PWG (kg)	0.17	0.07	-0.01 to 0.36
Serum 25(OH)D (nmol/L)	-0.04	0.15	-0.10 to 0.02
Sex [d]	1.00	0.34	-1.07 to 3.05
R^2=0.27			

[δ]n=69, 1 maternal variable missing for mom pregnancy weight gain
PWG pregnancy weight gain, 25(OH)D serum 25-hydroxyvitamin D
[a] 1=white 2=non-white.
[b] 1= < $70,000 2= ≥ 70,000 3=do not know/refuse to answer
[c] 2=high school 3=vocational school or apprenticeship training 4=CEGEP (pre-university) 5=university
[d] 1= male 2= female.

Table 3.6: Infant lean and fat mass among infant PUFA tertiles

	Lean mass (g)	Lean Mass (g/kg)	Fat mass (g)	Fat mass (%)
DHA (%)				
Lowest tertile (3.3 to 5.1 %)	2786.0±189.1	892.2±32.4	609.8±153.8	18.1±2.7
Highest tertile (6.7 to 6.9 %)	2887.8±166.4	915.1±31.2	484.6±148.8	15.5±2.6*
∑n-3 PUFA (%)				
Lowest tertile (3.8 to 6.2 %)	2871.6±189.9	892.0±30.5	610.1±147.5	17.5±2.6
Highest tertile (6.7 to 8.5 %)	2956.9±167.8	920.1±29.3[#]	481.9±143.2	14.8±2.6*
AA (%)				
Lowest tertile (11.6 to 14.2 %)	2707.8±193.6	858.7±33.2	672.8±170.7	20.6±2.5
Highest tertile (15.1 to 17.6 %)	2871.3±171.5	893.5±28.6*	577.8±144.8	18.0±2.2*
∑n-6 PUFA (%)				
Lowest tertile (25.7 to 28.8 %)	2926.2±180.5	918.3±35.3	478.6±168.2	15.2±3.0
Highest tertile (30.2 to 32.6 %)	2935.6±168.9	923.5±30.1	487.6±143.1	15.0±2.6
n-6:n-3 ratio				
Lowest tertile (3.3 to 4.4 %)	2956.5±170.4	930.9±29.2	379.4±142.6	13.3±2.6
Highest tertile (4.9 to 6.7 %)	2884.6±183.0	904.6±28.2	532.3±136.7*	15.9±2.5*

Values are mean±SD,
n=69, 1 maternal variable missing for mom pregnancy weight gain
Mixed model ANOVA adjusted for infant age, sex, family income, mother's education, race, maternal pregnancy weight gain, infant serum 25-hydroxyvitamin D concentration
*significantly different vs lowest tertile, $p<0.05$
[#] $p=0.0505$

Figure 3.1. Pearson correlation between infant a) AA and FM, b) DHA and FM, c) AA and FM%, d) DHA and FM%, e) AA and LM (g/kg), f) DHA and LM (g/kg) (n=70).

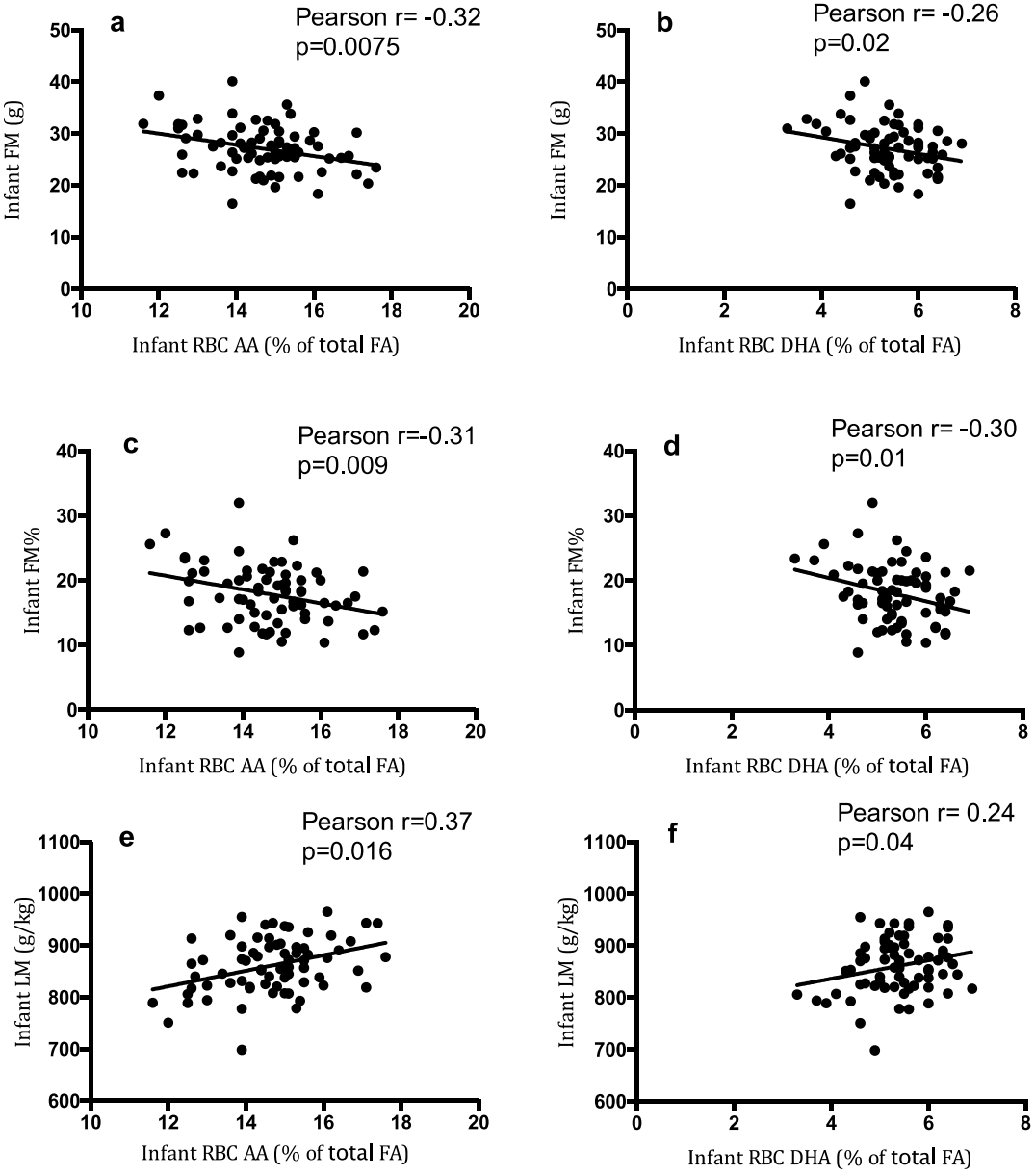
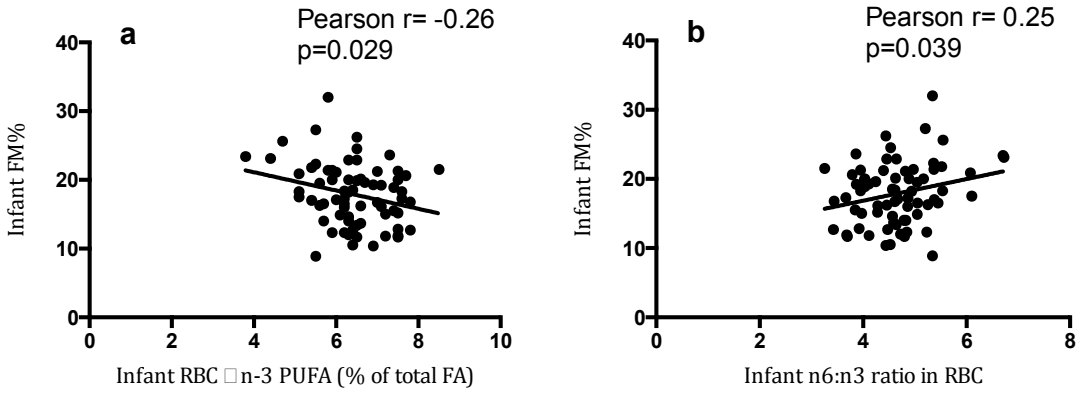


