Relationship between Breast Milk Fatty Acids and Infant Bone Mass and Metabolism

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Abstract

The primary objective of this thesis was to determine if long chain polyunsaturated fatty acids (LCPUFA) in maternal milk relates to infant bone mineral content, density and metabolism. A cohort of healthy singleton breastfed infants (n=120) were studied at 1 month post-partum. The measurements included anthropometric measurements of both mother and infant. Maternal dietary intake was assessed using both a 24-h recall and a food frequency questionnaire. A representative breast milk sample was collected for measurement of LCPUFA. Infant bone mineral content and density were assessed using dual-energy x-ray absorptiometry along with plasma parathyroid hormone, 25-hydroxyvitamin D and ionized calcium, plus urinary calcium:creatinine. Infant size at birth and 1 mo were within 2 SD of the World Health Organization growth standard. Maternal intake of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) during pregnancy (r = 0.42, p<0.0001; r = 0.46, p<0.0001; respectively) directly associated with milk LCPUFA composition. Maternal intake of DHA and EPA the day before showed similar results (r = 0.43, p<0.0001; r = 0.51, p<0.0001; respectively). Correlation analysis revealed a negative correlation between breast milk DHA and arachidonic acid (AA) with the infant lumbar spine vertebrate 1-4 bone mineral content (BMC) (r = - 0.18, p = 0.05; r = - 0.19, p = 0.04; respectively) but these relationships were not evident in multivariate analyses. In multivariate analysis, adjusted for infants' weight, age, gender, ethnicity, vitamin D supplementation and maternal smoking and pre-pregnancy body mass index (BMI), breast milk LCPUFA was not related to infant bone mineral density (BMD) nor BMC. Based on the multivariate analysis, breast milk LCPUFA do not explain the variance in bone mass early post-natally. However, since dietary intake may not reflect LCPUFA status, further studies are warranted using measures of infant LCPUFA status along with bone mass to confirm these observations.

Abstrait

L'objectif principal de cette thèse était de déterminer si longue chaîne d'acides gras polyinsaturés (AGPI-LC) dans le lait maternel se rapporte à la teneur en minéraux des os du nourrisson, la densité et le métabolisme. Une cohorte de santé Singleton nourrissons allaités (n = 120) ont été étudiés à 1 mois post-partum. Les mesures comprenaient des mesures anthropométriques de la mère et du nourrisson. Maternelle apport alimentaire a été évaluée en utilisant à la fois un rappel de 24 h et un questionnaire de fréquence alimentaire. Un échantillon de lait maternel représentatifs ont été recueillis pour la mesure de AGPILC. Le contenu minéral osseux et la densité du nourrisson ont été évaluées en utilisant la bi-énergie absorptiométrie à rayons X avec l'hormone parathyroïdienne plasma, 25-hydroxyvitamine D et le calcium ionisé, plus de calcium urinaire: créatinine. La taille du nourrisson à la naissance et 1 mois étaient à moins de 2 SD de la norme de croissance mondiale de la Santé Organisation. Apport de la mère de l'acide docosahexaénoïque (DHA) et l'acide eicosapentaénoïque (EPA) pendant la grossesse ($r = 0,42$, $p < 0,0001$, $r = 0,46$, $p < 0,0001$; respectivement) directement associés avec le lait AGPILC composition. Apport de la mère de DHA et d'EPA la veille a montré des résultats similaires ($r = 0,43$, $p < 0,0001$, $r = 0,51$, $p < 0,0001$; respectivement). L'analyse de corrélation a révélé une corrélation négative entre le lait maternel DHA et acide arachidonique (AA) avec la colonne vertébrale lombaire infantile vertébrés 1-4 contenu minéral osseux (CMO) ($r = - 0,18$, $p = 0,05$, $r = - 0,19$, $p = 0,04$; respectivement), mais ces relations ne sont pas évidentes dans les analyses multivariées. En analyse multivariée, ajustée pour le poids des nourrissons, âge, sexe, origine ethnique, supplémentation en vitamine D et le tabagisme maternel et pré-grossesse indice de masse corporelle (IMC), le lait maternel AGPILC n'était pas liée à la densité minérale osseuse du nourrisson (DMO), ni BMC. Basé sur l'analyse multivariée, le lait maternel AGPILC n'expliquent pas la variance de la masse osseuse précoce post-natale. Toutefois, depuis l'apport alimentaire peut ne pas refléter l'état AGPILC, nouvelles études sont justifiées par des mesures d'AGPILC état infantile ainsi que la masse osseuse pour confirmer ces observations.

Author's Contributions

S. AlSaleh was the primary author of the publication included in this thesis and was a large contributor to the work included. S. AlSaleh assisted in the recruitment of infants. S. AlSaleh was present for infant visits and assisted in collection of anthropometric data from mothers and infants, blood and urine samples and dual-energy x-ray absorptiometry (DXA) bone scans.

S. AlSaleh administered 24 hour recalls and audited dietary data from infants and mothers; she also conducted all entry and/or auditing of DXA data and statistical analyses. Analysis of breast milk fatty acids was also conducted by S. AlSaleh.

C. Vanstone was responsible for the day to day coordination and, in part, the conception of the project. C. Vanstone was also responsible for blood procurement and was the primary technician for the DXA.

S. Gallo is the doctoral student working on the primary outcomes of the vitamin D response study.

S. Agellon trained the candidate how to analyze breast milk fatty acids.

C. Rodd was the co-investigator on this project. C. Rodd also served as a safety officer, coordinating and monitoring laboratory results from the Montreal Children's Hospital communicating with both parents and study coordinators.

H. Weiler was the principle investigator of the vitamin D response study. H. Weiler was responsible for the conception and overall coordination of all authors involved. H. Weiler was also S. AlSaleh direct supervisor.

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Key Terms

Acronyms and Abbreviations

1,25(OH)$_2$D, 1,25-dihydroxyvitamin D;

25(OH) D, 25-hydroxyvitamin D;

AA, Arachidonic acid;

AGA, Appropriate size for gestational age;

ALA, α-Linolenic acid;

ALP, Alkaline phosphatase;

AOAC, Association of Analytical Communities;

AOCS, American Oil Chemists' Society;

BMC, Bone mineral content;

BMD, Bone mineral density;

BMI, Body mass index;

C, Carbon;

CCHS, Canadian Community Health Survey;

Chol, Cholesterol;

COX, Cyclooxygenase;

CTx, C-teleopeptide;

CV, Coefficient variation;

DHA, Docosahexaenoic acid;

DPA, Docosapentaenoic acid;

DXA, Dual energy x-ray absorptiometry;

EFA, Essential fatty acids;

EPA, Eicosapentaenoic acid;

FA, Fatty acid;

FAME, Fatty acid methyl esters;

GC, Gas chromatograph;

GLA, Gamma linolenic acid;

H_0, Null hypothesis;

H_1, Alternative hypothesis;

IL-1, Interleukin-1;

IL-6, Interleukin-6;

ISCD, International Society for Clinical Densitometry;

LA, Linoleic acid;

LCPUFA, Long chain polyunsaturated fatty acid;

LNA, Linolenic acid;

LOX, Lipoxygenase;

MCH, Montreal Children's Hospital;

MUFA, Monounsaturated fatty acids;

n-3, Omega 3 fatty acids;

n-6, Omega 6 fatty acids;

NA, Nervonic acid;

NF-κB, Nuclear factor-κB;

NO, Nitric oxide;

NTx, N-teleopeptides;

OPG, Osteoprotegerin;

PCB, Polychlorinated biphenyls;

PGE_2, Prostaglandin E_2;

PL, Phospholipids;

PPARs, Peroxisome proliferator activator receptors;

PTH, Parathyroid hormone;

RANKL, Receptor activator of nuclear factor-kB ligand;

RBC, Red blood cells;

RDIs, Recommended Dietary Intakes;

ROS, Reactive oxygen species;

SFA, Saturated fatty acids;

SGA, Small for gestational age;

TFA, Trans fatty acids;

TG, Triglycerides;

TNF-α, Tumor necrosis factor-α;

WHO, World Health Organization

1 Introduction

In 2009, nearly 88% of Canadian women between the ages of 15 and 55 who had given birth in the past five years breastfed their most recent infant, even if only for a short time, according to the Canadian Community Health Survey (CCHS) [1]. Over half (54%) of new mothers who initiated breastfeeding matched the 2001 World Health Organization (WHO) and 2004 Health Canada guidelines of exclusive breastfeeding for the first six months of life, while 16% breastfed for more than a year [1-2].

Human milk reflects a dynamic physiological system wherein fat composition is influenced by factors such as maternal diet, duration of pregnancy, or stage of lactation [3-4]. The composition of breast milk is highly variable with the energy and macronutrient contents changing significantly with increasing post-partum age until they stabilize by the end of the first month [5]. There are three sources of fatty acids in human milk: diet, mammary gland synthesis and mobilization from adipose, liver and other tissues [4, 6]. The regulation of fatty acid transfer through breast milk is a complex mechanism involving hormones, local synthesis, infant demand and maternal supply [7]. The increase in the lipid concentration especially omega 3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) from the beginning of all daily feedings (foremilk) to the complete expression of the breast (hindmilk) is well documented [3-4, 7]. Certain LCPUFA, such as docosahexaenoic acid (DHA), is present in human milk regardless of maternal dietary intake, although consumption of fish has a positive effect on the amount in milk [7]. Since 1929, dietary intakes of the essential fatty acids (EFA), which are the base structure for synthesis of the LCPUFA, arachidonic acid (C20:4 n-6, AA) and DHA (C22:6 n-3) have been manipulated experimentally to reveal their role in growth and development [8].

Bone mineral accrual during both childhood and adolescence is thought to play a vital role in preventing osteoporosis [9-10]. Human infant bone mass at birth is affected by a number of factors including maternal intake of calcium [11], magnesium, potassium, phosphorous [12], maternal physical activity and

smoking [13]. In humans, child cohort studies suggest that in utero exposure to maternal diet, particularly fat, has long lasting effects on bone mass to at least 8 years of age [12].

Evidence to date indicates that n-3 fatty acids, especially DHA, are positively associated with bone mineral accrual and with peak bone mineral density (BMD) in young men [9, 14]. The anabolic effects of n-3 fatty acids on bone health may be multifaceted [9, 15]. Postulated mechanisms include enhanced intestinal calcium absorption, reduced urinary excretion of calcium, reduced bone resorption, enhanced synthesis of bone collagen and inhibited production of cytokines such as interleukin 6 (IL-6) and tumor necrosis factor (TNF), which are implicated in the pathogenesis of bone loss [9, 14-15]. The LCPUFA such as AA and DHA are associated with bone mass by reducing bone resorption in animals and human adults [9, 16-17], yet no data exist for human infants beyond birth. Thus, the primary objective of this thesis was to determine if n-3 LCPUFA in maternal milk relates to infant bone mineral content (BMC), density and bone metabolism at 1 month of age. The secondary objectives were to: 1) quantify AA, eicosapentaenoic acid (C20:5 n-3, EPA) and DHA in human milk; and 2) describe the relationship between dietary LCPUFA intake and breast milk fatty acid composition.

2 Literature Review

2.1 Human Breast Milk

Human breast milk is species-specific therefore its composition is highly variable in energy and macronutrient content in addition to significant changes over the first month post-partum as is required to meet infant needs [5]. There are three phases of milk production: colostrum (1 to 5 d postpartum), transitional milk (6 to 15 d postpartum), and mature milk (after 15 d) [3, 18].

Breast milk is composed of 90 percent water and 10 percent solids including the macronutrients (fat, carbohydrate and protein) along with vitamins, minerals, enzymes, growth factors and anti-infective properties [19-20]. Human colostrum has a high level of antibodies and greater percentages of protein, fat-soluble vitamins and minerals than in transitional and mature milk [21]. Mature human milk is 3–5% (w/w) lipids that provide approximately 50% of the total energy (60-75 kcal/100 ml) value in support of the energy and tissue growth needs of the fast growing infant [4, 7, 22].

2.1.1 Breast Milk Carbohydrates

Lactose is the primary carbohydrate in human milk, although small quantities of fructose and galactose also are present [20]. The lactose levels in mature breast milk are relatively constant at 7.0 g/dL.[19]. Lactose enhances calcium absorption and is metabolized readily to galactose and glucose, which supply energy to the infant [20].

2.1.2 Breast Milk Proteins

Protein content in mature breast milk is about 0.8 to 0.9 g/dL; to support the infant energy metabolism, enzymatic reactions, development of the gastrointestinal tract and immunological purposes [19, 23]. Breast milk provides high quality protein, predominantly whey protein but also free amino acids, including essential amino acids, as well as nucleotides [19-20]. Whey

protein plays important roles in immunologic defense which is a unique quality of breast milk [19-20].

2.1.3 Breast Milk Vitamins

The amount of vitamins and micronutrients varies, depending on the diet and genetic differences of mothers. Generally, as lactation progresses, the level of water-soluble vitamins increases and the level of fat-soluble vitamins declines [20]. Human milk is a good source of vitamin A and vitamin E, but has insufficient amounts of fat-soluble vitamin D [20, 24]. The low levels of vitamin D in breast milk vitamin D increases the infant's risk of vitamin D deficiency that results in bone diseases, such as rickets [20]. Health policy in Canada recommends that all breastfed, healthy term infants in Canada receive a daily vitamin D supplement of 10 µg (400 IU) beginning at birth until the infant reaches one year of age [25].

2.1.4 Breast Milk Fatty Acids

In mature human milk, the lipid fraction (3–5%) is emulsified in globules suspended in the aqueous phase (87%) of milk [22]. The lipids are triacylglycerols (98%, TG), phospholipids (0.8%, PL), cholesterol (0.5%, Chol), and the remaining includes carotenoids, fat-soluble vitamins, some free fatty acids and cholesterol esters [4, 20, 22, 26]. The lipids of human milk and formula are of critical importance during the first year of life for several reasons as a major energy source to support appropriate growth and maturation of numerous organ systems [27-28].

There are three sources of fatty acids in human milk: diet, de novo synthesis by the liver or breast tissue and mobilization of endogenous fatty acids stores [4, 6, 29]. Several studies have shown that maternal dietary habits may have an important long- and short-term impact on milk fatty acid composition [4, 7, 22]. Consumption of a high dairy fat diet resulted in a greater milk lipid concentration compared with a low dairy diet [4.6 ± 0.5 vs. 3.8 ± 0.2 % milk lipid], respectively [30]. Anderson et al. stated that the consumption of regular margarine, compared with low trans fatty acids (TFA) margarine, decreased

milk fat in lean women [31]. The amount of short- and medium-chain fatty acids in the milk is influenced by high maternal carbohydrate intake and energy supply, while the PUFA are derived from mobilization of maternal stores or directly from the diet [4, 6]. Certain LCPUFA, such as DHA, is present in human milk regardless of the maternal dietary intake, although consumption of fish has a positive effect on its amount in the milk [7, 22, 32]. For example, Francois et al studied the effects of 6 dietary fats including menhaden oil and herring oil on breast-milk fatty acids after ingestion of a single fat rich meal; DHA increased significantly in human milk within 6 h of consumption ($P < 0.001$) reaching a peak in 24 h and remained significantly elevated for 2 d ($P < 0.05$) [33]. Thus, cultural traditions, social and economic status and the lactating mother's metabolism (individuality) play important roles in milk composition [4].

The fatty acids can be divided into two categories: saturated fatty acids (SFA) usually solid at room temperature and unsaturated fatty acids which are liquid at room temperature. The unsaturated fatty acids are of two classes: monounsaturated fatty acids (MUFA) with one double bond only, and polyunsaturated fatty acids (PUFAs) with multiple double bonds [28]. According to chemical structure, PUFA are divided into two categories depending on where the double bonds reside. The n-3 (i.e., omega-3) and n-6 (i.e., omega-6) denote the double bonds begin at the third and sixth carbon, respectively, from the methyl terminal [10], Figure 2-1. These lipids include essential fatty acids such as α-linolenic acid (C18:3 n-3, ALA) and linoleic acid (C18:2 n-6, LA) [4]. Both ALA and LA are substrates for LCPUFA such as EPA, DHA or AA; moreover these metabolites are important for fluidity of membrane lipids, prostaglandin synthesis and their presence in brain and visual cells suggests a critical role [27, 32, 34]. A smaller portion of breast milk fatty acids are esterified in the form of PL that surround and stabilize the lipidic core membrane of the fat globule of milk. PL also perform a nutritional function as suppliers of LCPUFA, nervonic acid (C24:1 n-9, NA), and choline which are needed to achieve optimal development and function in the newborn [3].

2.1.4.1 Diurnal Variation in Breast Milk Composition

The lipid content of human milk changes diurnally, also called "cyclic changes". Milk lipid increases as nursing proceeds and as Jensen reported, in the majority of breast feeding women the lipid content reaches a maximum about 8 h after a meal [22]. Hartmann et al. showed that the degree of breast emptiness/fullness is the primary factor that influences milk lipid concentration and explains almost 70% of the variation in fat content [7, 35]. The composition of breast milk changes significantly during the lactation period as a result of normal physiological events in the mother [23].

These fluctuations in lipid could may, in part, stem from differences in methods of collection of milk samples. The increase in the lipid concentration from the beginning of any feeding (foremilk) to the complete expression of the breast (hindmilk) is well documented [3-4, 7]. The increased availability of n-3 LCPUFA in hind milk provides an insight on the complex mechanism involving hormones, local synthesis, infant demand and maternal supply in the regulation of fatty acid transfer through breast milk [7]. Triglycerides (TG) concentration during nursing, rapidly increases from foremilk to hind milk [36]. According to da Cunha et al., the milk fat increases up to 52% due to feeding stimulus and in boys the hind milk fat content is higher as compared to girls due to the higher suckling power which provides higher volumes of milk that are more energy dense [7]. Therefore, studies regarding milk LCPUFA should employ standardized milk collection methodologies along with consideration for gender effects.

2.1.4.2 LCPUFA in Breast Milk

Linoleic acid (C18:2 n-6, LA) and α-linolenic acid (C18:3 n-3, ALA) are considered as the parent compounds of PUFA fatty acids families [27, 37]. ALA and LA are converted to longer chain, more highly unsaturated fatty acids through enzymatic chain elongation (elongases ELOVL2 and ELOVL5) and desaturation (Δ6-desaturase and Δ5-desaturase) [25, 30], Figure 2-1. ALA is converted to EPA (C20:5 n-3) then on to DHA (C22:6 n-3), whereas LA is converted to gamma linolenic acid (C18:3 n-6, GLA) and AA (C20:4 n-6)

[28]. Human milk provides LA, ALA, DHA, AA, and other LCPUFA to breastfed infants. The AA level is relatively constant on a worldwide basis whereas the level of DHA is more variable and depends on maternal diet and lifestyle. Population means of AA in human milk range between 0.35 – 0.7 weight % of total fatty acids, whereas means of DHA ranges between 0.17% – 1.0% of total fatty acids [27].

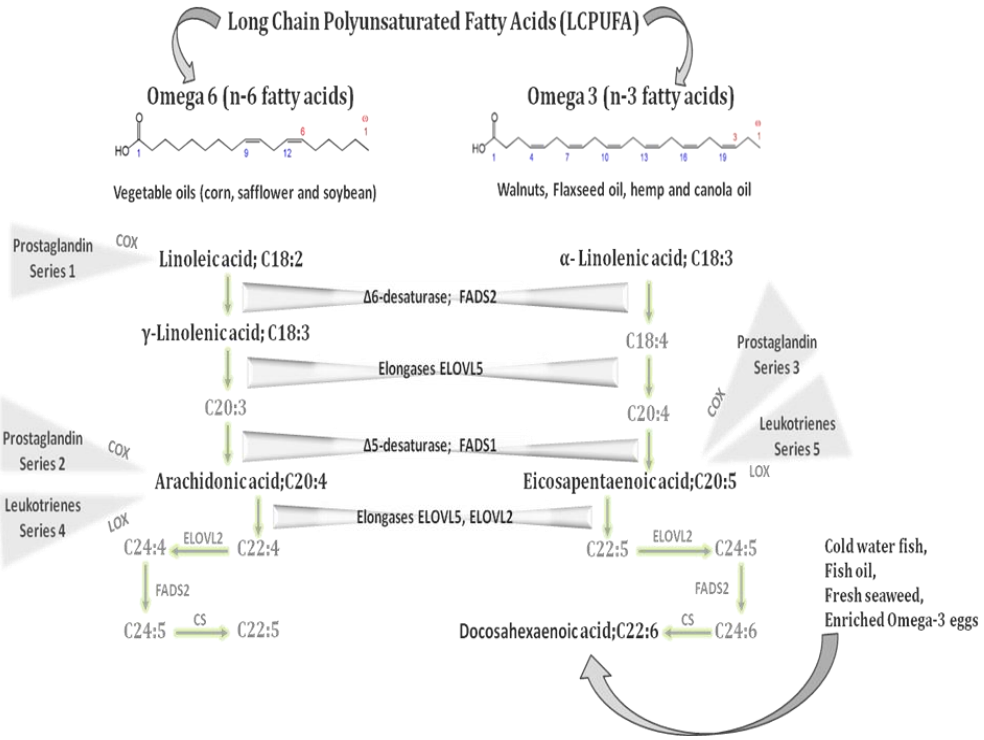

Figure 2-1 Dietary Sources and Metabolism of Long-Chain Polyunsaturated Fatty Acids and it Mediators (LCPUFA); COX (Cyclooxygenase); LOX (Lipoxygenase), adapted from [15, 27-28, 37-38].

2.1.4.3 Sources of LCPUFA

Sources of LCPUFA during the first year of life include human milk, infant or follow-on formula enriched with LCPUFA and complementary foods such as egg, meat and fatty fish [27]. Maternal dietary changes are reflected in breast milk composition, which at the population level exist as decreasing n-3 LCPUFA content and increasing n-6 LCPUFA levels over a 20-y period [39].

Vegetable oils are rich in LA, while seed oils are the richest sources of ALA, notably those of rapeseed (canola), soybeans, walnuts, flaxseed (linseed), perilla, chia and hemp [37-38]. The n-3 LCPUFA, EPA and DHA are found in

fish (salmon, herring), fish oils and n-3 enriched or fortified products such as n-3 enriched eggs [28, 37], Figure 2-1.

The dietary sources of AA are meats, poultry and eggs, but it is obtained largely by endogenous synthesis from LA [27, 37]. In undernourished women the dietary intake of AA is generally low, but the relative content in their milk is similar to that of well nourished women [40-41]. This observation suggests that the secretion of n-6 LCPUFA into milk lipids does not depend solely on maternal dietary intake. Del Prado et al, showed that the major source of LA and AA in human milk originates from maternal body stores (70% and 90%, respectively) [32].

2.1.4.4 Recommendation of Breast Milk LCPUFA

Numerous studies have evaluated the effects and safety of LCPUFA supply to pregnant and lactating women; taking into account the use of DHA alone or fish oils with various levels of DHA and EPA [27]. The Workshop on the Essentiality of and Recommended Dietary Intakes (RDIs) for Omega-6 and Omega-3 Fatty Acids, recommended reduction of n-6 PUFA even as the n-3 PUFA are increased in the diet of adults and newborns for optimal health and to reduce adverse effects of excesses of AA and its eicosanoid products [42]. However, a recent European Commission consensus recommendation based on systematic literature review and an expert consensus process advised that pregnant and lactating women should aim to achieve an average daily intake of at least 200 mg DHA [27]. Whereas the Workshop on the Essentiality of and RDIs for n-6 and n-3 FA, recommended an intake of 300 mg/d [42]. Intakes of up to 1 g/day of DHA or 2.7 g/day of n-3 LCPUFA have been used in randomized trials without occurrence of significant adverse effects. Women of childbearing age can meet the recommended intake of DHA (200 to 300 mg) by consuming 1 to 2 portions of sea fish per week, including fatty fish, which is a good source of n-3 LC-PUFA. Fish consumption may increase the exposure of the mother and fetus to contaminants such as methylmercury, polychlorinated biphenyls (PCB) and dioxins and also increase the levels of these contaminants in breast milk. The recommended intake of fatty fish,

which is supported by the World Association of Perinatal Medicine and the Early Nutrition Academy and the Child Health Foundation, rarely exceeds the tolerable intake of environmental contaminants [27]. Dietary fish should be selected from a wide range of species without undue preference for large predatory fish, which are more likely to be contaminated with methylmercury [27].

Even though some experts share concerns about excess AA and its eicosanoids which may result in adverse health, this is not likely as applicable to neonates since mother's milk AA is maintained relatively constant [27]. The adverse effects, however, are potentially applicable to infant formula. These can be avoided by two interdependent dietary changes; reduced amount of n-6 and/or increase the n-3 PUFAs in the diet [42]. Therefore, recommending a balance (n-6):(n-3) ratios is important in artificial milk replacers or formula. In Canada, the recommended ratio of (n-6):(n-3) PUFA for infant formula is between 16:1 and 4:1 which is similar to the recommendations of the United States (16:1 to 6:1) and in Europe (15:1 to 5:1) [8]

2.1.4.5 LCPUFA as Mediators of Cellular Signalling

Long-chain PUFA serve as precursors in the production of pro-resolving lipid mediators, including lipoxins synthesized from AA, E-resolvins synthesized from EPA and D-resolvins synthesized from DHA [43-44]. Lipoxins and resolvins appear to have a myriad of effects that promote the resolution of inflammation and lipid mediators have been found to reduce bone loss induced by periodontitis in animal models [45-47], Figure 2-1.

The predominant precursor fatty acid is AA; due to its high concentration in membrane phospholipids, AA is highly regarded as an important precursor of eicosanoids [27]. AA can be oxygenated by three different enzymatic systems: cyclooxygenases [forming prostaglandins and thromboxane], lipoxygenases [forming leukotrienes] and cytochrome P450 monooxygenases [forming 19- and 20 HETE] [27]. The biological activities of eicosanoids are extensive, for example, prostaglandins and leukotrienes participate in the local control of bone metabolism [27, 48-51]. Prostaglandin E_2 (PGE_2) is an important

product of AA and the major prostaglandin affecting bone metabolism [27, 48-51]. PGE_2 influences both bone formation and resorption and its effect is dose dependent. At low levels, it enhances bone formation by osteoblasts, while at higher levels PGE_2 suppresses osteoblast differentiation [51] and promotes bone resorption by osteoclasts [52], refer to section 2.2.2. Excess arachidonic acid and its eicosanoids can cause adverse effects which can be avoided by interdependent dietary changes [42]. Hence, eicosanoids derived from n-3 FA have much less biological potency than those derived from n-6 FA (AA). In addition, n-3 FA are potent inhibitors of cyclooxygenase [53]. Thus, the n-6:n-3 ratio defines the net balance of eicosanoids derived from n-6 and n-3 and the biological response elicited after eicosanoids release. Reduction of dietary n-6 has been associated with lower PGE_2 synthesis and increased bone formation in growing rats [54], decreased loss of bone weight and strength in ovariectomized adult rats [55] and reduced osteoclastic activity [56].

2.2 Bone Health and Metabolism

Knowledge about fetal and neonatal bone development in humans is based mainly on the study of nonhuman vertebrate species due to ethical and methodological difficulty in examining bone [57-58]. However, findings of animal models may not be applicable to the human situation because they fail to replicate key characteristics of the human feto-placental unit or the postnatal adaptation process [57]. The bones of the skeleton provide structural support for the whole body and protection for its organs, maintenance of mineral homeostasis and acid-base balance, a reservoir of growth factors and cytokines, and provide the environment for hematopoiesis within the marrow spaces [59-61]. There are four general categories of bones: short, long, flat and irregular bones [61]. Bone is generally classified into two types: cortical bone (compact bone) and trabecular bone (cancellous or spongy bone) which is metabolically more active than cortical bone [61]. Cortical and trabecular bones are normally formed in a lamellar pattern, in which collagen fibrils are laid down in alternating orientation, but this pattern is absent in woven bone where the collagen fibrils are laid down in a disorganized manner. Therefore,

woven bone is weaker than lamellar bone. Woven bone is normally produced during formation of primary bone and may also be seen in high bone turnover states [61].

During the fetal and neonatal periods, bone development is characterized by extremely rapid growth. In the 7^{th} month of fetal life, body length increases by ~45 cm. After a brief deceleration at around birth time, linear growth resumes at a rate that is about twice as fast as at the peak of the pubertal growth spurt [57]. Longitudinal and radial growths occur during childhood and adolescence [61]. Longitudinal growth occurs at the growth plates, where cartilage proliferates in the epiphyseal and metaphyseal areas, before subsequently undergoing mineralization to form primary new bone [61].

Bone mass in adulthood depends upon the peak attained during childhood and adolescence, and on the subsequent rate of bone loss [62]. Calcium, phosphorus, vitamin D, magnesium, proteins and fluoride have different effects on bone mass along with exercise which elicits osteogenic responses in bone development [63]. At all ages, the dietary essentials for skeletal health are calcium, vitamin D, protein along with zinc, copper, vitamin A, C, D and K [12, 28, 63-64]. The fetus accumulates 80% of the bone calcium, phosphate, and magnesium during the last trimester [65]. Recently the balance of n-6 to n-3 fatty acids has been acknowledged as beneficial to skeletal growth and mineralization [10, 54, 66] and may also associate with neonatal bone.

2.2.1 Calcium / Vitamin D

Calcium is an essential ion in all organisms and participates in a variety of structural and functional roles, it accounts for 1 to 2% of adult human body weight [60, 67]. 99% of body calcium is located in bone and teeth in the form of hydroxyapatite (Ca_{10} $(PO_4)_6$ $(OH)_2$) [59-60]. The remaining 1% of body calcium is present in blood, extracellular fluid, muscle, and other tissues, where it plays a role in mediating vascular contraction and vasodilation, muscle contraction, nerve transmission, and glandular secretion [59-60, 68].

The path of calcium metabolism involves ingestion, digestion, absorption and excretion in feces, urine or sweat [68]. Calcium is absorbed across the enterocyte by active transport mechanisms or by passive diffusion between enterocytes [60]. At low and moderate intake levels, most of calcium absorption occurs through active transport of calcium which is dependent on the active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), and its intestinal receptor. While at high calcium intakes, passive diffusion becomes the main absorption mechanism, it represents the movement of calcium between mucosal cells and is dependent on the luminal to serosal calcium concentration gradient [60, 68]. Through the lifespan the fractional calcium absorption varies. It is highest (about 60%) in infancy and rises again in early puberty. In young adults fractional absorption remains at about 25%, with the exception that it increases during the last two trimesters of pregnancy [60]. The urinary loss of calcium decreases with aging possibly because of an age-related decrease in intestinal calcium absorption efficiency and an associated reduction in filtered calcium load while the endogenous fecal calcium excretion does not change appreciably with aging [60]. The body maintains plasma calcium around ~2.5 mmol/L, regulated primarily by the parathyroid hormone (PTH) [60, 68]. High calcium demand is present throughout the course of lactation to accommodate the 300 mg calcium that is required daily to meet the demands of breast-milk production in exclusively breastfeeding women. Low calcium intake during pregnancy is associated with reduced BMC in newborns [69].

2.2.2 Bone Remodeling and "Calcium Homeostasis"

Bone is a dynamic tissue, which is constantly undergoing osteoclastic bone resorption and osteoblastic bone formation throughout life [10, 15, 60, 62, 70]. Bone remodeling begins before birth and continues until death [61]. This permanent renewal process is essential to maintain bone strength and mineral homeostasis [10, 15, 61]. The remodeling cycle is composed of four sequential phases: activation precedes resorption; which precedes reversal; which precedes formation [61]. Osteoclastogenesis is regulated by various systemic

and local factors including hormones, growth factors, eicosanoids and immune mediators [15, 61, 71]. The receptor activator of nuclear factor-κB ligand (RANKL) present on the osteoblast cell membrane as well as its receptor, which belongs to the tumor necrosis factor-α (TNF-α) family, and their activation leads to osteoclastogenesis [15, 71-72]. Osteoprotegerin (OPG) is a decoy receptor for RANKL which prevents RANKL/RANK induced osteoclastogenesis; therefore increased OPG levels reduce osteoclast number. Both RANKL and OPG are produced by osteoblasts [15]. Mature osteoblasts release the lipid mediator prostaglandin E_2 (PGE_2); it promotes osteoclastogenesis by stimulating expression of both RANKL and RANK, and inhibiting expression of OPG [15, 71]. The inflammatory cytokines IL-1 and TNF-α are potent stimulators of bone resorption and inhibitors of bone formation and also stimulate IL-6 production. It is well established that reactive oxygen species (ROS) and oxidative stress are key factors in regulating bone metabolism [10], Figure 2-2.

During bone growth, resorption and formation are not coupled whereas after cessation of growth they should occur in a balanced ratio for maintenance of bone mass [10, 60]. Peak bone mass is reached between the ages of 20 to 30 y, followed by a gradual decrement in bone density at a rate of about 0.5‑1% annually [10]. The rate of cortical (compact) bone remodeling can be 50% per year in young children and about 5% per year in adults. In adults, trabecular (cancellous) bone remodeling is about five-fold higher than cortical remodeling [60].

Several factors influence the accumulation of bone mineral during childhood and adolescence including heredity, sex, diet, physical activity, endocrine status, and sporadic risk factors such as cigarette smoking [62]. Genetic factors are responsible for ≈70% of the variance in bone mass [9-10], but Cole and Cooper suggested that although peak bone mass is inherited, current genetic markers explain only a small proportion of the individual variation in bone mass or fracture risk [62]. The remaining 30% depends on the phenotype; in which physical activity and/or nutrition can induce physiological responses

that support attainment of higher bone mass [10, 62-63]. Ethnic differences in bone mass in adults and children are widely reported [73].

The combination of vitamin D and calcium, which are arguably the most important nutrients in bone metabolism, has a clear synergistic effect on bone mass in all age groups [63]. The hormonally active form of vitamin D, calcitriol ($1,25(OH)_2D$), acts primarily on the small intestine to promote absorption of calcium and phosphate from ingested foods [74-75]. Genetic and environmental factors have also been shown to affect the vitamin D status of newborns, for example, infants of mothers with darker skin [73] or those who are born during the winter/early spring [76] have low 25 hydroxyvitamin D (25(OH)D). Recent recommendations for adequate intake of vitamin D_3 have been set at 5 - 10 μg/day for children of all ages in the absence of exposure to sun [63, 77]. The other main component of bone is phosphorus, which together with calcium composes hydroxyapatite, the main crystalline salt of bone. Calcium homeostasis is controlled by some substances like PTH, calcitonin, OPG, and the active form of vitamin D [59, 75, 78-79]. The Ca:P ratio associated with maximum BMC and BMD depends on the influence of other factors, such as low vitamin D intake, caffeine intake, drug and alcohol intake and age [63]. Moderate-to-high dietary protein intake has positive effects on bone health, most obviously increased bone growth and peak bone mass in children and increased BMD and a reduced rate of bone loss in adults [74]. Over the past years evidence has been growing on the effects of dietary fatty acids on bone health [10].

2.3 Fatty Acids and Bone Health

2.3.1 Adult Animal Intervention Studies

Many animal studies showed a positive influence of n-3 fatty acids or a low ratio of n-6 to n-3 fatty acids on bone. In 1994, Sakaguchi et al. concluded that EPA inhibited bone loss in ovariectomized rats that were maintained on a low calcium diet [55]. Shortly after, Yamada et al. reported that both EPA and DHA prevented bone fragility in diabetic rats and that EPA prevented

osteopenia even in diabetic rats fed a low zinc diet which was used as a potent accelerator of diabetic osteopenia [80]. In rats, n-3 PUFA deficiency caused severe osteoporosis. Moreover, when the deficient animals were replenished with n-3 PUFA, the ratio of n-3 to n-6 PUFA in bone compartments was restored and the process of bone loss was reversed [81]. However, not all ovariectomized rats studies showed beneficial skeletal effects of n-3 [82]. Long-term intake of n-3 fatty acids, especially EPA, improved structural and mechanical properties of cortical bone in the femur in intact female mice without detectable effects on age related loss of trabecular bone or BMD [83] .