Figure 3.2: Pearson correlation between infant a) ∑n-3 PUFA and FM%, b) n-6:n-3 ratio and FM% (n=70).



Supplemental table 3.1: maternal and infant fatty acid profile as % of total fatty acid in RBC

	Mothers (n=69)*	Infants (n=70)
ALA (18:3 n-3)	0.2±0.1	0.1±0.03
EPA (20:5 n-3)	0.5±0.2	0.2±0.1
DHA (22:6 n-3)	4.5±0.8	5.4±0.7
∑n-3	7.0±1.1	6.4±0.9
LA (18:2 n-6)	8.6±1.0	6.7±0.9
AA (20:4 n-6)	12.6±1.0	14.7±1.3
∑n-6	27.8±1.3	29.4±1.5
n-6:n-3 ratio	4.1±0.7	4.7±0.7
∑PUFA	46.7±1.7	46.6±2.2
∑SFA	42.6±1.4	43.9±2.4
∑MUFA	15.8±1.1	13.4±1.1

Values are mean±SD

* the PUFA values from 1 maternal RBC were excluded from analysis as extreme outliers

ALA α-linolenic acid, EPA eicosapentaenoic acid, DHA docosahexaenoic acid, n-3 omega-3, LA linoleic acid, AA arachidonic acid, n-6 omega-6, PUFA polyunsaturated fatty acid, SFA, saturated fatty acid, MUFA monounsaturated fatty acid.

Supplemental table 3.2: Spearman correlations between infant RBC (% of total fatty acids) and maternal PUFA intake during pregnancy

Infant RBC fatty acid (% of total fatty acids)	Maternal PUFA intake during pregnancy (g/d)									
	ALA		DHA		∑n-3		LA		∑n-6	
	r	p	r	p	r	p	r	p	r	p
DHA (%)	-0.30	**0.02**	0.05	0.71	-0.004	0.98	-0.29	**0.02**	-0.31	**0.02**
∑n-3 (%)	-0.27	**0.04**	0.11	0.39	-0.02	0.90	-0.26	0.05	-0.27	**0.04**
AA (%)$^{\delta}$	-0.05	0.71	0.05	0.70	-0.05	0.68	-0.09	0.50	-0.08	0.54
∑n-6 (%)	0.06	0.68	0.08	0.54	-0.03	0.83	0.009	0.95	0.03	0.84
n-6:n-3 ratio	0.28	**0.04**	-0.10	0.47	0.02	0.89	0.24	0.06	0.26	**0.047**

n=58,

ALA:α-linolenic acid, LA: linoleic acid,

Significant p values are bolded

Supplemental table 3.3: Spearman correlation between infant body composition and maternal PUFA intake during pregnancy

	Maternal PUFA intake during pregnancy (g/d)									
	ALA		DHA		∑n-3		LA		∑n-6	
Infant Measures	r	p	r	p	r	p	r	p	r	p
Weight (g)	-0.04	0.79	-0.11	0.41	-0.05	0.73	-0.02	0.90	0.001	0.99
Length (cm)	-0.13	0.31	-0.13	0.35	-0.12	0.36	-0.01	0.92	0.002	0.99
BMI (kg/m^2)	0.01	0.93	-0.09	0.51	-0.005	0.97	-0.07	0.61	-0.05	0.70
BMI z-score	-0.01	0.93	-0.04	0.75	-0.04	0.78	-0.15	0.28	-0.13	0.32
LS BMD(g/cm^2)	-0.19	0.15	-0.19	0.15	-0.22	0.10	-0.14	0.30	-0.15	0.25
LS BMC (g)	-0.24	0.07	-0.25	0.06	-0.27	**0.04**	-0.14	0.29	-0.15	0.25
Whole body BMC (g)	0.07	0.58	0.11	0.42	0.10	0.48	0.14	0.31	0.15	0.25
BMC/weight (g/kg)	0.16	0.24	0.21	0.12	0.16	0.22	0.19	0.15	0.20	0.14
FM (g)	0.21	0.12	0.12	0.39	0.20	0.13	0.17	0.19	0.17	0.20
FM (%)	0.27	**0.04**	0.18	0.17	0.26	**0.047**	0.22	0.09	0.21	0.11
LM (g)	-0.19	0.16	-0.22	0.09	-0.19	0.16	-0.14	0.30	-0.11	0.41
LM (g/kg)	-0.18	0.18	-0.11	0.40	-0.17	0.20	-0.12	0.36	-0.11	0.40

n=58,

ALA:α-linolenic acid, LA: linoleic acid, LS: lumbar spine, FM: fat mass, LM: lean mass, BMD: bone mineral density, BMC: bone mineral content

Significant p values are bolded

Supplemental table 3.4: Relation between infant baseline body composition and mothers' RBC fatty acid (% of total fatty acids)

	Maternal RBC Fatty Acids													
	DHA (%)$^{\gamma}$		∑n-3 (%)$^{\#}$		AA (%)		∑n-6(%)$^{\#}$		n-6:n-3 ratio		∑SFA (%)		∑MUFA(%)	
Infant Measures	r	p	r	p	r	p	r	p	r	p	r	p	r	p
Weight (g)	-0.11	0.38	-0.10	0.41	-0.07	0.59	-0.04	0.72	0.08	0.52	0.31	**0.009**	-0.02	0.85
Length (cm)	-0.18	0.14	-0.16	0.20	-0.008	0.95	-0.03	0.79	0.12	0.32	0.35	**0.004**	-0.05	0.69
BMI (kg/m^2)	0.01	0.94	-0.005	0.97	-0.10	0.40	-0.04	0.72	0.003	0.98	0.17	0.17	0.02	0.90
BMI z-score	0.07	0.57	0.07	0.58	-0.12	0.33	-0.05	0.65	-0.06	0.60	0.18	0.15	-0.03	0.81
LS BMD(g/cm^2)$^{\gamma}$	-0.03	0.84	-0.014	0.91	0.01	0.92	0.01	0.91	0.02	0.84	-0.03	0.80	0.17	0.15
LS BMC (g)	-0.07	0.58	-0.07	0.57	-0.03	0.81	-0.06	0.60	0.05	0.71	0.11	0.36	0.22	0.08
Whole body BMC (g)$^{\delta}$	-0.12	0.32	-0.05	0.71	-0.12	0.34	0.03	0.78	0.04	0.76	0.26	**0.03**	0.05	0.70
BMC/weight (g/kg)$^{\gamma}$	-0.10	0.43	-0.02	0.86	-0.07	0.54	0.16	0.20	0.04	0.73	0.02	0.89	0.17	0.16
FM (g)	-0.12	0.32	-0.10	0.41	-0.11	0.37	0.05	0.68	0.10	0.40	0.32	**0.008**	-0.05	0.67
FM (%)	-0.09	0.45	-0.08	0.54	-0.12	0.34	0.07	0.54	0.09	0.48	0.28	**0.02**	-0.07	0.55
LM (g)	-0.10	0.41	-0.09	0.47	-0.006	0.96	-0.06	0.60	0.06	0.65	0.19	0.11	0.03	0.78
LM (g/kg)	0.03	0.80	0.04	0.75	0.15	0.23	0.003	0.98	-0.04	0.72	-0.31	**0.01**	0.08	0.69

n=69, the PUFA values from 1 maternal RBC were excluded from analysis as extreme outliers,

LS: lumbar spine, FM: fat mass, LM: lean mass, BMD: bone mineral density, BMC: bone mineral content

$^{\gamma}$Maternal DHA are transformed using square root, infant LS BMD and BMC/weight are transformed using log before correlation analysis

$^{\#}$ Maternal ∑n-3 PUFA and ∑n-6 PUFA were transformed using the inverse of values before correlation analysis (the coefficient transformed back to reflect original units)

$^{\delta}$Spearman correlation is calculated for whole body BMC, since they were not normally distributed after transformation.

Significant p values are bolded

Supplemental table 3.5: Relation between infant baseline body composition and infant RBC fatty acid (% of total fatty acids)

	Infant RBC Fatty Acids									
	DHA (%)		∑n-3 (%)		AA (%)		∑n-6 (%)		n-6:n-3 ratio	
Infant measures	r	p	r	p	r	p	r	p	r	p
Weight (g)	-0.07	0.54	-0.03	0.84	-0.27	0.02	-0.07	0.56	0.02	0.87
Length(cm)	0.02	0.88	-0.04	0.72	-0.12	0.34	-0.02	0.85	-0.06	0.65
BMI(kg/m^2)	-0.13	0.28	-0.07	0.54	-0.33	**0.005**	-0.10	0.43	0.08	0.51
BMI z-score	-0.10	0.37	-0.07	0.57	-0.15	0.22	-0.02	0.83	0.10	0.41
LS BMD (g/cm^2)$^{\gamma}$	0.06	0.61	0.05	0.69	0.06	0.61	-0.08	0.50	-0.09	0.47
LS BMC (g)	-0.03	0.80	-0.03	0.79	-0.02	0.85	-0.10	0.41	-0.01	0.94
Total BMC (g)$^{\delta}$	-0.17	0.16	-0.15	0.22	-0.16	0.20	-0.09	0.47	0.11	0.37
BMC/weight (g/kg)$^{\gamma}$	-0.19	0.12	-0.20	0.10	0.01	0.92	-0.06	0.63	0.19	0.12
FM (g)	-0.26	**0.03**	-0.22	0.07	-0.32	**0.008**	-0.08	0.49	0.21	0.08
FM (%)	-0.30	**0.01**	-0.26	**0.03**	-0.31	**0.009**	-0.10	0.41	0.25	**0.039**
LM (g)	0.07	0.55	0.11	0.37	-0.12	0.34	-0.01	0.93	-0.11	0.37
LM (g/kg)	0.25	**0.04**	0.21	0.09	0.37	**0.002**	0.16	0.20	-0.20	0.11

n=70,
LS: lumbar spine, FM: fat mass, LM: lean mass, BMD: bone mineral density, BMC: bone mineral content
$^{\gamma}$Infant LS BMD and BMC/weight are transformed using log before correlation analysis
$^{\delta}$Spearman correlation is calculated for whole body BMC, since it was not normally distributed after transformation.
Significant p values are bolded

Supplemental table 3.6: Relationship between infant RBC ∑n-3 PUFA (% of total fatty acids), n-6:n-3 ratio and body composition