2.3.2 Infant Animal Intervention Studies

Dietary supplementation with combinations of AA, EPA or DHA is positively associated with bone mass in infant animals [66, 84-85]. Among these studies, the LCPUFA supplementation amounts ranged from ~1 g/100 g fat using semi-purified AA and DHA combined in piglets to 17 g/100 g fat using fish oil in chicks [84, 86]. However, feeding large amounts n-3 LCPUFA as fish oil (>80 g/100 g fat) during rapid growth postweaning is detrimental to bone growth and biomechanics of tibia of male rabbits and spine of female rats [87-88]. In another study, during late gestation and throughout lactation rat dams were fed soybean oil (n-6 and n-3 essential fatty acids in a 9:1 ratio) compared with linseed oil (predominantly n-3 essential fatty acids) or sunflower seed oil (predominantly n-6 essential fatty acids). The soybean oil group offspring had higher femur length (mm), cortical cross-sectional area and BMC at 30 wk of age compared to the other groups [89].

2.3.3 Adult Human Epidemiological Studies

In spite of the strong evidence of the dietary fats positive effects on bone metabolism from animal and *in vitro* studies, few studies have been conducted in humans [10]. In a longitudinal study of 891 women aged between 45–55 years were followed up 5–7 years later to evaluate the influence of diet on postmenopausal bone loss. Greater loss of femoral neck BMD was observed with increased intake of PUFA (r = -0.11, P<0.01) and MUFA (r = -0.069,

P<0.05) suggesting that diet may influence early postmenopausal bone loss [90]. In the Rancho Bernardo cohort of elderly, community dwelling men and women, self-reported food-frequency demonstrated that an increasing ratio of dietary n-6 to n-3 fatty acids was significantly associated with lower BMD [91]. Another cohort study evaluated role of serum fatty acids on bone accumulation and attainment of peak bone mass in 78 healthy young men at three age points (16, 22 and 24 years). The results showed that n-3 fatty acids, especially DHA, are positively associated with total body and spine BMD at 16 and 22 years of age. In addition, BMD of the spine measured at 22 years of age showed an inverse association with the ratio of serum n‑6 to n‑3 fatty acids (5.4:1) which emphasizes the role of n‑6 to n‑3 ratio [9]. A study on premenopausal Japanese women showed that the highest BMD scores was among those who consumed more fish and shellfish than those women with greater intake of meat, fats, and oils [92]. Thus there is ample epidemiological evidence of a relationship between dietary LCPUFA and MUFA with bone.

2.3.4 Adult Human Intervention Studies

Intervention trials in humans are limited and the results of human experimental studies on the effects of PUFA supplementation and bone are inconclusive and vary in type, concentration and dosing of n-3 [28]. Randomized trial of 40 osteoporotic patients were supplemented with n-3 PUFA (fish oil) for 16 week showed better calcium absorption and stimulation of osteoblast activity, detected by a rise in osteocalcin and procollagen as markers of bone formation while the placebo subjects showed no improvement [93]. To control for confounders due to background diets, a three-period cross-over feeding trial was designed with three test diets: average American diet (AAD), Linoleic Acid Diet (LA) and α-Linolenic Acid Diet (ALA). Results indicated dietary n-3 PUFA may have a protective effect on bone metabolism via a decrease in bone resorption since bone turnover marker; the serum N-telopeptides (NTx) levels were significantly lower following the ALA diet (13.20 ± 1.21 nM BCE) ($p < 0.05$) [94].

Over an 18-month period, 65 osteopenic postmenopausal women were supplemented with 600 mg/day calcium as the carbonate and divided into active treatment group receiving [LA (60%), ALA (8%), EPA (4%) and DHA (3%)] and placebo group receiving capsules [contained 6 g of coconut oil (97% saturated fat and 0.2% LA)]. Results indicated a decrease in bone turnover; the osteocalcin and deoxypyridinoline levels fell significantly in both groups, whereas lumbar spine density remained the same in the treatment group, but decreased 3.2% in the placebo group. Femoral BMD increased 1.3% in the treatment group, but decreased 2.1% in the placebo group. Twenty-one patients continued on treatment for a second period of 18 months; lumbar spine BMD (36 months) increased 3.1% in patients who remained on active treatment and 2.3% in patients who switched from placebo to active treatment, and femoral BMD in the latter group increased by 4.7% [95]. In contrast, 12-month randomized trial of 43 premenopausal women and 42 postmenopausal women received either Efacal® (containing 4 g of primrose oil, 1 g of calcium, and 440 mg of marine fish oil) or placebo failed to show an effect on BMD [96].

2.3.5 Infant Human Studies

No studies of the possible role of n-3 fatty acids in bone mineral accrual in human infants beyond term birth have been conducted. Only one infant human study was conducted on 30 mother-infant pairs to study LCPUFA status association with bone mass in full-term new born infants. Weiler et al. measured maternal and cord blood red blood cells (RBC) for AA, EPA and DHA. Cord RBC AA and maternal RBC AA were positively correlated with whole-body BMC (r = 0.61, p = 0.0032; r = 0.52, p = 0.014, respectively). AA:EPA positively correlated with lumbar spine 1–4 BMC (r = 0.44, p = 0.0206) and femur BMC (r = 0.39, p = 0.044) [16]. Mother's blood cells DHA was negatively associated with infant's spine and femur BMC (r = -0.46, p = 0.012; r = -0.45, p = 0.018, respectively) but that was not observed at the whole body level. The authors suggested that the maternal diet should be

balanced in n-6 and n-3 LCPUFA because the imbalances among the n-6 and n-3 LCPUFA by term gestation was associated with lower bone mass [16].

2.4 Mechanism of Fatty Acids Effect on Bone Health

The mechanisms by which PUFA influence bone health are wide ranging: opposing effects on inflammatory cytokines [97], modulation of PGE_2 production [49], enhancement of calcium transport and reducing urinary calcium excretion [98-99], Figure 2-2. PUFA n-3 and n-6 FA and their derivatives have been shown to serve as ligands for peroxisome proliferator–activator receptor-α and -γ [100], which have been found to inhibit the function of nuclear transcription factor-κB (NF-κB) [101-102] and to be involved in the differentiation of mesenchymal stem cells to adipocytes or osteoblasts [103-104], respectively.

One possible mechanism of the n-3 fatty acids anabolic effect on bone is by enhancing intestinal calcium absorption as shown in the rat models [98, 105]. However, this is not as likely to occur in neonates since calcium absorption is already high [106].

Another mechanism shown by several human studies is the dramatic decrease in cytokine production following n-3 fatty acid supplementation [28]. In human/animal supplementation trials using n-3 LCPUFA the findings were significant reduction in the production of IL-1β, IL-1α and TNF-α in peripheral blood mononuclear cells, and also lack of elevation of RANKL expression [107-109]. These studies underlie one of the mechanisms by which dietary n-3 fatty acids reduces bone loss by inhibition of osteoclast generation and activation by decreasing proinflammatory cytokine production (IL-1, IL-6 and TNF-α) which result in decreasing NF-κB expression and RANKL signaling [110]. In MC3T3-E1 osteoblast-like cells, the inhibited proinflammatory cytokine production down regulates cyclooxygenase (COX)-2-dependent prostaglandin (PG) synthesis which promotes osteoclastogenesis by: 1) stimulating expression of both RANKL and RANK [111], 2) down-regulating the mRNA levels of OPG [111-112], and 3) expression of insulin-

like growth factors (IGFs) [113], with a net diminution of bone resorption [15, 48-49, 71].

In contrast, n-6 PUFA enhance the production of pro-inflammatory cytokines and reactive oxygen species such as the inducible nitric oxide (NO) that has the potential to mediate some of the deleterious effects associated with cytokines on bone resorption [114]. Therefore the ratio of total n-6 to total n-3 PUFAs is important [10].

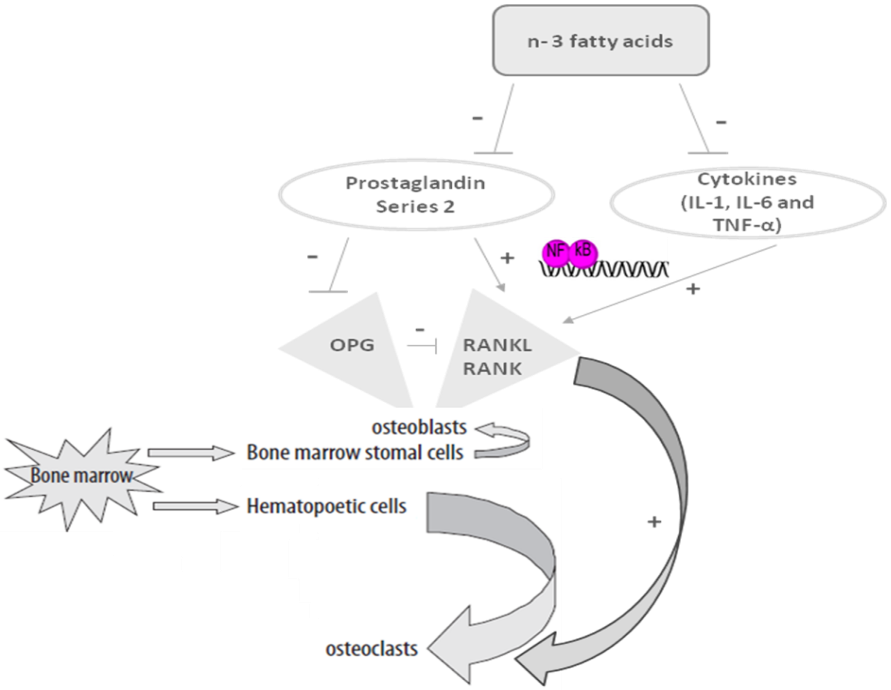

Figure 2-2 Mechanism of Fatty Acid Effects on Bone Health; OPG (Osteoprotegerin); RANKL (Receptor Activator of Nuclear Factor-κB Ligand); RANK (Receptor Activator of Nuclear Factor-κB); IL-1 (Interleukin-6); IL-6 (Interleukin-1); TNF-α (Tumor Necrosis Factor-α); NF-κB (Nuclear Factor-κB), adapted from [10, 110-112].

2.5 Ratio of n-6 to n-3 effect on Bone

The dietary n-3 LCPUFA EPA and DHA, partially replace the n-6 fatty acids, particularly AA, in the membranes of platelets, erythrocytes, monocytes and liver cells leading to a change in the ratio of n-6 to n-3 fatty acids in membranes, and a change in their function which can decrease the production of IL-1, IL-6 and TNF-α [115-116]. Therefore, it is speculated that these changes might underlie the negative association between n-6:n-3 fatty acids

with BMD in adults [37] and newborn infants [16]. In neonatal piglets low levels of dietary AA:DHA (0.5:0.1 g/100 g of fat) elevate bone mass, but higher amounts are not beneficial [17]. People who habitually consume a high-fish (high n-3 LCPUFA) diet, such as the Japanese and Greenland Eskimos, have a very low incidence of osteoporosis [15]. Although a negative association between total LCPUFA intake and BMD was observed in one study in postmenopausal women [82], a more recent study that examined dietary intake of the 2 families of LCPUFAs reported that postmenopausal women with a high dietary ratio of n-6:n-3 fatty acids had the lowest BMD [82]. Therefore, high n-6 LCPUFA intake rather than high total LCPUFA intake may be detrimental to bone mass. In a longitudinal study in adolescent males, concentration of n-3 LCPUFAs in the phospholipid fraction of serum was positively correlated with change in total body and spine BMD [14]. The association was greatest between serum phospholipid DHA concentration and BMD, which may indicate that specific LCPUFAs have anabolic effects on bone [15]. Furthermore, fish intake is associated with higher BMD [117]. However, it is important for exclusively breastfed infants to receive a balance between n-6 and n-3 fatty acids [118]. The ratio of total n-6 to total n-3 PUFA in breast milk is reported to range between 5 and 15 [118]. Imbalances among the n-6 and n-3 LCPUFA by term gestation are associated with lower bone mass, suggesting that the maternal diet should be balanced in n-6 and n-3 LCPUFA [16]. A negative association between higher ratios of n-6 to n-3 fatty acids and BMD was also found in a study of elderly men and women [37]. Whether higher maternal fish intake, and therefore n-3 LCPUFA enriched maternal milk, has an effect on infant bone has not been investigated. Such study is of high importance in view of recommendations that pregnant and lactating women consume DHA as part of their daily routine in support of infant development.

3 Manuscript

Relationship between Breast Milk Fatty Acids and Infant Bone Mass and Metabolism

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3.1 Abstract

Long chain polyunsaturated fatty acids (LCPUFA) including arachidonic (AA) and docosahexaenoic (DHA) acids are associated with bone mass by reducing bone resorption in animals and human adults, yet no data exist for human infants beyond birth. The primary objective of this study was to determine if human milk LCPUFA relates to infant bone mineral content (BMC), density (BMD) and metabolism. A cohort of healthy breastfeeding infants (n=120) was studied at 1 month post-partum for anthropometric measurements and BMC and BMD were assessed using dual-energy x-ray absorptiometry (DXA). Plasma parathyroid hormone, 25-hydroxyvitamin D and ionized calcium, plus urinary calcium:creatinine were measured. Maternal dietary intake was assessed using 24 h recall and a 3 mo food frequency questionnaire. Representative breast milk samples were collected. At birth and 1 mo of age, all anthropometry was within the normal range of the World Health Organization growth standards. Maternal intake of DHA and eicosapentaenoic acid (EPA) during pregnancy (r = 0.42, p<0.0001; r = 0.46, p<0.0001; respectively) directly associated with milk LCPUFA composition. Maternal intake of DHA and EPA the day before showed similar results (r = 0.43, p<0.0001; r = 0.51, p<0.0001; respectively). Correlation analysis revealed a negative correlation between breast milk DHA (r = - 0.18, p = 0.05) and AA (r = - 0.19, p = 0.04) and infant lumbar spine vertebrate 1-4 BMC but these relationships were not evident in multivariate analyses. In multivariate analysis, adjusted for infants' weight, age, gender, ethnicity, vitamin D supplementation and maternal smoking and pre-pregnancy body mass index (BMI), breast milk LCPUFA was not related to infant bone mineral density (BMD) nor BMC. Breast milk LCPUFA do not explain the variance in bone mass early post-natally based on the multivariate analysis. However, since dietary intake may not reflect LCPUFA status, further studies are warranted using measures of infant LCPUFA status along with bone mass to confirm these observations.

3.2 Introduction

Bone mass in adulthood depends upon peak bone mass attained during childhood and adolescence, and on the subsequent rate of bone loss [9, 62]. Therefore, recognition of the influencing factors is important in preventing osteoporosis and its related fractures [10, 119]. Evidence has been growing over the past years that long chain polyunsaturated fatty acids (LCPUFA) are positively associated with bone mineral accrual [9, 14, 80]. Several mechanisms by which LCPUFA affect bone have been suggested including opposing effects on inflammatory cytokines [97], modulation of PGE_2 production [49], enhancement of calcium transport and reduced urinary calcium excretion [98-99].

During the 20th century, there was dramatic change in food formulations resulting in greater dietary intake of plant oils (e.g. corn, safflower and soybean oil) which are high in linoleic acid, the substrate of AA, and resulted in a elevated ratio of n-6:n-3 fatty acids [120]. Since AA is highly regarded as an important precursor of eicosanoids such as PGE_2 [27, 48-51]; the net result of those dietary changes is higher levels of PGE_2 which leads to suppression of osteoblast differentiation [51] and promotion of osteoclastogenesis [52], and eventually bone resorption. Such adverse effects can be avoided by balancing the ratio of n-6:n-3 fatty acids which appear to be beneficial to skeletal growth and mineralization [10, 54, 66].

Many studies done on adult/infant animals showed a positive influence of n-3 fatty acids or a low ratio of n-6 to n-3 fatty acids on bone [55, 80-81, 83-85]. In spite of the strong evidence that dietary fats positively affect bone metabolism in animal and *in vitro* studies, few studies have been conducted in humans [10]. Based on the limited studies in humans, the effects of PUFA supplementation on human bone are inconclusive [28]. Moreover, there is a disjoint between adult and fetal/neonatal studies and even though LCPUFA have beneficial effects on bone mass in animals and human adults [9, 16-17], the effect might be the reverse in the fetus or neonate. Infant bone (woven bones) is different from adult bone (lamellar bone) [61] and in contrast to

adults, bone resorption and formation are not coupled in infants [10, 60]. Since there is no data for human infants beyond birth, the primary objective of this research was to determine if LCPUFA in maternal milk relates to infant BMC, BMD and bone metabolism at 1 month of age. The secondary objectives were to: 1) quantify AA, EPA and DHA in human milk; and 2) describe the relationship between dietary intake and breast milk LCPUFA content.

3.3 Methods

The study was conducted at the Mary Emily Clinical Nutrition Research Unit, McGill University under the umbrella of ongoing clinical trial (NCT00381914). Using the baseline time-point, a cohort of n=120 mother-infant dyads at one month post-partum was studied, such age was chosen because human breast milk composition stabilizes by the end of the first month [5]. Recruitment was facilitated through pediatric clinics in Montreal where mothers and infants were prescreened by pediatricians at the first postnatal visit and referred to the study. The inclusion criteria included full term (between 37- 42 gestation weeks) healthy singleton infants, appropriate size for gestational age (AGA) between the 5^{th} - 95^{th} percentile for weight and gender using the Centers for Disease Control growth charts [121] and predominantly breastfed (80% of feeds) by healthy women.

Exclusion criteria included infants born to mothers with a medical condition that may have affected pregnancy outcomes, nutrient absorption, or maternal-fetal transfer of nutrients such as gestational diabetes, gestational hypertension, malabsorption syndromes (Crohn's disease or celiac disease), diabetes, alcohol use, liver disease or kidney disease. Additionally, mothers taking medications that may affect absorption or utilization of nutrients were also excluded (e.g. anticonvulsants and bile acid sequestrants). Infants included in this analysis represent the first 120 infants to enrol in the study.

Ethics approval was granted by the Institutional Review Board of Medicine, at McGill University.

3.3.1 General Demographics

General health information including supplement and medication use was collected using a researcher-administered survey (Appendix 1). Maternal age, pre-pregnancy weight, weight gain in pregnancy, previous pregnancies, live births, height and weight of mother, family income range and number of dependent members, employment, ethnicity and level of education were self-reported.

3.3.2 Anthropometric Measurements

Size at birth (weight, length, head circumference) and gestational age was obtained from the vaccine carnet. At the one month visit infants were weighed in standardized gowns and clean diapers, to the nearest 0.1 g using an electronic scale with a movement program (model SB 32000, Mettler-Toledo Inc., Greifensee, Switzerland), the weight of gown and diaper was subtracted from the infant's final weight. Recumbent length and crown-heel length were measured, to the nearest 0.1 cm, using an infant length board (O'Learly Length Boards, Ellard Instrumentation Ltd., Washington, USA). Head circumference was measured, to the nearest 0.1 cm, using a non stretchable tape (model 212, Seca, Hanover, USA). Using the data collected, z-scores were calculated for weight, length and head circumference indexed for age and sex using ANTHRO software which is based on the 1978 NCHS/CDC reference [122].

Mothers were weighed to the nearest 0.5 kg in their casual clothes, without shoes using a balance beam scale (model 242, Seca, Hanover, USA). Standing height to the nearest 0.1 cm was measured using a digital stadiometer (model 242, Seca, Hanover, USA). Body mass index (BMI; kg/m^2) was computed.

3.3.3 Dietary Data

Maternal dietary and supplement intake during pregnancy was assessed using a validated food frequency questionnaire, the modified Willett/Harvard (Appendix 2) [123-124]. This questionnaire was modified to assess intakes over the months of pregnancy and analyzed using the Canadian Nutrient File. Additionally, the 24-hour recall method was conducted in person by a registered dietitian using a multiple pass method, including item, quantity (food models were used to estimate portions consumed), time of day, and cooking method (Appendix 3). All dietary data was analyzed using Nutritionist Pro Software (Axxya Systems, Stafford, USA) which includes data from the Canadian Nutrient File 2007b. Use of prenatal vitamin and mineral supplements was included in the analysis.

3.3.4 Breast Milk Procurement and Measurement

Breast milk samples (30-50 ml) were collected to represent a full feed by the infant since hind milk contains most of the fat; the mothers were asked to pump from one breast while the infant was nursing from the other. If that was not feasible then she pumped for a standard duration of feed and a minimum of 5 minutes using an electronic breast pump since lipid content rises as a nursing proceeds [22, 125]. The samples were immediately preserved at –80˚C [39, 126].

3.3.4.1 Milk Fatty Acids Analysis Method

To decide upon the analytical method, a pooled sample was created and spiked with different amounts of unmethylated DHA (Sigma Chemical Co.; catalog no. D-2534, St. Louis, MO, USA) and C17 and C19-PC internal standards added. The spiked and non-spiked pooled samples were analyzed using two methods: modified Folch and Lopez-Lopez. The recovery of DHA, C17, C19-PC were similar in both methods with coefficients of variation (CV) of 9.7%, 6.9% and 6.5%; respectively. To evaluate DHA recovery, the pooled samples were analyzed using Lopez-Lopez method using three different methylation times (15 min, 30 min, and 1 h) since Lopez-Lopez method requires only 15 min for methylation compared to an hour in the Folch method. Sodium methoxide transesterifies milk tri-, di- and mono-glycerides completely in 15 minutes. Boron Trifluoride esterifies milk free fatty acids in 15 minutes as well [127]. The CV% was 5.4%. Therefore, the Lopez-Lopez (direct method 2) was used since it was rapid, highly accurate and precise due to the double methylation (Sodium Methoxide and Boron Trifluoride) which yields a high recovery of breast milk fatty acids such as DHA, EPA [127]. Aliquots of breast milk were thawed at 37º C in a water bath for 10 min since the mean melting point (MMP) of milk lipids ranges between 27 to 32º C [128]. Then 100 µl of breast milk, 400 µl of C17 standard (1 mg/ml in Methanol), 1000 µl methanol, 200 µl of sodium methoxide were mixed and heated at 90º C for 15 minutes. After cooling to room temperature (~25º C), 1 ml of boron trifluoride-methanol reagent was added and heated for 15 minutes at 90ºC.

After a second cooling to room temperature 400 µl of n-hexane was added, shaken (1 min) then 1 ml of a saturated solution of sodium chloride in distilled water was added, followed by centrifugation (8 min, 3000 g). The clear n-hexane top layer, containing the fatty acid methyl esters (FAME), was transferred to an auto injector vial equipped with a volume adapter of 300 µl. The sample was directly injected into the gas chromatograph or stored at –20ºC until injection.

For quality control purposes, each extraction and methylation assay contained: 1) Control; with DHA (20 µl, 1 mg/ml in methanol) mixed with 75 µl distilled water to mimic the aqueous breast milk, 2) Pooled sample; to measure inter-assay variability, and 3) Triplicates of two random samples; to measure intra-assay variability. The mean DHA, EPA and AA in the pooled samples were: 0.23 ± 0.017, 0.09 ± 0.004 and 0.47 ± 0.021 g/100 g, respectively, with a coefficient variation 7.6 %, 4.6 % and 4.7 %, respectively. While the DHA, EPA and AA in the triplicates samples were: 0.17 ± 0.005, 0.06 ± 0.003 and 0.34 ± 0.009 g/100g, with a CV of 3.3 %, 4.9 % and 2.5 %, respectively. The CV% for the DHA standard 6.9 % with a mean value of 88.3 ± 6.2.

3.3.4.2 Standards and Controls

In order to avoid variability within the assay; all chemicals and standards were prepared in advance. To identify fatty acid peaks; five mix standards were prepared in the laboratory obtained from individual unmethylated FA (Nu-Chek Prep, Inc.; Elysian, MN, USA). Another three fatty acid methyl esters were used for peak identification: Supelco™ 37 Component FAME Mix (catalog no. 47885-U; Bellefonte, PA), Supelco™ Linoleic Acid Methyl Ester Isomer Mix (catalog no. 47791; Bellefonte, PA), and Supelco™ Linolenic Acid Methyl Ester Isomer Mix (catalog no. 47792; Bellefonte, PA).

3.3.4.3 GC Equipment and Conditions

Varian 3800 CP gas chromatograph (Walnut Creek, California, USA) was used, equipped with a flame ionisation detector, split injector, CP-SIL 88 capillary column (catalog number CP7489) (100 m, 0.25 mm, 0.20 µm)

(Chrompack, Netherlands) and Varian's Galaxie software. The chromatographic conditions were modified based on Mazalli [129]: detector temperature 280º C; injector temperature 250º C; initial column temperature 120º C for 10 min, programmed to increase at a rate of 15º C per minute up to 160º C and then at 4º C per minute up to 195º C, maintaining this temperature for 12 min and then increasing again at 15º C per minute up to the final temperature of 220º C, maintained for 18 min. The carrier gas was hydrogen at 30 ml/min with linear velocity of 33.95 cm/s, with a make-up gas of nitrogen at 30 ml/min and synthetic air at 300 ml/min. Standard injection and a volume of 1 µl were used. The standard injection mode was to empty the needle by quick injection and a 0.5 µl/s dwell time. Fatty acids were identified by comparing the retention times of the standards with those of the samples. The quantification was using the C17 internal standard. The results are expressed as mg/100 g of the sample according to American Oil Chemists' Society (AOCS) [130].

3.3.5 Biochemical Analysis

Blood and urine samples were typically collected in the morning to limit diurnal variation.

Infant blood was collected by heel lance using a heparinized capillary tube (100 µl) for blood gas analysis at the Montreal Children's Hospital (MCH) to measure ionized calcium using an ABL 725 series blood gas analyzer (Radiometer America, Copenhagen, Denmark). An additional sample (0.9 ml) was collected in heparinized microtainer centrifuge tubes then separated into plasma and erythrocyte fractions by centrifugation for 20 min at 3000 g, 4°C. Resulting plasma was stored at -80°C until analysis of 25(OH)D and PTH. A 200 µl aliquot was removed prior to freezing and was analyzed by technicians at the MCH for total calcium and alkaline phosphatase (Beckman DxC600 California, USA). The MCH is a participant in the Programme de Controle Externe de Qualite DigitalPT provided by HealthMetrx Canada and a provincial quality assurance program by the Laboratoire de sante publique du Quebec.

Plasma 25(OH)D and PTH were measured at the McGill laboratory of Dr. Weiler. For both assays, all samples, standards and controls were analyzed in duplicate. Total 25(OH)D was measured using a RIA (25-50 μl; Diasorin) that is known to measure both D_2 and D_3 in plasma [131]. The assay is sensitive to 3.75 nmol/L and has a CV% of <10% and the laboratory is registered with DEQAS (vitamin D external quality assurance scheme). Serum intact PTH was also measured using an ELISA, for determination of both bioactive PTH as well as truncated fragments (50 μl; Immutopics International, San Clemente, USA). This assay is sensitive to 0.09 pmol/L and has a CV% <8%.

3.3.5.1 Urine Procurement and Measurements

Urine sample bags were affixed to the infant's genitals after cleaning with a commercial baby wipe. Urine samples were transferred to tubes, 1.5 ml of which was sent to MCH for analysis of calcium and creatinine (Beckman DxC600 California, USA).

3.3.6 Dual-Energy X-Ray Absorptiometry

BMC and BMD [132-133] were measured using DXA (QDR 12.1, 4500A Discovery Model, Hologic, Bedford, USA). Infants wore a diaper, a standardized gown without plastic or metal components and were wrapped in a single receiving blanket [134-136]. The infants were scanned while sleeping. DXA has been validated for measuring infant whole body and regional BMC and it delivers minimal radiation (6 μSv) in comparison to standard pediatric x-rays (~60 μSv) [135]. The best predictor of infant BMC is weight for whole body [137] and lumbar spine [134, 138-139] with minimum to no effect of gender or ethnicity [140]. Other regions such as femur are not typically measured; but it could provide information regarding linear growth [134]. Femur scans were included as an exploratory measure since in piglets, LCPUFA was related to femur BMC [84].

Using the DXA array mode; infants were scanned for the whole body using the infant software, vertebrae (L1-4) lumbar spine using the low density analysis software feature and finally femur was scanned using the subregion

analysis of the left hip scan mode. Whole body BMC was also corrected to body weight (expressed a g/kilogram) and length (expressed as g/centimeter). Correction to weight or length is endorsed by International Society for Clinical Densitometry (ISCD) [141] since normative data for infants is not currently available.

Since infant positioning is difficult to standardize, BMC is used only for whole body and femur. For lumbar spine, BMD (g/cm^2) was also examined since standardized positioning is feasible.

3.3.7 Data Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences for Windows (SPSS version 19) including assumption for normality, identification of multicollinearity, Pearson correlation, multiple regression analysis and ANOVA. A p-value ≤ 0.05 was accepted as significant. The null hypothesis (H_0) was that LCPUFA (AA, EPA and DHA) in maternal milk are not related to infant BMC, BMD and metabolism. While the alternative hypothesis (H_1) was that LCPUFA (AA, EPA and DHA) in maternal milk are related to infant BMC, BMD and metabolism.

The estimated sample size (n=36) was calculated to detect a correlation of 0.45 [16] with a two sided alpha (0.05) and a power of 80% [142]. A sample of 120 participants would enable the detection of $r \geq 0.25$.

Preliminary diagnostics of the outcome data included normality testing using Q-Q plots and studentized residual to identify outliers. Multicollinearity within model was tested using variance inflation factor (VIF) with a cut-off point of 10. Possible confounders were assessed by Pearson correlation between potential confounder and outcome. The Non-Gaussian variables were transformed. Dietary DHA, EPA were logarithmically transformed while the breast milk DHA, EPA and AA were square root transformed. Normally distributed continuous variables were presented as mean ± standard deviation, median and minimum-maximum values for those parameters with non-Gaussian distribution.

The relationship between maternal dietary intakes and infant bone was examined by dividing the intakes into quartiles and bone mass and metabolism values for infants in each quartile compared using one way ANOVA (Bonferroni's Multiple Comparison Test) between all groups.

To test if there was an age effect the results of each bone parameter (lumbar spine, femur and whole body BMC) were divided into quartiles according to the infant's age (< 28 d, 28-35 d, 36-42 d and > 42 d). The values in each quartile were compared using one way ANOVA (Bonferroni's Multiple Comparison Test) between all groups; there was no significant statistical difference.

3.4 Results

3.4.1 Subject Characteristics

The sample population came primarily from white, dual parent families. Mean age of the mothers was 32.9 years old and 88.3% of them completed a college/university program (Table 3-1). Pre-pregnancy BMI was on average 24.6 kg/cm² and within normal range [143]. Household income approximated the Canadian national median of $63,900 per annum after taxes for dual parent families with children [144]. More than 68.3% of families had household incomes above the national median and no families reported incomes less than $30,000 per annum after taxes (Table 3-1).

By design, all infants were born full term, gestational age 39.5 weeks (Table 3-2). The majority of the infants were receiving vitamin D supplementation 81.7% (Table 3-2) and none of them were deficient according to the infant cut off value of plasma 25(OH)D concentration of <27.5 nmol/L [60, 145].

3.4.2 Anthropometric Measurements

At birth and 1 mo of age, z-scores for weight (0.57 and 0.21), length (1.05 and 0.00) and head circumference (0.52 and 0.24) were within the healthy range (Table 3-2).

3.4.3 Dietary Data

Mother's intake of DHA and EPA was directly associated with milk LCPUFA content (p <0.0001) (Figures 3-1, 3-2). The mothers barely met the 2008 Perilip statement recommendation for DHA (200-1000 mg/d) and EPA (100-700 mg/d) during pregnancy/lactation (Table 3-3) [146]. During pregnancy, eight mothers took omega 3 supplements two of which were ≤25 mg/d and the rest ≤ 101mg/d. Mothers intake of fish during pregnancy included tuna 3-5 oz was 16.7% (≤ once/month), 20.8% (1-3 times/month), 26.7% (once/week), 5.8% (2-4 times/week), 0.8% (5-6 times/week) and 29.2% never had fish.

3.4.4 Breast Milk Data

The average of total fat in was 39.6 g/L; within the typical range (22.3–61.6 g/L) [147], which indicates that the collected samples were representative of full feed (fore and hindmilk). Medians for breast milk DHA, EPA and AA were 0.17 (0.06 - 0.91), 0.06 (0.02 - 0.31) and 0.43 (0.27 - 0.78), respectively (Table 3-3).

3.4.5 Other Biochemical Measurements

Values for 25(OH)D, PTH, ALP and urinary Ca:Cr were within normal limits (Table 3-4). Breast milk fatty acids did not relate to bone metabolism with exception of a weak positive association between AA and PTH (r = 0.21, p = 0.02) (Table 3-6). However, this was not observed when using multivariate regressions adjusted for infant weight, age, gender, vitamin D supplementation, maternal smoking and pre-pregnancy BMI.

3.4.6 Infant Bone and Relationships to LCPUFA

Values for BMC and BMD are presented in Table 3-5. Correlation analysis (Figures 3-3, 3-4, 3-5) revealed that breast milk DHA and AA were negatively correlated with infant lumbar spine vertebrate 1-4 BMC (r = - 0.18, p = 0.05; r = - 0.19, p = 0.04; respectively). Breast milk AA was negatively correlated with lumbar spine vertebrate 1-4 BMD (r = - 0.19, p = 0.04); no other correlations were observed.

In multivariate analysis (Tables 3-7, 3-8) fatty acids were not related to spine vertebrate 1-4 BMC nor BMD although there was a trend between breast milk EPA and lumbar spine vertebrate 1-4 BMD (r = - 0.13, p = 0.06). Femur and whole body BMC were not related to mother's milk fatty acid composition regardless of univariate or multivariate analysis. No significant relationship was observed in n-6:n-3 and bone mass regardless of univariate or multivariate analysis (Figures 3-6, 3-7, 3-8; Tables 3-7, 3-8).

The maternal dietary intakes during pregnancy relation to bone are presented in (Figures 3-9, 3-10, 3-11, 3-12, 3-13, 3-14). There was no significant relation

with exception of the ratio of AA:EPA to whole body BMC and BMC/kg (p = 0.02, p = 0.003; respectively) and AA:DHA to BMC/kg (p = 0.02).

3.5 Discussion

This is the first study to examine the relationships among maternal diet, human milk LCPUFA and neonatal bone. Maternal LCPUFA status in humans [16] and intake in animals [87-89] appear to limit bone mineralization outcomes. For example, Weiler et al found that mother's RBC DHA was negatively associated with infant's spine and femur BMC [16]. In animals, intervention with large amounts of fish oil (>80 g/100 g fat) during rapid growth postweaning was negatively associated with bone growth and biomechanics of tibia of male rabbits [87] and on vertebral strength and on length growth of female rats [88]. The current study adds that in humans, higher maternal intake of LCPUFA is reflected with higher milk LCPUFA and based on the multivariate analysis, breast milk LCPUFA do not explain the variance in bone mass early post-natally. However, it suggests that EPA may be negatively associated with lower spinal bone mass in infants (r = - 0.13, p = 0.06) which matches the observations of Weiler et al. [16] and Sirois et al. [88]. The present study observed no effect on linear growth while Korotokova et al. reported a negative relationship between maternal n-3 PUFA and rat offspring linear growth including femur length (mm), cortical cross-sectional area and BMC [89]; suggesting limited modeling as would be expected due to less resorption. In intact and ovariectomized female mice, long-term intake of n-3 fatty acids, especially EPA, reduces bone resorption cortical bone in the femur [55, 83]. These studies suggest that EPA limits bone resorption making it noteworthy that infant formula now contains only DHA and not EPA as sources of n-3 LCPUFA. Piglet studies suggested that very low amounts of DHA and AA enhance bone, while when higher amounts were used, benefits were negated [17, 84, 148]. Thus not only is it important to consider that DHA be considered for effects on bone, but that the amount be carefully considered.

The results of studies focused on the end of fetal development or early neonatal period appear somewhat contradictory to those conducted later in life. Adult human and animal studies using EPA or DHA supplementation showed enhanced bone by way of limiting bone resorption [55, 80, 83, 93-94]. Even

though it appears contradictory, both adult and neonatal mechanisms appear to rely on bone resorption. The effect in growth is different since bone resorption and formation are not coupled [10, 60] and the bone goes under rapid modeling [58] to reshape during growth. While in healthy adults, after cessation of growth, resorption and formation occur in a balanced ratio for maintenance of bone mass [10, 60], and lags behind resorption with aging in men and women especially after menopause [60] with a gradual decrement in bone density at a rate of about 0.5‑1% annually [10]. Thus in adults, strategies to limit resorption often yield higher BMD, but these may not be as beneficial during growth.