Variables	Regression coefficients	p value	95% confidence interval
BMC/ weight (n=69) [δ]	Intercept=33.46		
∑n-3 PUFA (%)	-0.91	**0.03**	-1.73 to -0.09
Mothers' race[a]	-0.75	0.34	-2.30 to 0.80
Family income[b]	0.28	0.64	-0.92 to 1.48
Mothers' education[c]	0.04	0.92	-0.71 to 0.78
Age at visit (mo)	-7.08	0.0008	-11.07 to 3.09
Mothers' PWG (kg)	0.18	0.004	-0.06 to 0.31
Serum 25(OH)D (nmol/L)	-0.008	0.67	-0.05 to 0.03
Sex[d]	-0.70	0.34	-2.14 to 0.74
R^2=0.31			
FM (%) (69) [δ]	Intercept=21.91		
∑n-3 PUFA (%)	-1.10	0.08	-2.35 to 0.14
Mothers' race[a]	0.04	0.97	-2.30 to 2.39
Family income[b]	-1.09	0.24	-2.91 to 0.73
Mothers' education[c]	-0.05	0.93	-1.18 to 1.08
Age at visit (mo)	5.55	0.07	-0.49 to 11.58
Mothers' PWG (kg)	0.21	0.03	0.02 to 0.39
Serum 25(OH)D(nmol/L)	-0.03	0.28	-0.09 to 0.03
Sex[d]	0.05	0.96	-2.12 to 2.23
R^2=0.24			
FM (%) (69) [δ]	Intercept=7.52		
n-6:n-3 ratio	1.34	0.09	-0.19 to 2.88
Mothers' race[a]	0.0007	1.00	-2.34 to 2.34
Family income[b]	-0.94	0.31	-2.77 to 0.89
Mothers' education[c]	0.005	0.99	-1.13 to 1.14
Age at visit (mo)	6.06	0.05	0.05 to 12.08
Mothers' PWG (kg)	0.22	0.02	0.03 to 0.40
Serum 25(OH)D(nmol/L)	-0.03	0.28	-0.09 to 0.03
Sex[d]	0.15	0.89	-1.99 to 2.30
R^2=0.24			

[δ]n=69, 1 maternal variable missing for mom pregnancy weight gain
PWG: pregnancy weight gain, 25(OH)D serum 25-hydroxyvitamin D
[a] 1=white 2=non-white.
[b] 1= < $70,000 2= ≥ 70,000 3=do not know/refuse to answer
[c] 2=high school 3=vocational school or apprenticeship training 4=CEGEP (pre-university) 5=university
[d] 1= male 2= female.

Supplemental table 3.7: Relationship between infant RBC ∑n-6 PUFA (% of total fatty acids) and body composition

Variables	Regression coefficients	p value	95% confidence interval
BMC/weight (n=69) [δ]	Intercept=34.16		
∑n-6 PUFA (%)	-0.21	0.46	-0.76 to 0.35
Mothers' race [a]	-0.81	0.32	-2.43 to 0.81
Family income [b]	0.15	0.82	-1.16 to 1.46
Mothers' education [c]	-0.05	0.91	-0.82 to 0.73
Age at visit (mo)	-7.09	0.0015	-11.34 to -2.84
Mothers' PWG (kg)	0.17	0.01	0.04 to 0.30
Serum 25(OH)D (nmol/L)	-0.02	0.45	-0.05 to 0.02
Gender [d]	-0.27	0.71	-1.70 to 1.16
R^2=0.26			
LM (g/kg) (69) [δ]	Intercept=774.67		
∑n-6 PUFA (%)	4.35	0.37	-5.24 to 13.93
Mothers' race [a]	3.32	0.81	-24.54 to 31.18
Family income [b]	14.92	0.19	-7.68 to 37.53
Mothers' education [c]	4.19	0.53	-9.13 to 17.52
Age at visit (mo)	-79.83	0.03	-153.13 to -6.54
Mothers' PWG (kg)	-2.51	0.03	-4.77 to 0.26
25(OH)D (nmol/L)	0.17	0.63	0.52 to 0.85
Gender [d]	-6.56	0.60	-31.16 to 18.05
R^2=0.24			
FM (%) (69) [δ]	Intercept=23.66		
∑n-6 PUFA (%)	-0.28	0.50	-1.11 to 0.55
Mothers' race [a]	-0.01	0.99	-2.43 to 2.40
Family income [b]	-1.27	0.20	-3.23 to 0.69
Mothers' education [c]	-0.15	0.79	-1.31 to 1.00
Age at visit (mo)	5.49	0.09	-0.86 to 11.83
Mothers' PWG (kg)	0.20	0.05	0.0006 to 0.39
25(OH)D (nmol/L)	-0.04	0.18	-0.10 to 0.02
Gender [d]	0.56	0.60	-1.57 to 2.69
R^2=0.21			

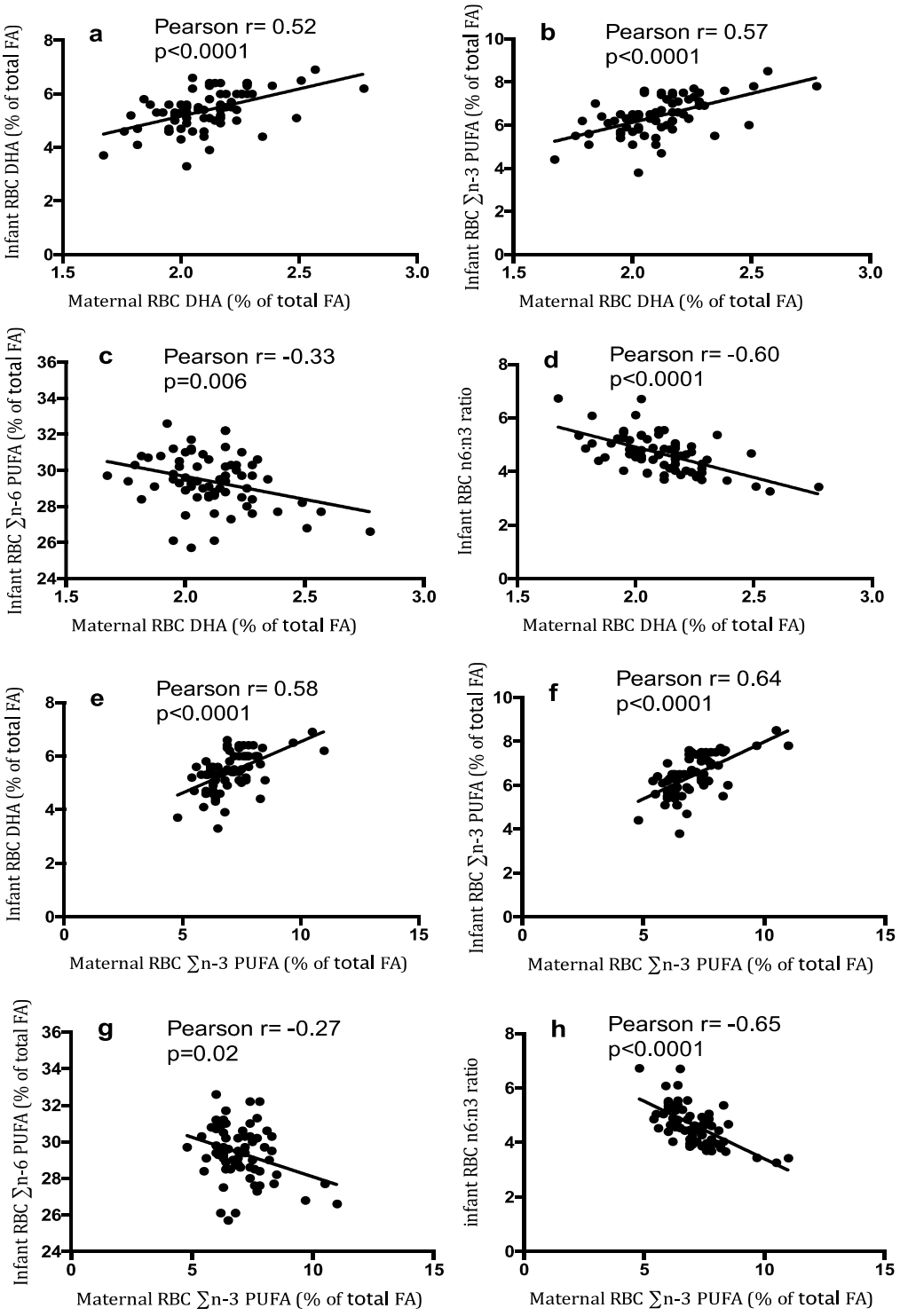
[δ]n=69, 1 maternal variable missing for mom pregnancy weight gain
PWG: pregnancy weight gain, 25(OH)D serum 25-hydroxyvitamin D
[a] 1=white 2=non-white.
[b] 1= < $70,000 2= ≥ 70,000 3=do not know/refuse to answer
[c] 2=high school 3=vocational school or apprenticeship training 4=CEGEP (pre-university) 5=university
[d] 1= male 2= female.

Supplemental table 3.8: Infant bone measurements among infant PUFA tertiles

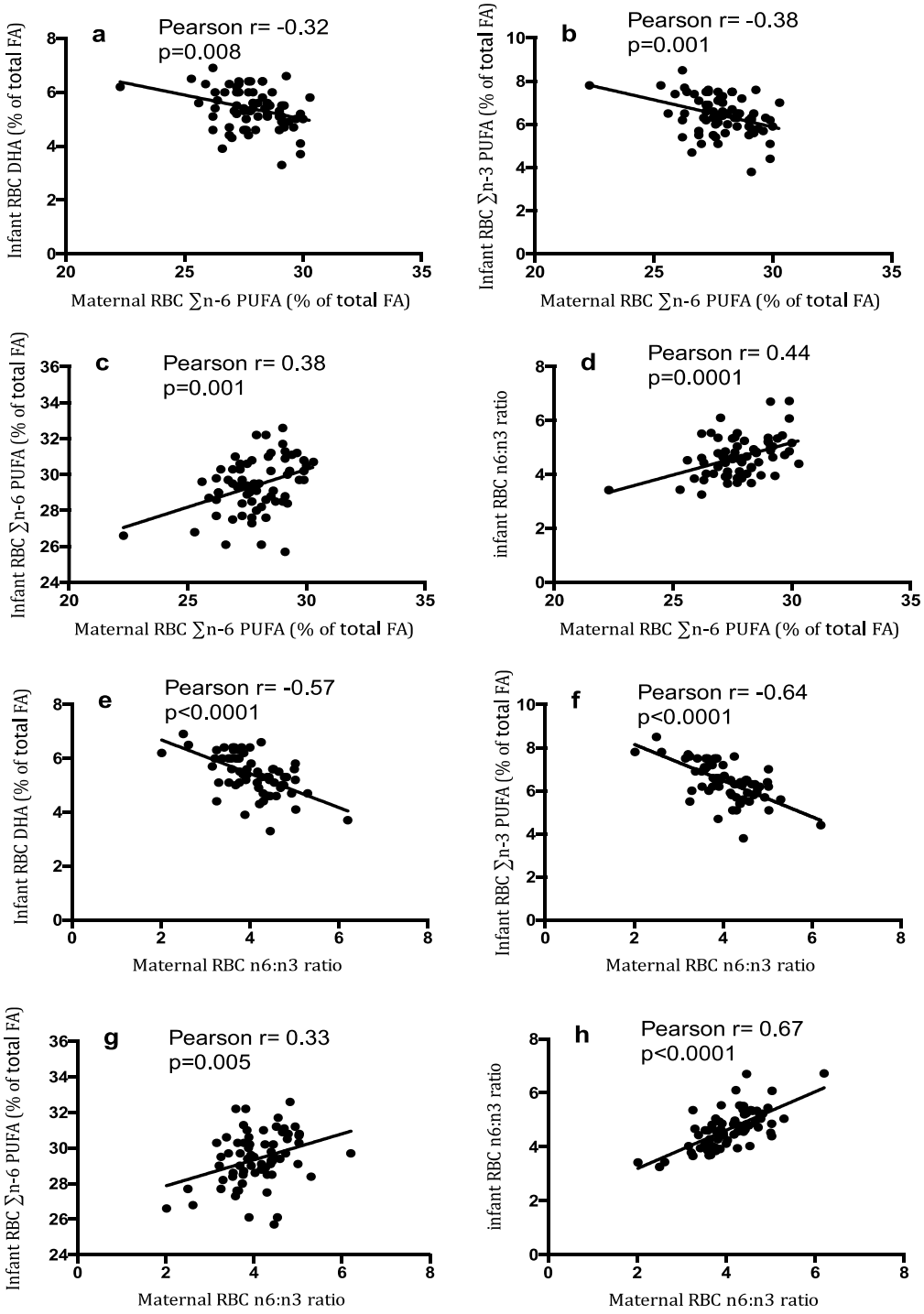
	LS BMC (g)	LS BMD (g/cm^2)	Whole BMC (g)	BMC/weight (g/kg)
DHA(%)				
Lowest tertile (3.3 to 5.1 %)	1.834±0.166	0.175±0.025	93.8±6.2	27.3±1.6
Highest tertile (6.7 to 6.9 %)	1.825±0.173	0.191±0.024	87.2±6.0	25.7±1.5
∑n-3 PUFA (%)				
Lowest tertile (3.8 to 6.2 %)	1.834±0.163	0.169±0.025	94.4±6.2	26.3±1.6
Highest tertile (6.7 to 8.5 %)	1.850±0.168	0.188±0.023	88.1±5.9	25.0±1.5
AA (%)				
Lowest tertile (11.6 to 14.2 %)	1.795±0.158	0.183±0.023	89.6±6.2	26.6±1.9
Highest tertile (15.1 to 17.6 %)	1.851±0.151	0.196±0.021	87.9±6.4	26.2±1.6
∑n-6 PUFA (%)				
Lowest tertile (25.7 to 28.8 %)	2.110±0.198	0.220±0.024	88.9±6.1	27.0±1.7
Highest tertile (30.2 to 32.6 %)	2.029±0.175	0.212±0.021	87.3±5.7	26.4±1.5
n-6:n-3 ratio				
Lowest tertile (3.3 to 4.4 %)	1.911±0.168	0.208±0.024	88.9±6.0	25.1±1.7
Highest tertile (4.9 to 6.7 %)	1.893±0.155	0.190±0.024	93.4±6.1	25.9±1.6