Even though the mothers barely met the 2008 Perilip statement recommendation for DHA (200-1000 mg/d) and EPA (100-700 mg/d) during pregnancy/lactation (Table 3-3) [146], median values for breast milk DHA, EPA and AA [0.17 (0.06 - 0.91), 0.06 (0.02 - 0.31), 0.43 (0.27 - 0.78), respectively] were within typical ranges that are observed worldwide [DHA 0.32 (0.06 –1.4), AA 0.47 (0.24 –1.0)] [149]. Such observation confirms that diet is not the only source of fatty acids in milk and that perhaps the observed LCPUFA content also reflects mobilization from adipose tissue [4, 6, 29, 150]. Nonetheless, dietary LCPUFA intake the day prior to collection was significantly related to milk LCPUFA (Figure 3-1, 3-2). Both methods of dietary assessment are well accepted and the FFQ has been validated [123]. Furthermore, the measurement of milk LCPUFA was based on a high recovery method [127]. Francois et al. suggested that within as few as 8 h of consuming a dietary or supplemental source of LCPUFA, milk LCPUFA peaks and remains elevated for at least 24 h [33]. The observations of the present study closely align with others [16, 89], moreover that total milk fat content was within typical ranges attests to the completeness and quality of milk collection and analysis.

3.5.1 Conclusion

Further understanding of this relationship is important. Pregnant and lactating women are recommended to enhance their omega 3 intake [27, 42]. Based on the multivariate analysis, breast milk LCPUFA do not explain the variance in bone mass early post-natally. However, since dietary intake may not reflect LCPUFA status, further studies are are required using biochemical assessment of fatty acid status of the mother and infant, and using a larger sample size or randomized dose-response studies, to establish if higher maternal intakes of n-3 LCPUFA are truly detrimental to neonatal bone.

3.6 Tables

Table 3-1 Characteristics of Mothers

Characteristic		
Age (years)		32.9 ± 3.9
Pre-pregnancy Body Mass Index (kg/m^2)		24.6 ± 5.1
Education		
	High School	7 (5.8%)
	Vocational/ Apprentice Training	7 (5.8%)
	College/University	106 (88.3%)
Race		
	Caucasian	91 (86.7%)
	Black	1 (0.8%)
	Hispanic	1 (1.9%)
	Asian	7 (6.7%)
	Pacific Islander	4 (3.8%)
Family Income (Canadian Dollars/Annum)		
	30,000 - 44,999	10 (8.3%)
	45,000 - 59,999	11 (9.2%)
	60,000 - 74,999	23 (19.2%)
	75,000 - 89,999	70 (58.3%)
	Above 90,000	6 (5.0%)

Continuous variables are presented means ± SD, categorical variables are presented as number (percent), (n= 120).

Table 3-2 Characteristics of Infants according to Sex

	Girls		Boys	
Characteristic	x ± SD	Z-score	x ± SD	Z-score
At Birth				
Gestational Age (weeks)	39.6 ± 1.1		39.5 ± 1.2	
Birth Weight (kg)	3.5 ± 0.4	71.6% (0.57)	3.6 ± 0.4	69.4% (0.51)
Birth Length (cm)	51.1 ± 2.6	85.3% (1.05)	52.4 ± 2.0	86.6% (1.12)
Birth Head Circumference (cm)	34.5 ± 1.5	70% (0.52)	34.9 ± 1.5	63.5% (0.34)
At One Month of Age				
Infant Age (d)	34 ± 5.2		35 ± 5.9	
Weight (kg)	4.4 ± 0.5	58.2% (0.21)	4.8 ± 0.7	61.5% (0.63)
Length (cm)	54 ± 1.9	49.9% (0.00)	55 ± 2.3	45.8% (-0.10)
Head circumference (cm)	37 ± 1.1	59.5% (0.24)	38 ± 1.2	65.6% (0.40)
Vitamin D Supplements (yes)	39 (78%)		59 (84.3%)	
Plasma 25(OH)D (nmol/L)	47.8 ± 16		46.5 ± 18.8	

Continuous variables are presented means ± SD, categorical variables are presented as number (percent), (n= 50 in girls and; n= 70 in boys).

Table 3-3 Dietary and Milk LCPUFA

Parameter	Mean ± SD	Median (Min-Max)
Mothers Intake (during Pregnancy) ∫		
Dietary DHA (mg/d)	212.8 ± 302.0	122.4 (1.2 – 1344.0)
Dietary EPA (mg/d)	129.8 ± 273.3	49.3 (0.4 – 1167.0)
Dietary AA (mg/d)	175.2 ± 90.0	167.6 (9.03 - 585.2)
Mothers Intake (Day Prior to Breast Milk Collection) ∫		
Dietary DHA (mg/d)	132.8 ± 334.3	20.0 (1.0 – 1893.0)
Dietary EPA (mg/d)	76.1 ± 173.6	10.0 (1.0 – 939.0)
Breast Milk §		
DHA (g/100g)	0.21 ± 0.14	0.17 (0.06 - 0.91)
EPA (g/100g)	0.08 ± 0.05	0.06 (0.02 - 0.31)
AA (g/100g)	0.44 ± 0.10	0.43 (0.27 - 0.78)

Continuous variables are presented means ± SD, categorical variables are presented as median (Min-Max), ∫ non-Gaussian distribution transformed to (Log of mg/d), § non-Gaussian distribution transformed to (square root of g/100g).

Table 3-4 Infant Bone Homeostatis at One Month of Age

Parameter	Girls	Boys
Blood		
Ionized Calcium (mmol/L)	1.4 ± 0.03	1.4 ± 0.04
Plasma Total Calcium (mmol/L)	2.6 ± 0.1	2.5 ± 0.07
Plasma Phosphorous (mmol/L)	2.1 ± 0.1	2.0 ± 0.1
Plasma Creatinine (μmol/L)	27 ± 8.8	29 ± 8.4
Plasma Alkaline Phosphatase (U/L)	298.7 ± 81.9	321 ± 88.9
Parathyroid Hormone (pg/ml)	19.3 ± 10.8	21.7 ± 12.7
Urine		
Urine Calcium (mmol/L)	1.9 ± 1.1	2.2 ± 1.7
Urine Phosphorous (mmol/L)	3.2 ± 1.2	3.9 ± 4.9
Urine Creatinine (mmol/L)	1.0 ± 0.5	1.1 ± 0.6
Urine Ca:Cr (mmol/L)	1.9 ± 0.9	1.9 ± 0.9

Continuous variables are presented means ± SD.

Table 3-5 Infant Lumbar Spine (Vertebrae 1-4), Femur and Whole Body BMC and BMD

	Girls		Boys	
Parameter	Mean ± SD	Median (Min-Max)	Mean ± SD	Median (Min-Max)
Spine-BMC (g)	2.9 ± 0.7	2.8 (1.7 – 4.3)	2.6 ± 0.5	2.6 (1.5 – 3.7)
Spine-BMC (g/kg)	0.65 ± 0.15	0.61 (0.42 – 1.0)	0.56 ± 0.13	0.55 (0.27 – 0.86)
Spine-BMD (g/cm²)	0.27 ± 0.06	0.26 (0.17 – 0.38)	0.23 ± 0.04	0.24 (0.11 – 0.36)
Femur-BMC (g)	3.4 ± 0.6	3.5 (2.2 – 4.8)	3.7 ± 0.8	3.6 (1.9 – 5.9)
Femur-BMC (g/kg)	0.76 ± 0.13	0.78 (0.47 – 1.0)	0.77 ± 0.13	0.76 (0.49 – 1.0)
Whole Body-BMC (g)	97.7 ± 14.9	97.8 (65.7 – 126.4)	102.4 ± 18.0	101.2 (57.3 – 151.5)
Whole Body-BMC (g/kg)	21.9 ± 1.9	22.2 (18.0 – 25.8)	21.4 ± 2.2	21.5 (16.7 – 26.6)

Continuous variables are presented means ± SD.

Table 3-6 Breast Milk LCPUFA Relationship to Biomarkers of Calcium Homeostatis in Infants

	25(OH)D (nmol/L)		Plasma PTH (pg/ml)		Plasma ALP (U/L)		Urine Ca:Cr (mmol/L)	
Breast Milk FA	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value	*r*	*P*-value
DHA (g/100g) §	- 0.02	0.82	0.08	0.42	0.05	0.59	0.07	0.50
EPA (g/100g) §	0.01	0.89	- 0.01	0.90	- 0.02	0.82	0.07	0.52
AA (g/100g) §	- 0.03	0.76	0.21	0.02*	0.14	0.14	- 0.08	0.43
AA:DHA	0.01	0.95	- 0.09	0.36	- 0.06	0.53	- 0.08	0.45
AA:EPA	- 0.03	0.77	0.08	0.42	0.07	0.49	- 0.08	0.45
AA: EPA+DHA	- 0.05	0.60	0.01	0.92	0.08	0.37	0.02	0.84

Relationships are presented as correlation coefficients (*r*), § non-Gaussian distribution which was transformed to (square root of g/100g) prior to analyses, * Significant at $p \leq 0.05$.

Table 3-7 Multivariate Analyses for Breast Milk LCPUFA Relationships to Infant Lumbar Spine (Vertebrae 1-4) BMC and BMD

	Spine-BMC (g)		Spine-BMC (g/kg)		Spine-BMD (g/cm²)	
Breast Milk LCPUFA	β	*P*- value	β	*P*- value	β	*P*- value
DHA (g/100g) §	-0.713†	0.250	-0.153	0.258	-0.077	0.130
EPA (g/100g) §	- 1.078	0.221	- 0.214	0.264	-0.132	0.065
AA (g/100g) §	- 0.806	0.433	- 0.166	0.461	-0.106	0.209
AA: DHA	0.034	0.629	0.008	0.594	0.003	0.635
AA: EPA	0.019	0.441	0.003	0.516	0.002	0.368
AA: EPA+DHA	0.092	0.267	0.020	0.261	0.006	0.395

† All β-values were adjusted for infants' (weight, age, gender, vitamin D supplementation, ethnicity), maternal smoking and pre-pregnancy BMI, and presented as (unstandardized β), § denotes non-Gaussian distribution which was transformed (square root of g/100g), * Significant at $p \leq 0.05$.

Table 3-8 Multivariate Analyses for Breast Milk LCPUFA Relationships to Infant Femur and Whole Body BMC and BMD

	Femur-BMC (g)		Femur-BMC (g/kg)		Whole Body-BMC (g)		Whole Body-BMC (g/kg)	
Breast Milk LPUFA	β	*P*-value	β	*P*-value	β	*P*-value	β	*P*- value
DHA (g/100g) §	0.554†	0.372	0.125	0.348	4.453	0.678	1.124	0.613
EPA (g/100g) §	0.835	0.344	0.203	0.286	5.684	0.708	1.755	0.576
AA (g/100g) §	0.548	0.594	0.104	0.640	-12.201	0.495	- 2.322	0.530
AA: DHA	-0.052	0.454	-0.010	0.526	-0.563	0.639	-0.118	0.633
AA: EPA	-0.009	0.725	-0.002	0.640	-0.150	0.721	-0.041	0.639
AA:EPA+DHA	0.108	0.194	0.022	0.225	0.270	0.850	0.042	0.887

† All β-values were adjusted for infants' (weight, age, gender, vitamin D supplementation, ethnicity), maternal smoking and pre-pregnancy BMI, and presented as (unstandardized β), § denotes non-Gaussian distribution which was transformed (square root of g/100g), * Significant at $p \leq 0.05$.

3.7 Figures

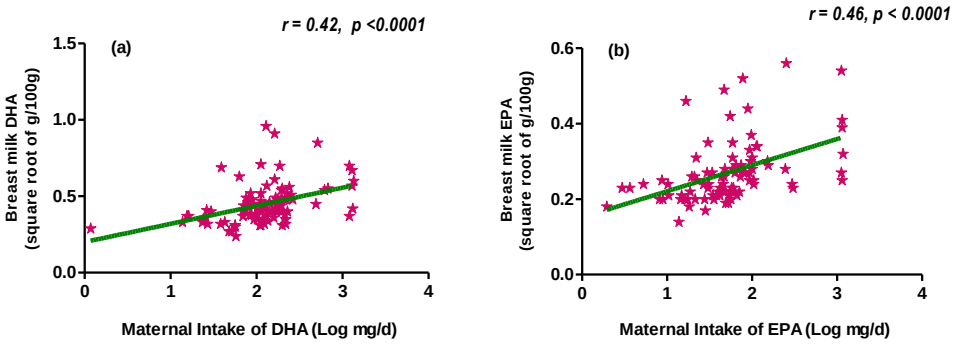

Figure 3-1 Maternal Intake of (a) DHA and (b) EPA during Pregnancy Correlation to her Breast Milk Composition.

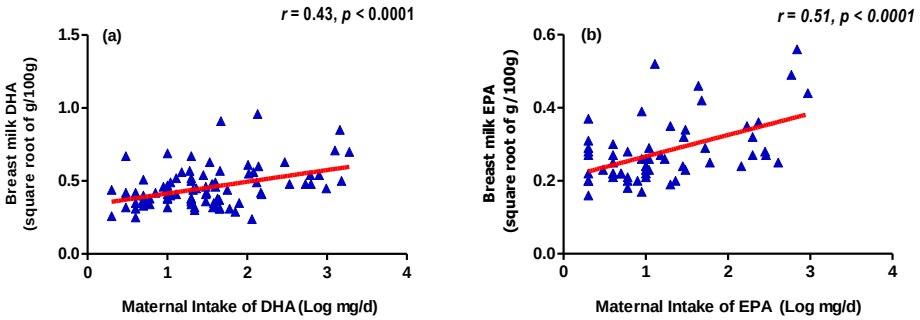

Figure 3-2 Maternal Intake of (a) DHA and (b) EPA during the Day before Breast Sample Collection Correlation to the Breast Milk Composition.