Values are mean ± SD
n=69, 1 maternal variable missing for mom pregnancy weight gain
Mixed model ANOVA adjusted for infant age, sex, family income, mother's education, race, maternal pregnancy weight gain, infant serum 25-hydroxyvitamin D concentration.

Supplemental figure 3.1. The correlation between maternal a) DHA and infant DHA, b) DHA and infant ∑n-3 PUFA, c) DHA and infant ∑n-6 PUFA, d) DHA and infant n-6:n-3 ratio, e) ∑n-3 PUFA and infant DHA, f) ∑n-3 PUFA and infant ∑n-3 PUFA, g) ∑n-3 PUFA and infant ∑n-6 PUFA, h) ∑n-3 PUFA and infant n-6:n-3 ratio. (n=69, the PUFA values from 1 maternal RBC were excluded from analysis as extreme outliers)



Supplemental figure 3.2. The correlation between mother's a) ∑n-6 PUFA and infant DHA, b) ∑n-6 PUFA and infant ∑n-3 PUFA, c) ∑n-6 PUFA and infant ∑n-6 PUFA, d) ∑n-3 PUFA and infant n-6:n-3 ratio, e) n-6:n-3 ratio and infant DHA, f) n-6:n-3 ratio and infant ∑n-3 PUFA, g) n-6:n-3 ratio and infant ∑n-6 PUFA, h) ∑n -3 PUFA and infant n-6:n-3 ratio. *Correlations remained significant after removing outliers. (n=69, the PUFA values from 1 maternal RBC were excluded from analysis as extreme outliers)



4. Extended discussion

4.1 Interpretation of findings

According to the research available, this study is novel in that it evaluated the neonatal polyunsaturated fatty acid (PUFA) profile by using red blood cell (RBC) membranes and examined its relation to infant body composition variables, which were measured by dual-energy X-ray absorptiometry (DXA). Our findings supported our primary hypothesis that higher infant docosahexaenoic acid (DHA) is associated with lower fat percentage (FM%). Interestingly, an unexpected inverse association between infant arachidonic acid (AA) and FM% was observed as well. Although the design of this study may not enable us to compare our findings with the results from the studies that examined maternal PUFA status, the relationship between AA and FM% in infants is not supported by previous studies, which demonstrated maternal n-6 PUFA (total n-6 PUFA, AA) was positively associated with offspring FM% [159, 161]. Additionally, the hypothesis that a higher level of n-6:n-3 ratio is associated with higher FM% was supported. Therefore, the preliminary deduction from these findings could be that both AA and DHA may play beneficial roles in regulating adiposity during infancy, and controlling n-6:n-3 ratio may help prevent excessive fat deposition in infants.

Beside the encouraging results of infant AA and DHA in fat deposition, infant AA and DHA were observed to be positively associated with lean mass (LM) (g/kg). Hence, it supports our hypothesis that infants with higher DHA will have higher LM. Likewise, several animal studies reported that rats fed with DHA had higher LM [215]. From mechanistic aspects, n-3 PUFA is found to activate regulatory kinases and promote muscle protein synthesis [127]. The anti-catabolic and anabolic effects of n-3 PUFA could help prevent muscle loss and promote LM accretion [18]. However, very few studies described the relationship between AA or n-6 PUFA status and body composition. As the literature regarding the association between n-3 PUFA, n-6 PUFA and LM is limited, the relationships between AA, DHA and LM in neonates we found are not conclusive.

Based on the discussed findings above, higher proportions of AA and DHA in infants seems to be associated with lower fat mass (FM) and higher LM; in other words, both may support healthy growth and regulate body composition in infants. This implication is different from the findings in several previous animal studies and infant studies. In animal studies, DHA and EPA

intake are associated with lower body fat compared with saturated fat or LA [138]. In contrast, AA promotes adipose tissue development in some [118], but not all studies [216]. Although maternal n-3 PUFA supplementation is found to reduce offspring adiposity in several trials, few studies have investigated infant AA status after n-3 PUFA supplementation and its relation to infant body composition. Still, the beneficial effect of AA + DHA supplementation on infant body composition is supported by a randomized clinical trial conducted in preterm infants, reporting that preterm infants supplemented with AA and DHA in formula had higher LM and lower FM compared with those who had control formula [154]. However, in this study only the role of the combination of AA and DHA in altering body composition was investigated. The separate effects of AA and DHA on infant body composition, and whether these effects are synergistic or antagonistic, need further research to confirm. Moreover, those effects of AA and DHA in the context of healthy infants should be clarified as well.

It is worth noting that we observed statistically significant relationships between DHA or AA with infant body composition variables, but observed much weaker relationships for both ∑n-3 PUFA and ∑n-6 PUFA. This implies that the functional roles of longer chain PUFAs, DHA and AA, could be more active or effective than their precursors in modulating infant body composition. The importance of longer chain PUFA in infant health has been debated by researchers. The limited capability among infants to synthesize DHA and AA from their precursor α-linolenic acid (ALA) and linoleic acid (LA), two common PUFA supplied from formula milk, has been demonstrated [26]. However, the differences in visual and cognitive development between infants fed formula with and without longer chain PUFA from previous trials are inconsistent, making the essentiality and functional role of AA and DHA in infants not clearly defined [217]. Comparing the efficacy of AA, DHA and their precursors in altering infant body composition should be conducted in future studies.

We also observed an inverse association between infant DHA and whole body bone mineral content (BMC)/weight. A similar relationship was reported in previous studies. For instance, in rats from dams fed with a n-3 PUFA enriched diet, femur BMC was reduced compared with those from dams fed a balanced n-6:n-3 diet [210]. It was also found that maternal DHA is associated with lower lumbar spine (LS) BMC in infants, while maternal AA is positively related with whole body BMC [171]. The potential mechanistic explanation behind this might be that a

certain concentration of prostaglandin 2 (PGE-2) derived from AA in the infants stimulates bone formation and resorption which might have interfered with the production of PGE-2 by n-3 PUFA [218]. Nonetheless, AA, or n-6 PUFA, was not significantly associated with infant BMC in the present study. Hence, the mechanism is not known as we did not measure infant PGE-2 concentration particularly in bone, which was not practical in the present study. Still, there are considerable evidence that indicates n-3 PUFA can suppress nuclear factor-κB (NF-κβ) activation and PGE-2 production when it is at a high level, supporting a positive role of n-3 PUFA in bone health [219]. However, the expected positive relation between infant n-3 PUFA and LS bone mineral density (BMD), BMC was not found in this study. Since it takes a period of time for infants to develop bone homeostasis with bone formation and resorption, the true relationship between n-3 PUFA and bone mass in infants may require a long-term observational study to detect. Previous studies also emphasized the role of n-6:n-3 ratio in maintaining bone mass [171]. However, in the present study, no significant relationship was observed between infant n-6:n-3 ratio and infant bone mass variables.

The relationship of maternal PUFA intake and status to infant body composition variables was investigated in this study as well. Unlike previous studies that used maternal plasma PUFA concentration during pregnancy as an indicator of maternal PUFA supply, we measured maternal RBC PUFA after the birth of the infants. Since maternal PUFA concentration might fluctuate dramatically due to the selective PUFA transfer from maternal to fetal circulation and into breastmilk [212, 213], the maternal data we recorded at the collect time point could not fully reflect the PUFA status in the infants. Hence, although most but not all maternal PUFA variables were correlated with infant PUFA variables, there were no significant associations between maternal RBC n-3 PUFA or n-6 PUFA ratios and infant body composition variables. Moreover, only maternal ALA and n-6 PUFA intake during pregnancy were observed to be related with infant RBC n-3 PUFA and n-6:n-3 ratio. Breastmilk was the primary resource of PUFA for the infants in our study after birth. Beside maternal diet, the PUFA content in breastmilk can be affected by endogenous PUFA synthesis in liver and breast tissue and mobilization of PUFA stores [201]. Furthermore, certain PUFA, such as DHA, can be present in breastmilk without intake of a dietary source, although its amount is related with the DHA in diet [220]. Hence, maternal PUFA intake is a significant factor, but may not be a sufficient predictor of infant

PUFA status. This could be the reason why the observed relations between maternal n-3 PUFA intake and infant FM% variables were opposite to the relationship between infant n-3 PUFA and FM%, adding to the disadvantages in food frequency questionnaire (FFQ) analysis we performed, which will be discussed in next section.

This thesis project was also designed to explore the role of interactions between PUFA and vitamin D in infant body composition. Vitamin D is suggested as a potential factor that may affect infant fat deposition and expenditure, since it plays a role in regulating circulating PTH and leptin concentrations [174, 175]. There are a few studies reporting that vitamin D absorption might be affected by PUFA status [177, 178], indicating the interaction between vitamin D and PUFA may result in an effect on body composition. However, in this study, infant serum 25-hydroxyvitamin D 25(OH)D was not a significant covariate in each regression model. The interaction between PUFA and 25(OH)D was also tested in mixed model ANOVA and regression model. Overall, it did not change the corresponding results or their interpretation. This may be because the only vitamin D metabolite assessed was 25(OH)D. Its metabolites, such as 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is more hormonaly active and could be the form that impacts infant fat deposition. Moreover, n-3 PUFA was suggested to regulate the activity of enzyme 1α-hydroxylase, that catalyzes 25(OH)D transformation to $1,25(OH)_2D$ [179]. Therefore, further study should include $1,25(OH)_2D$ in the analysis and examine the interaction between vitamin D hormonal action and PUFA in relation to infant body composition.

4.2 Strengths and limitations

Study design

The present study provided supportive evidence of the relationships between infant RBC n-3, n-6 PUFA ratios and body composition. However, its observational design cannot indicate the effect of PUFA profile in altering body composition. The target sample size for the partial correlation coefficient was 67 according to Spearman (r) =0.2, an alpha of 0.05 and 80% power [156]. It was also estimated that 21 participants were needed in each tertile in order to observe an effective size of 5% difference in fat mass [221] caused by PUFA tertile with an alpha of 0.05 and 80% power. There for 63 mother-infant pairs were needed for the tertile analyses. Both estimated

sample sizes were fulfilled in the present study. The sufficient sample size allowed us to perform our statistical analysis with sufficient power.