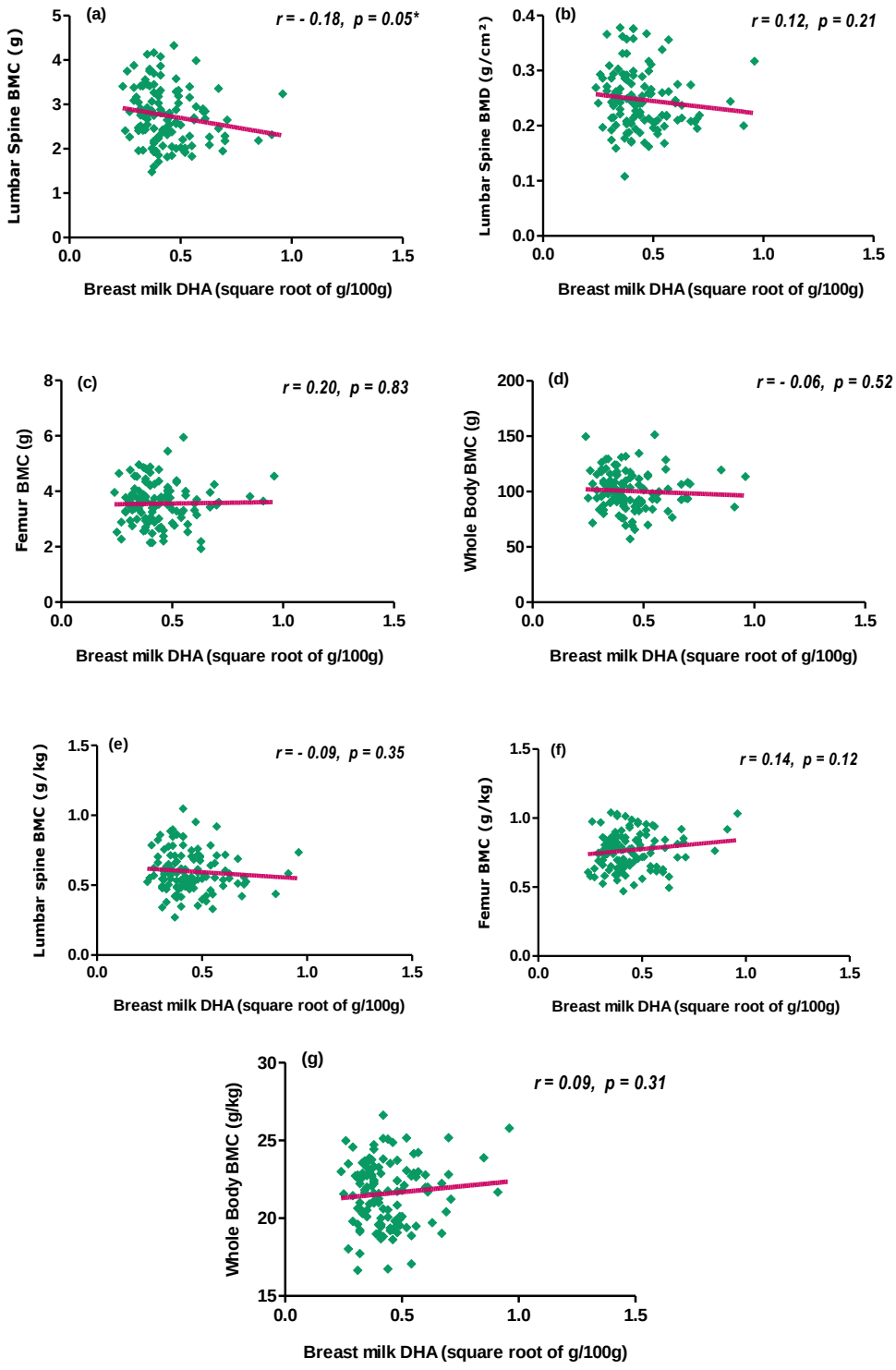

Figure 3-3 Breast Milk DHA Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

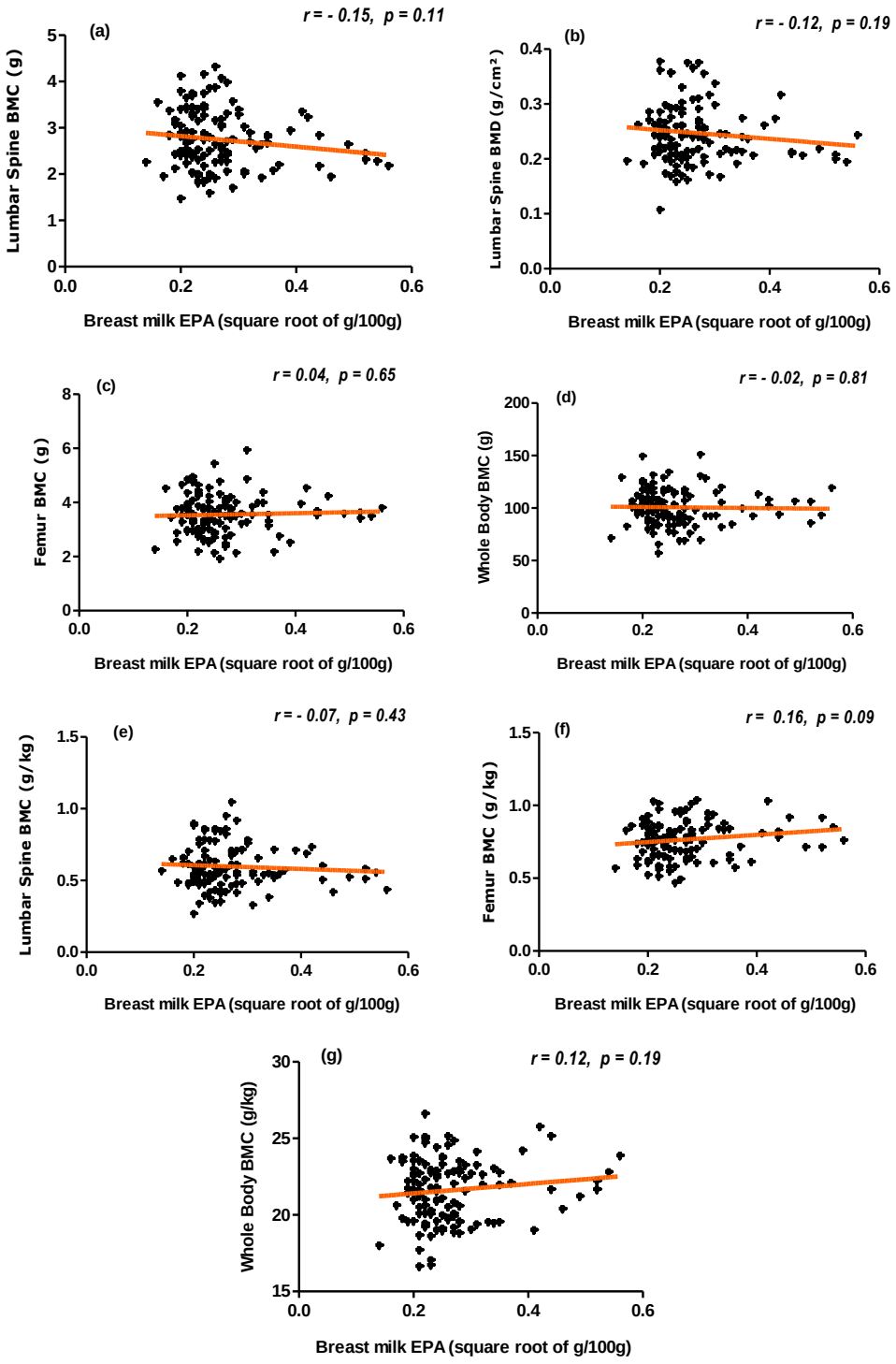

Figure 3-4 Breast Milk EPA Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

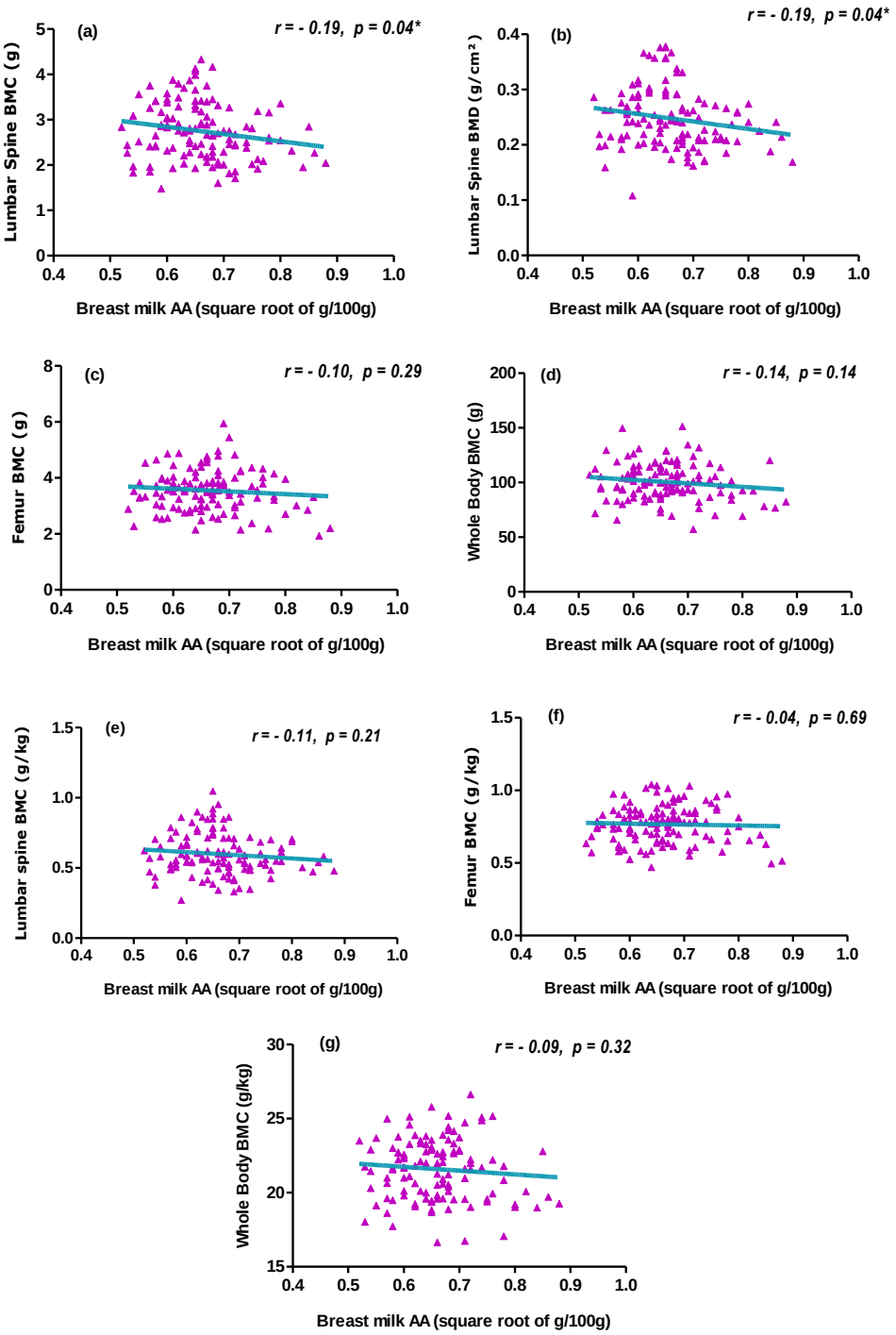

Figure 3-5 Breast Milk AA Correlation to Infant's Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

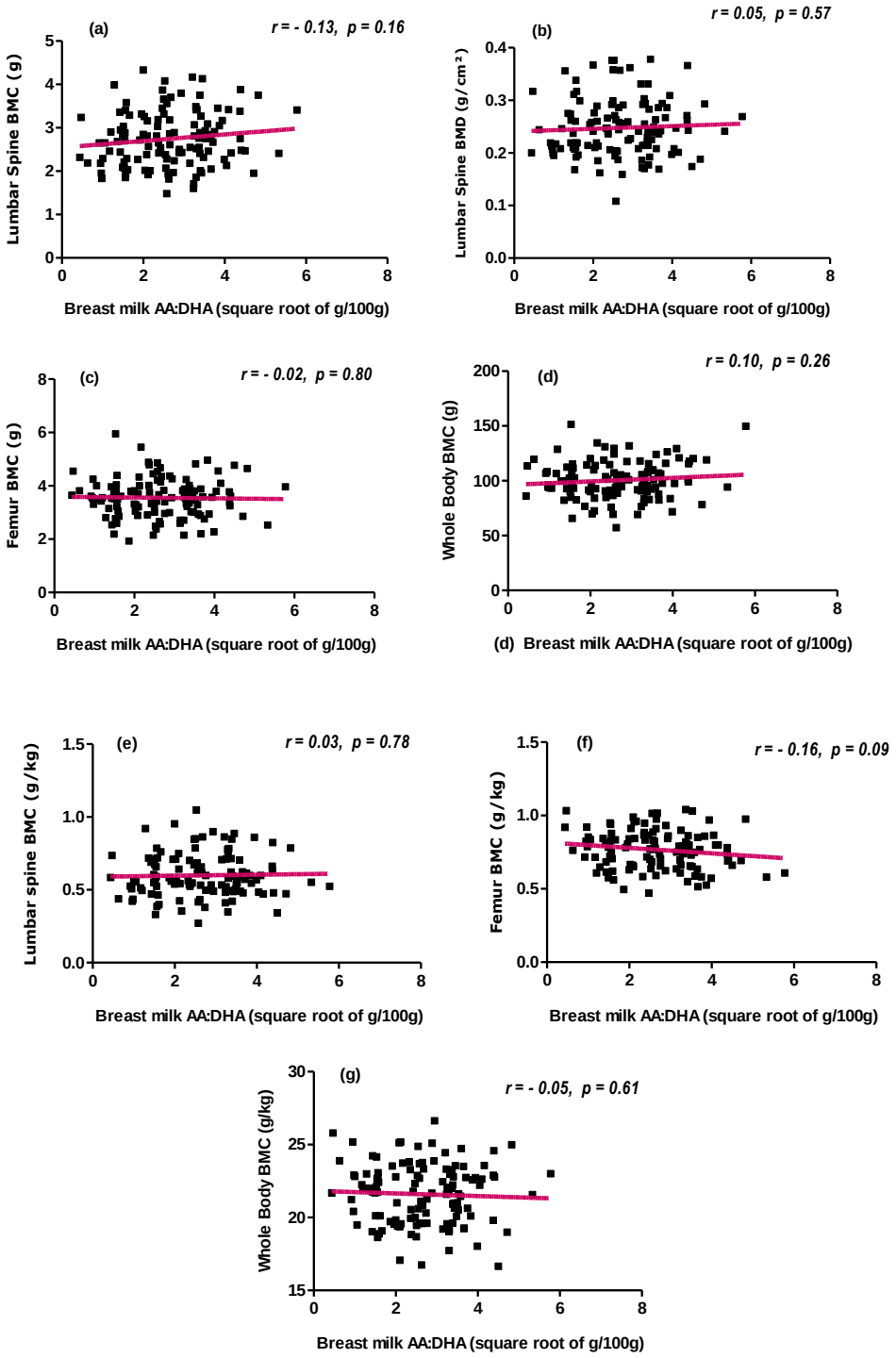

Figure 3-6 Breast Milk n-6 to n-3 Ratio (AA:DHA) Correlation to Infant's Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

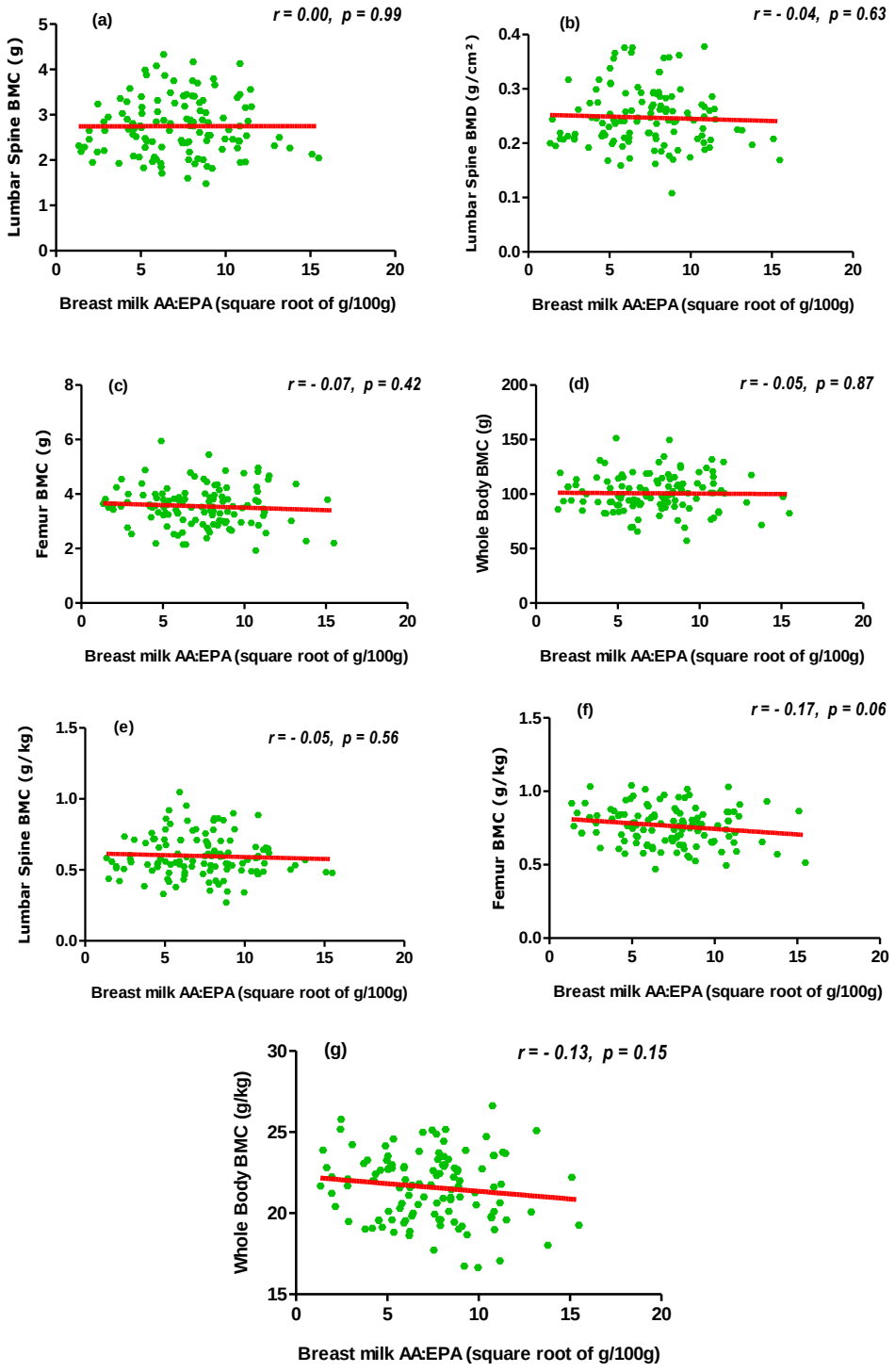

Figure 3-7 Breast milk n-6 to n-3 ratio (AA:EPA) Correlation to Infant's Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

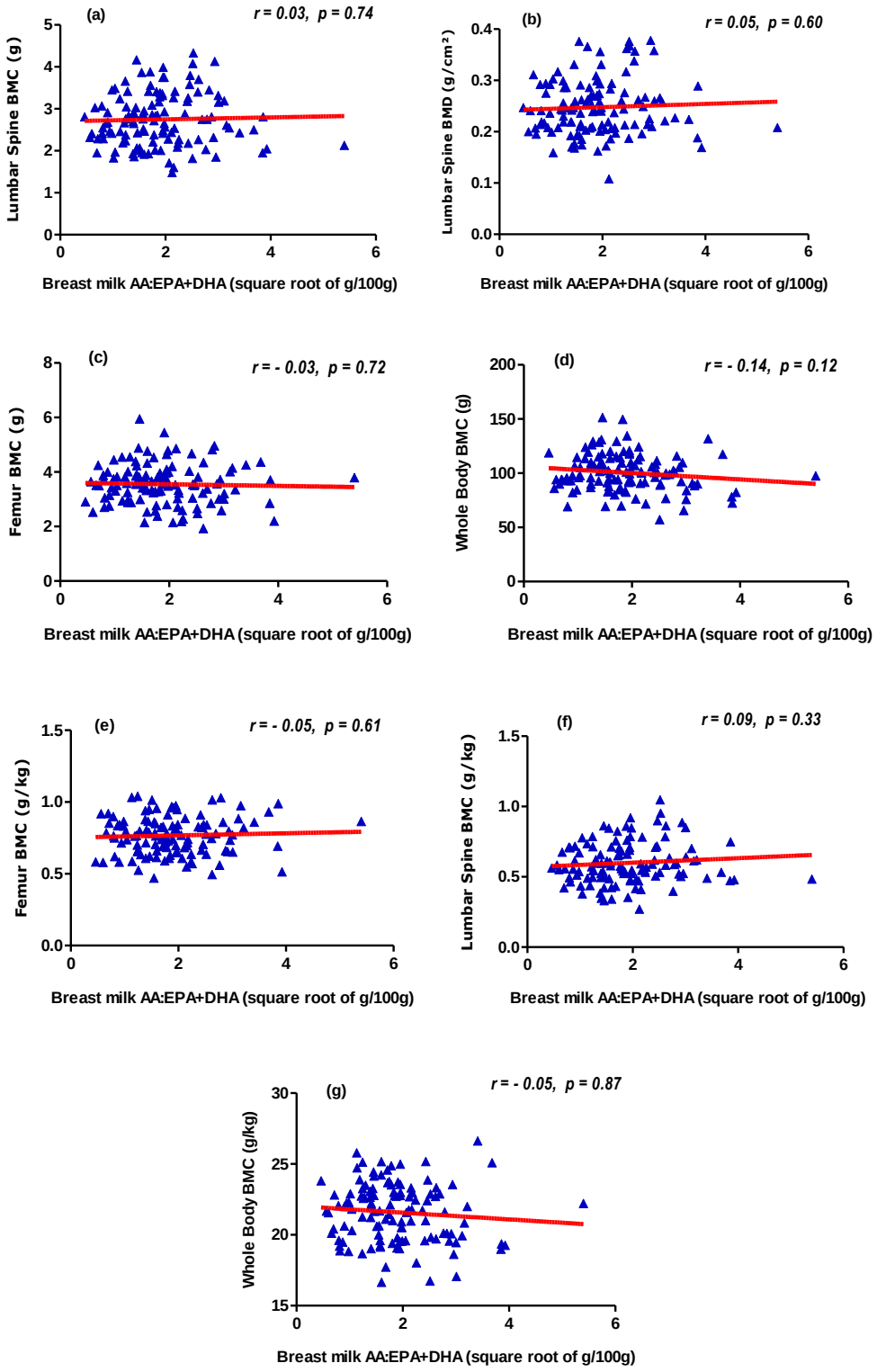

Figure 3-8 Breast milk n-6 to n-3 ratio (AA:EPA+DHA) Correlation to Infant's Correlation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

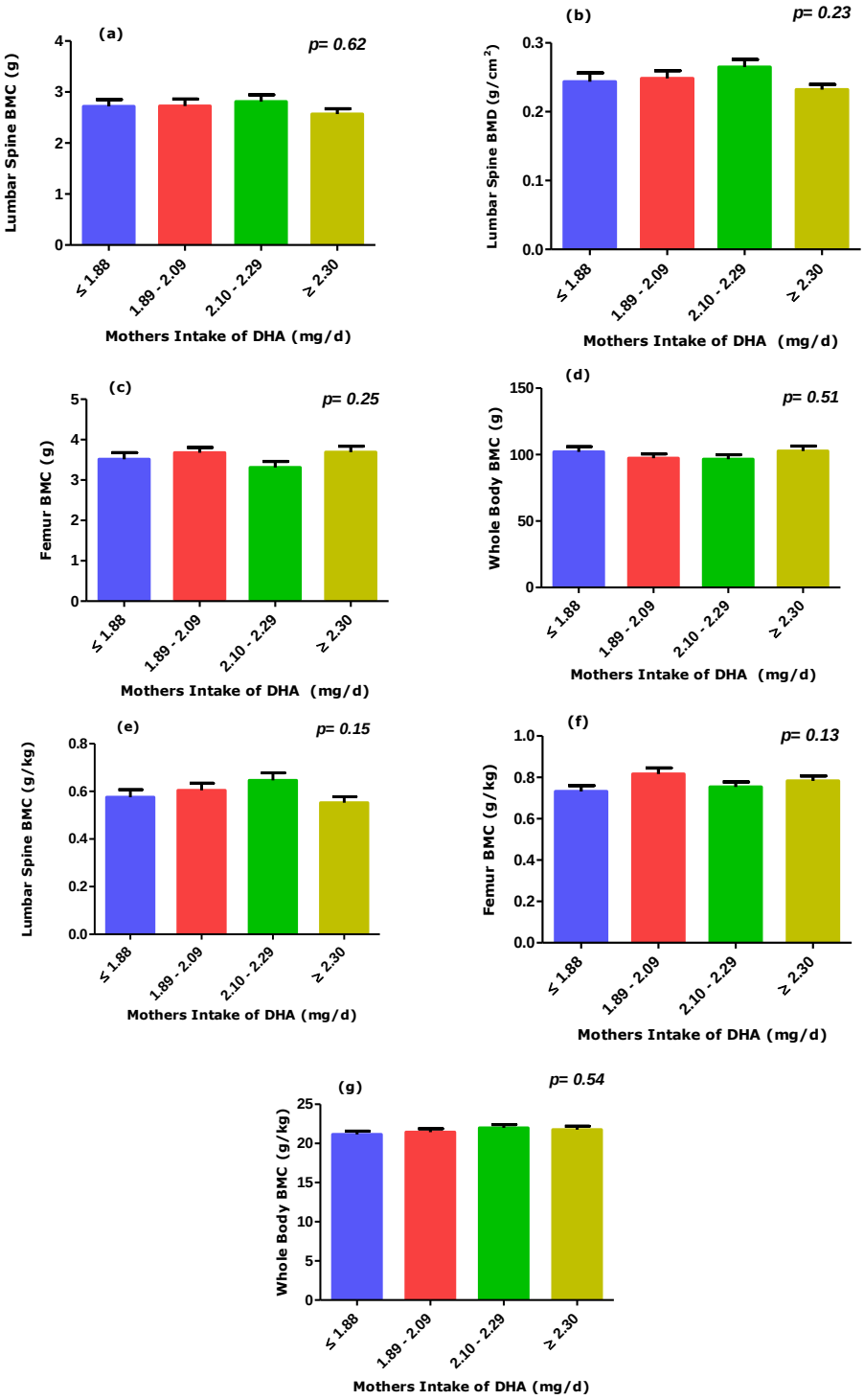

Figure 3-9 Mothers Intake of DHA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

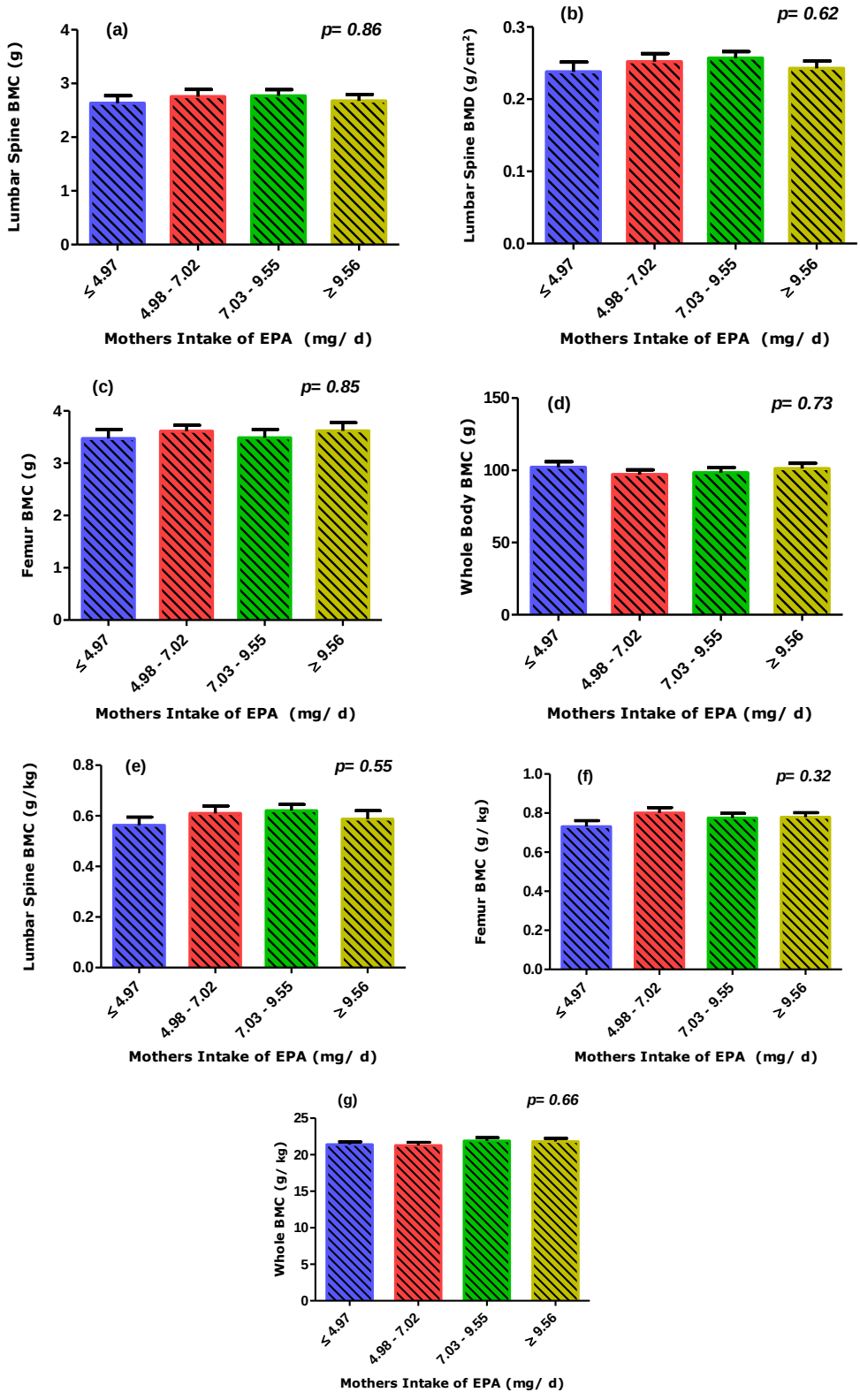

Figure 3-10 Mothers Intake of EPA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

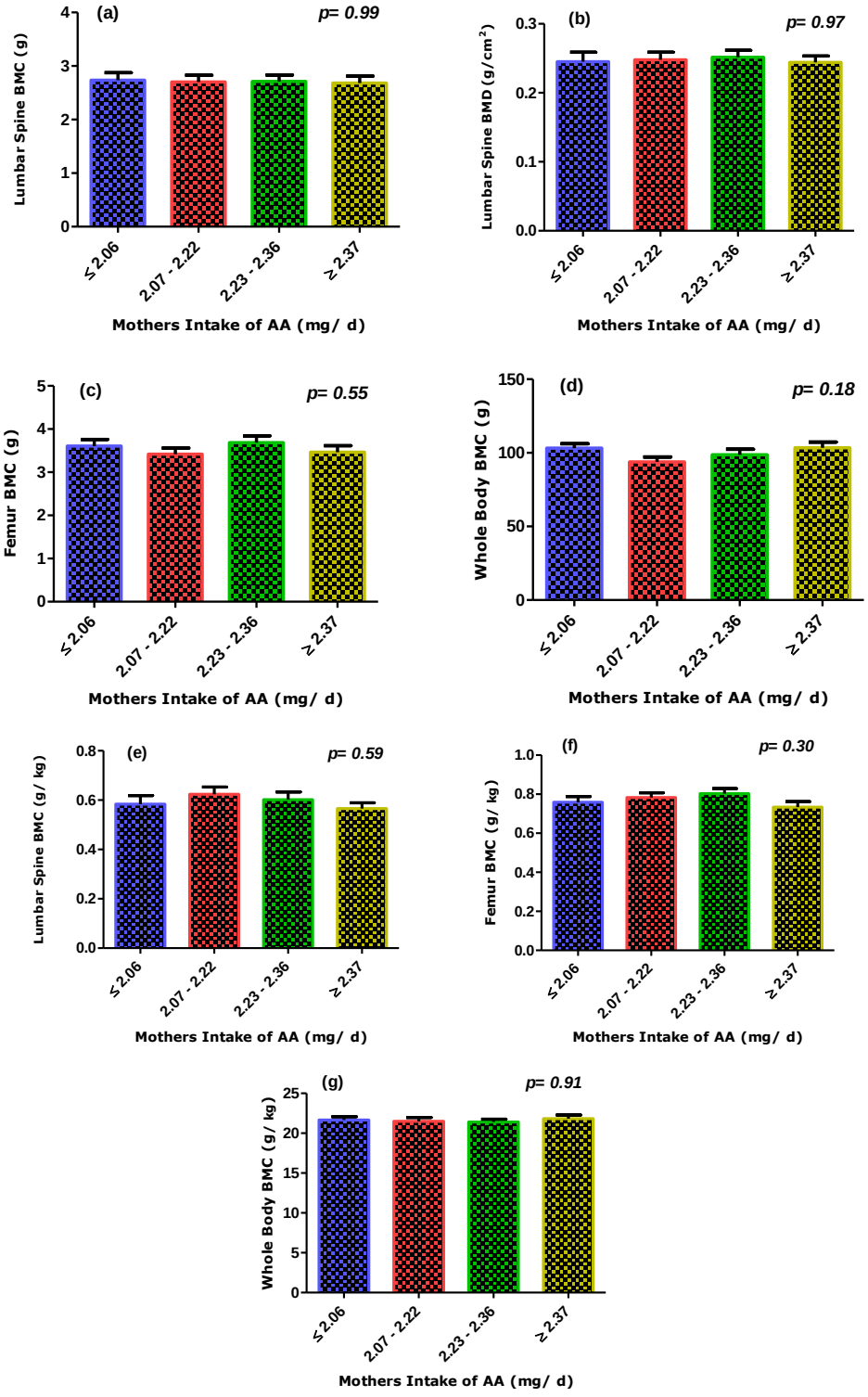

Figure 3-11 Mothers Intake of AA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

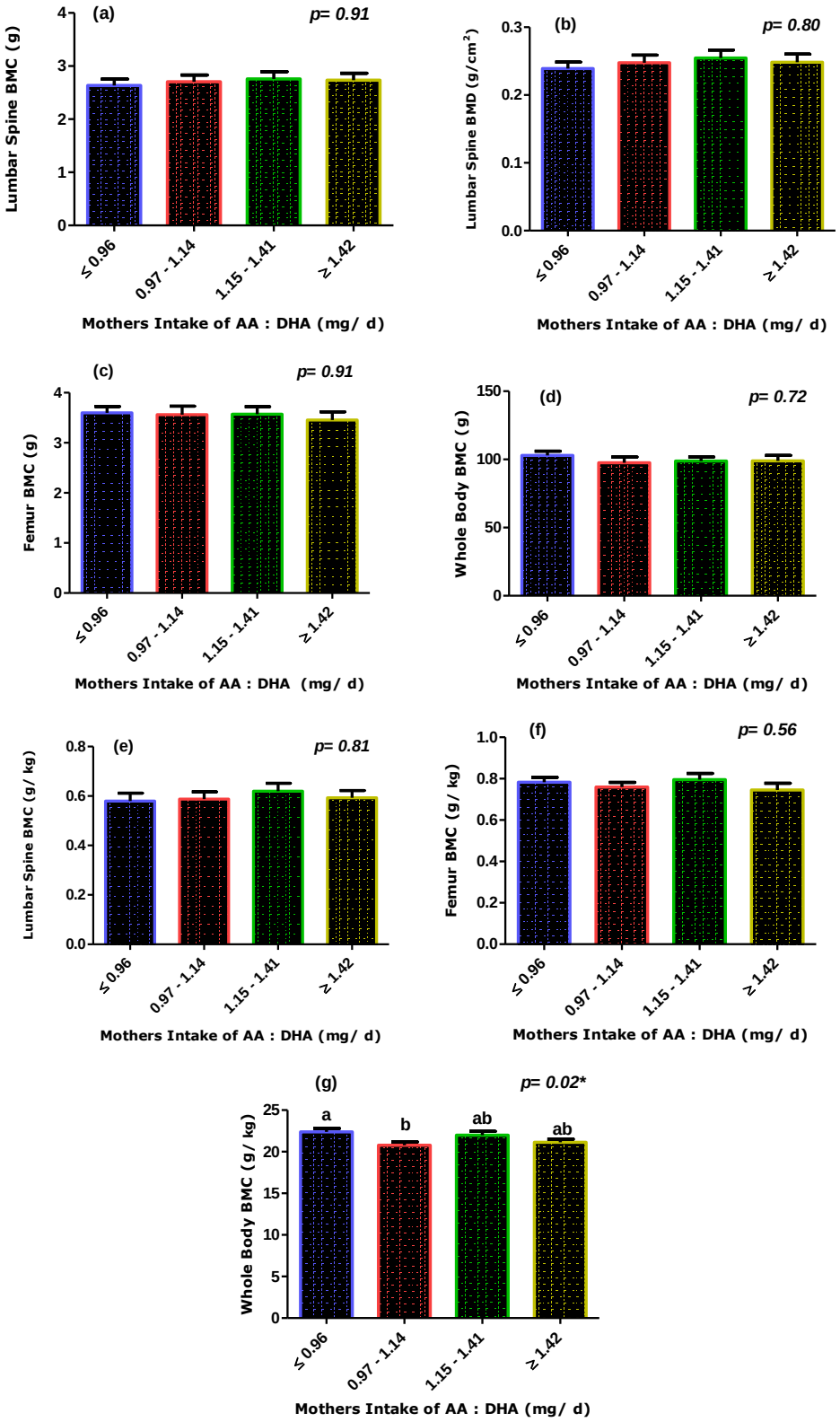

Figure 3-12 Mothers Intake of AA:DHA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