Study population

Since all of the participants were recruited from a hospital located in Montreal, the generalizability could be limited in different regions and ethnic groups. In the present study, infants who had low vitamin D status (<50 nmol/L) were not excluded on the basis of maternal pre-pregnancy body mass index (BMI). Although we performed our analyses considering maternal pre-pregnancy BMI as a covariate, not excluding the specific group of participants with high maternal pre-pregnancy BMI may have caused potential bias and may interfere in examining our proposed hypothesis.

There were 14 mothers with a pre-pregnancy BMI greater than 27 kg/m^2. After excluding them from data analysis, the previously mentioned relationships of infant AA to LM (g/kg) and FM% were not significant in both regression model and tertile analysis, while AA was negatively related with BMI (p=0.01) and BMI z-score (p=0.03) after adjusting covariates. Still, the results regarding the relation between infant DHA, n-3 PUFA, n-6:n-3 ratio and FM% did not change much after excluding the 14 mothers who had higher BMI. Additionally, infants in the highest tertile of n-6:n-3 ratio also had significantly lower LM than those in lowest tertile (p=0.04). Since the main findings were similar whether mothers with high BMI were included or not, it appears that this was not a major factor in interpretation of the study data.

FFQ data

Although the FFQ we used for assessing maternal PUFA intakes was validated, there were many food items in the Canadian Nutrient File that have missing values for PUFAs. Therefore, we may not have fully captured the maternal PUFA intake from those food items. This limitation might be an explanation why we observe less relationships of maternal PUFA intakes compared with maternal RBC PUFA ratios to infant RBC PUFA ratios.

Body composition assessment and RBC PUFA analysis

Unlike the anthropometric measurements used in previous studies, the tool we used to measure infant body composition, DXA, provides comprehensive assessment of LM, FM and BMC in a

three-compartment model [106]. It is a validated tool and promoted by researchers for studies investigating the relationship between infant n-3 PUFA and body composition [8]. However, the accuracy and precision of DXA results vary with different pediatric software and manufacturer [104]. Hence, our results should be interpreted with caution when comparing with the findings from other studies using a different DXA scanner or software or other techniques to measure infant body composition.

RBC membrane is considered as a preferable biomarker for quantifying PUFA in humans [222]. Biomarkers are effective indicators of individual nutrient status, since they can comprehensively reflect the dietary intake, biosynthesis and metabolism of nutrients. There are several types of biological samples to evaluate PUFA status, including adipose tissue, plasma and RBC [222]. PUFA in plasma is a widely used reference method, but a short-term reflection of PUFA intake. It could also provide insight on the state of lipogenesis with the information of circulating triacylglyceride and non-essential fatty acid pools. While compared to plasma, PUFA in RBC is a functional pool for accurately reflecting longer-term dietary intake and not affected by individual postprandial status [223]. Although measuring PUFA in adipose tissue can evaluate even longer-term PUFA status (the condition during least last year), the functional role of PUFA in adipose tissue has been suggested lower than those from other lipid pools [222]. Therefore, infant RBC PUFA ratio could be the most appropriate PUFA biomarker to investigate our research questions. Besides, the protocol that we used to extract RBC PUFA and prepare FAMEs has been validated as a rapid and reliable method for PUFA quantification, which is another advantage of present study [200]. Moreover, PUFA status was assessed using PUFA percentage relative to total fatty acids, which is commonly used and comprehensively reflects fatty acid metabolism, exogenous factors and the interaction between different PUFA pools [222]. It is suggested that specific PUFA concentration might be more appropriate when tissue exposure is important [224]. Estimation of PUFA concentration requires high quality internal standard with constant concentration. The potential for solvent evaporation and thus inaccurate standard concentration might cause errors and change the results of the observations. Nonetheless, the RBC PUFA concentration data in this thesis follows a similar pattern. For example, concentration of DHA in RBC followed the same correlation pattern with LM (g/kg), however it did not reach statistical significance ($r=0.25$, $p=0.08$). Future studies wishing to explore

concentration should estimate the sample size based on the variability of the measurement of PUFA concentration in RBC membranes.

While the primary objective of this thesis was set to examine the potential role of PUFA in infant body composition, other fatty acid groups, such as saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) should also be considered as fatty acids that modulate infant body composition. SFA consumption was found to increase fat deposition and correlated with higher BMI, waist-to-hip ratio and FM [225]. In line with previous evidence, maternal RBC SFA was positively associated with infant FM% and negatively associated with LM (g/kg) (Supplemental table 3.4) in this study. Moreover, MUFA content in adipocytes was inversely associated with fat cell number [226]. A MUFA enriched diet was reported to reduce weight gain and fat accumulation [225]. No relationship was found between infant body composition with either infant or maternal MUFA status in this study. The beneficial effect of MUFA on FM development might be limited or masked by the effect of PUFA for individuals at the earliest stage of life. Since there are limited studies that examined the specific role of n-7, n-9 MUFA and SFA in infant body composition, comprehensively evaluating the relationships between infant fatty acid profile and body composition should be considered for further research.

4.3 Future directions

The most updated recommended dietary intakes of n-3 PUFA and n-6 PUFA in infants were made based on the benefits of these fatty acid series in brain and visual development. Therefore, it is unknown whether the current recommendation is also appropriate for infants to establish and optimize infant body composition compartments. Future research should firstly be conducted by continuing to use advanced and validated body composition assessment tools, such as DXA, to assess infant body composition and examine its relation to infant PUFA biomarkers in a larger sample. After that, well-designed randomized clinical trials investigating the separate and combined effects of prenatal and postnatal maternal n-3 PUFA and n-6 PUFA supplementation on infants should be conducted.

4.4 Conclusion

Since excessive fat deposition in neonates may have irreversible and long-term effects on body composition and related health status later in life, it is important to investigate the nutrient factors that may help to control neonatal adiposity. The results from this thesis suggest that AA and DHA are inversely associated with FM% and positively correlated with LM (g/kg) in neonates. Meanwhile, a positive association between n-6:n-3 ratio and infant FM% as well as a negative association between DHA and BMC/weight were observed. Further studies are required to confirm the aforementioned relationships we observed and explored longer-term relationship and causal effect of changes in infant PUFA profile in regulating body composition.

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