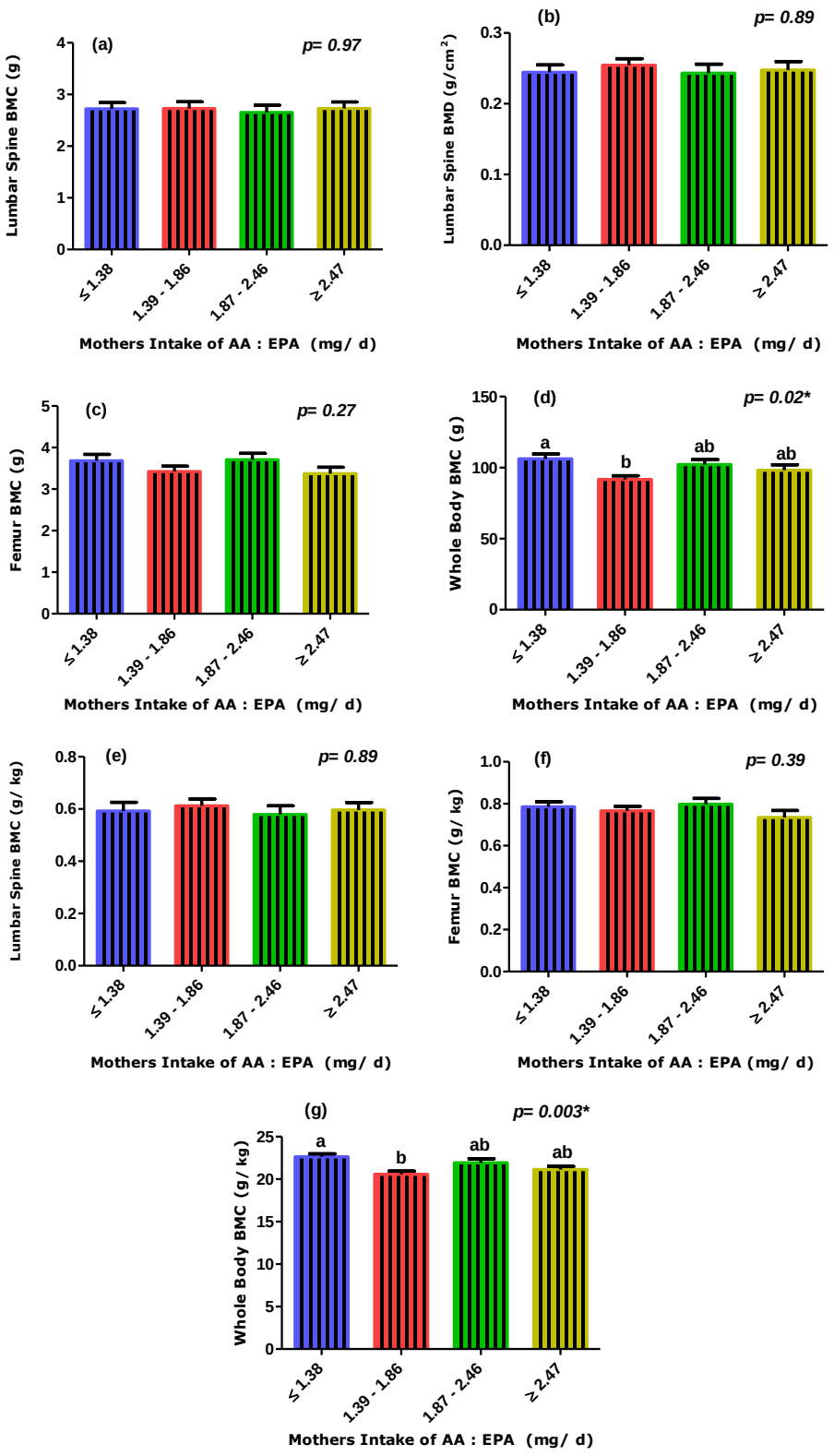

Figure 3- 13 Mothers Intake of AA:EPA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

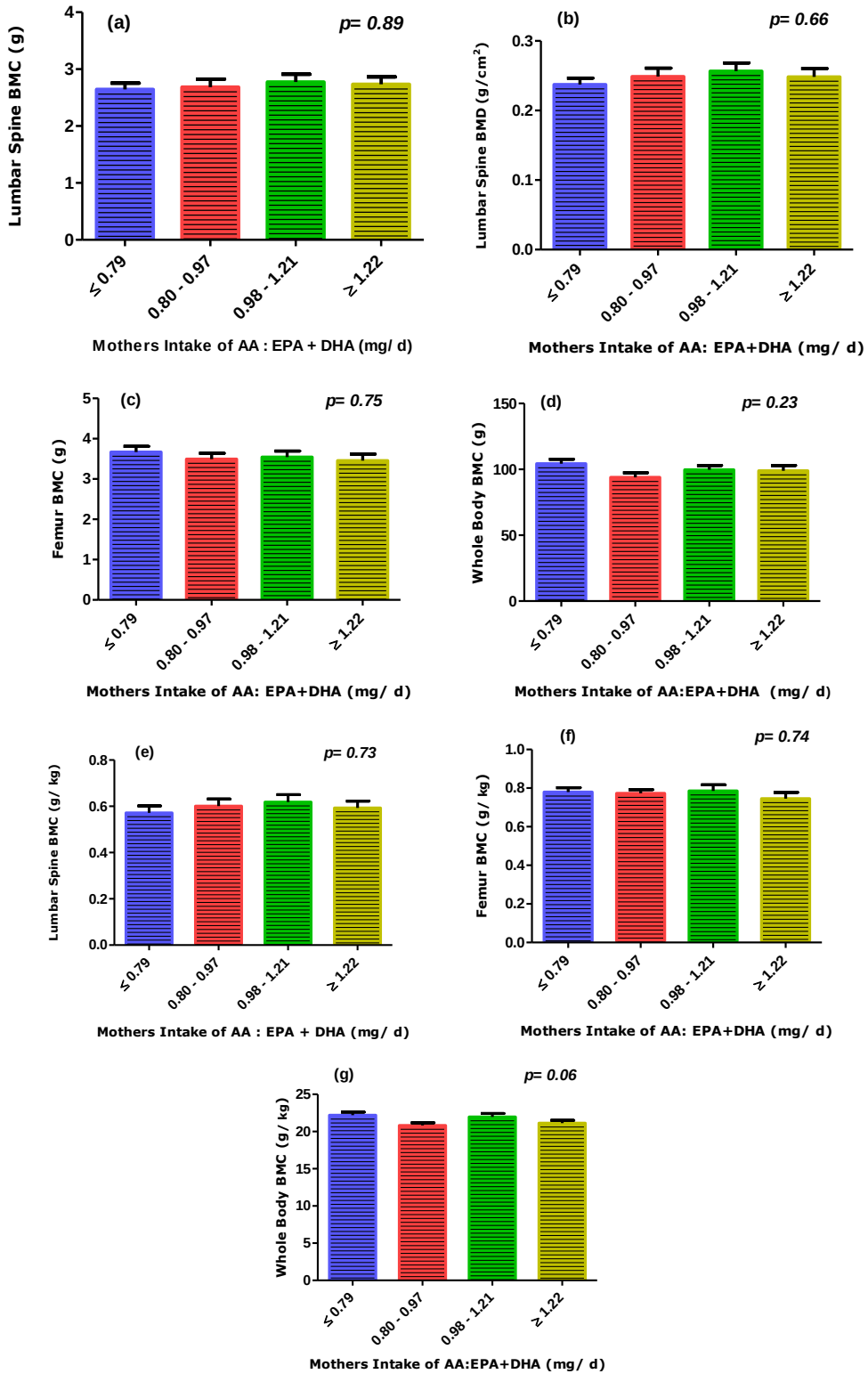

Figure 3-14 Mothers Intake of AA:EPA+DHA during Pregnancy Relation to Infant's (a) Lumbar Spine Vertebrae 1-4 BMC, (b) Lumbar Spine BMD, (c) Femur BMC, (d) Whole Body BMC, (e) Lumbar Spine BMC adjusted for Infant Weight, (f) Femur BMC adjusted for Infant Weight, (g) Whole Body BMC adjusted for Infant Weight. *Significant at $p \leq 0.05$.

4 Discussion

The primary hypothesis of this thesis was that human milk LCPUFA is associated with neonatal bone mass. The omega 3 LCPUFA relationship to bone is negative at the end of fetal development or early neonatal period [16] while later in the adult life it is positive [94-95]. Omega 3 LCPUFA decease bone resorption which is beneficial in adults to maintain bone strength and prevent osteoporosis; while early in infancy where bones are going under rapid modeling bone resorption is needed to reshape the growing bone whether it is linear or appositional (diameter). During the first months of life, the decrease in cortical thickness had been dubbed "physiological osteoporosis of infancy" [151]. This could be the reason why no relation was detected with femur BMC. Moreover, DXA interpretation during growth could be misleading. For example, during growth increases in "bone density" often are attributed uniformly to "bone mineralization," regardless of whether this represents greater cortical thickness, thicker trabeculae, or incorporation of additional mineralization [151]. The relationship with spine BMD is likely to be observed since it reflects a higher proportion of trabecular bone and since BMD could be assessed in addition to BMC due to feasibility of positioning in the neonate.

Correlation analysis revealed a negative relationship between breast milk DHA and infant lumbar spine vertebrate 1-4 BMC (r = - 0.18, p = 0.05), breast milk AA and infant lumbar spine vertebrate 1-4 BMC and BMD (r = - 0.19, p = 0.04; r = - 0.19, p = 0.04, respectively). Based on the multivariate analysis, breast milk LCPUFA do not explain the variance in bone mass early post-natally. However, it suggests that EPA may be negatively associated with lumbar spine vertebrate 1-4 BMD (r = - 0.13, p = 0.06). In healthy babies, the decline in BMD is not necessarily a sign of bone loss. It reflects a redistribution of bone tissue from the endocortical to the periosteal surface rather than bone loss. Therefore, bone mineral increases considerably during the first months of life despite the precipitous drop in BMD [151]. However, more studies are needed using measures of infant LCPUFA status along with

bone mass and as dose-response or randomized clinical trials to investigate cause/effect relationship

It is well documented that dietary or supplemental sources of LCPUFA the day before is reflected in the milk LCPUFA [33] which was observed in this study as well. Mother's intake of DHA and EPA was directly associated with milk DHA and EPA composition (p <0.0001). There are three sources of fatty acids in human milk: diet, de novo synthesis by the liver or breast tissue and mobilization of endogenous fatty acids stores [4, 6, 29]. This clarifies why even though dietary intake of LCPUFA was lower than recommendations, the milk LCPUFA were within typical ranges.

Agreeing with the literature, the values for neonatal BMC and BMD (Table 3-5) showed sex differences where boys whole body bone area, and femur bone were greater than girls [152].

4.1 Limitations

In addition to the limitations already mentioned, the major limitation is lack of biological assessment of fatty acid status in both infant and mothers, such as LCPUFA in RBC membranes and plasma phospholipids. Furthermore, the day-to-day variation in human milk LCPUFA is not clear. It is possible that milk samples obtained following consumption of fatty fish might have lead to non-representative milk LCPUFA composition. Therefore, future studies should examine average milk LCPUFA over a longer duration of time and establish if the relationship to infant bone is evident or not.

Future Directions

The current recommendations for n-3 LCPUFA supplementation of infants are designed for the brain/retina. However, these do not take into account the possible effect on bone health. Future research studies should incorporate measures of bone in neonates to confirm if the dietary intakes required for brain are also beneficial to bone. If adverse effects were observed,

recommendations would have to consider the trade-offs among various developing systems.

4.2 Conclusion

Breast milk LCPUFA do not explain the variance in bone mass early post-natally based on the multivariate analysis. However, since dietary intake may not reflect LCPUFA status, further studies are warranted using using biochemical assessment of fatty acid status of the mother and infant and a dose-response study of the effects of maternal supplementation on neonatal bone mass. This relationship is important based on the current recommendation to enhance n-3 LCPUFA intake during pregnancy and lactation.

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6 Appendices

6.1 Appendix 1

Vitamin D dose-response study to establish dietary requirements in infants.
Subject code:_____ Date:_____

Demographic Survey

Purpose: for prospective studies as a screening interview to clarify inclusion criteria and participant demographics.

Background: This survey was developed based on questions modified from the NHANES dietary questionnaire as well as Health Canada's National Infant Nutrition Survey. It was further modified for the current study.

Data Collection Mode: to be conducted either on own then verified by a researcher the same day/visit or by a researcher.

Requirements: the survey is to be completed by the biological mother and the researcher.

[Mother], I'm going to ask you questions about you and your family. Please answer to the best of your ability. You have the choice to not answer all questions. If you do not know the answer, let us know.

MOTHER'S INFORMATION

Q. 001 What was the highest grade you completed?

Elementary school	□ grade:_____
High School	□ grade:_____
Vocational school or apprenticeship training	□ years:_____
College/University	□ years:_____
Refused to answer □	

Q.002 What would you say is your profession/career?

_____

Refused to answer □

Q.003 How old were you when [Participant] was born?

_____(years)

Refused to answer □

Does not know □

Q.004 What is your current weight?

_____(circle lbs/kg)

Refused to answer □

Does not know □

Q.005 What is your height?

_____(circle cm/inches)

Refused to answer □

Does not know □

Q.006 How many times have you given birth?
_____

Q.007 How many of your births were at term (37-42 weeks gestation)?
_____

Q.008 How many of your births were pre-term (<37 weeks gestation)?
_____

Q.009 How many pregnancies were spontaneously or medically terminated?
_____

Q.010 How many pregnancies resulted in live births?
_____

Q.011 How much personal breastfeeding/pumping experience do you have, include all children you've breastfed?
|___|___|___| Unit:_____
Enter in days, weeks, months (indicate which one)
None ☐
Refused to answer ☐
Does not know ☐

Q.012 Are you currently taking birth control pills?
Yes ☐
No ☐
Refused to answer ☐
Does not know ☐

Q.013 Are you currently taking medications?
Yes ☐ If yes, which one(s):_____
No ☐
Refused to answer ☐
Does not know ☐

Q.014 Are you currently taking vitamins?
Yes ☐ If yes, which one(s):_____
No ☐
Refused to answer ☐
Does not know ☐

Q.015 Are you currently taking dietary supplements (including fish oil)?
- Yes ☐ If yes, which one(s):_____
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.016 Do you drink coffee?
- Yes ☐ _____cups/day
- No ☐
- Refused to answer ☐
- Does not know ☐

FATHER'S INFORMATION

Q. 017 What was the highest grade [Participant's] Father completed?
- Elementary school ☐ grade:_____
- High School ☐ grade:_____
- Vocational school or apprenticeship training ☐ years:_____
- College/University ☐ years:_____
- Refused to answer ☐
- Does not know ☐

Q.018 What would you say [Participant's] Father's profession/career?
_____
- Refused to answer ☐
- Does not know ☐

Q.019 Was this profession/career that same when you were pregnant with [participant]?
- Yes ☐
- No ☐_____(list previous)
- Refused to answer ☐
- Does not know ☐

Q.020 How old was [Participant's] Father when (he/she) was born?
_____(years)
- Refused to answer ☐
- Does not know ☐

Q.021 What is [Participant's] Fathers weight?
_____(circle lbs/kg)
- Refused to answer ☐
- Does not know ☐

Q.022 What is [Participant's] Father's height?
_____(circle cm/inches)
Refused to answer ☐
Does not know ☐

FAMILY INFORMATION

Q.023 Since [Participant] was born, how would you describe your family for the most part?
Single parent ☐
Dual parent ☐
Refused to answer ☐

Q.024 What was your family's income (household) the year [Participant] was born?
Less than 15, 000 ☐
15, 000 - 29, 000 ☐
30, 000 - 44,999 ☐
45,000 - 59,999 ☐
60,000 - 74,999 ☐
75,000 or more ☐
Refused to answer ☐
Does not know ☐

Q.025 What is your family's income (household) now?
Less than 15, 000 ☐
15, 000 - 29, 000 ☐
30, 000 - 44,999 ☐
45,000 - 59,999 ☐
60,000 - 74,999 ☐
75,000 or more ☐
Refused to answer ☐
Does not know ☐

PREGNANCY

Q.026 When was your last menstrual period (LMP)?
_____
(DA / MO / YEAR)
Refused to answer ☐
Does not know ☐

Q.027 What was you pre-pregnancy weight?
_____(circle lbs/kg)
Refused to answer ☐
Does not know ☐

Q.028 How much weight did you gain during pregnancy with [Participant]?
_____(circle lbs/kg)
Refused to answer ☐
Does not know ☐

Q.029 Did you take any vitamin or mineral supplements while pregnancy with [Participant]?
Yes ☐
No ☐
Refused to answer ☐
Does not know ☐

Q.030 Do you recall which supplement you took?
_____
No ☐
Refused to answer ☐
Does not know ☐

Q.031 When did you start taking the supplement?
Before you became pregnant ☐
When you found out you were pregnant ☐ _____weeks pregnant
Refused to answer ☐
Does not know ☐

Q.032 How often did you take the supplement?
1 x weekly ☐
2 x weekly ☐
3 x weekly ☐
4 x weekly ☐
5 x weekly ☐
6 x weekly ☐
7 x weekly ☐
Every other day ☐
Less than 1x/week ☐
Refused to answer ☐
Does not know ☐

Q.033 When did you stop taking the supplement?

- 1st trimester ☐
- 2nd trimester ☐
- 3rd trimester ☐
- Delivery ☐
- Weaning ☐
- Other_____
- Refused to answer ☐
- Does not know ☐

Q.034 Did you smoke during pregnancy with [Participant]?

- Yes ☐
- No ☐ (if no, go to Q.038)
- Refused to answer ☐
- Does not know ☐

Q.035 If you quit during pregnancy with [Participant], how far along was the pregnancy?

_____(weeks)

- Refused to answer ☐
- Does not know ☐

Q.036 How many cigarettes did you smoke per day?

_____(circle cigarettes/packages)

- Refused to answer ☐
- Does not know ☐

Q.037 Did you reduce the number of cigarettes you smoked per day?

- Yes ☐
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.038 Did you exercise while pregnant with [Participant]?

- Yes ☐
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.039 How many hours per week did you typically exercise?

- Less than 1 hr ☐
- 1-2 hr ☐
- 2-3 hr ☐
- More than 3 hr ☐
- Refused to answer ☐
- Does not know ☐

Q.040 How would you describe the exercise intensity?
- Low intensity/not out of breath ☐
- Moderate intensity/slightly elevated breathing/able to talk ☐
- Heavy intensity/out of breath/sweating ☐
- Refused to answer ☐
- Does not know ☐

Q.041 Did you attend prenatal or pregnancy classes when pregnant with [Participant]?
- Yes ☐
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.042 Did you ever attend prenatal or pregnancy classes for an earlier pregnancy?
- Yes ☐
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.043 Did you drink coffee when pregnant with [Participant]?
- Yes ☐ _____cups/day
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.044 Did you take any medications while you were pregnant with [Participant]?
- Yes ☐ If yes, which one(s):_____
- No ☐
- Refused to answer ☐
- Does not know ☐

NUTRITION KNOWLEDGE

Q.045 Had you ever heard of vitamin D?
- Yes ☐
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.046 Where did you hear of vitamin D?
_____(source)
- No ☐
- Refused to answer ☐
- Does not know ☐

Q.047 What is vitamin D important for?

_____

Refused to answer ☐
Does not know ☐

Q. 048 Where do we get vitamin D?

_____

Refused to answer ☐
Does not know ☐

EXPOSURE TO SUNSHINE - *when you were pregnant* between April 1st and October 31st.

Q.049 Did you use sunscreen on yourself?

Yes ☐
No ☐
Refused to answer ☐
Does not know ☐

Q.050 Did you spend time outdoors?

Yes ☐
No ☐
Refused to answer ☐
Does not know ☐

Q.051 If yes, was skin exposed to direct sunlight?

Yes ☐
No ☐
Refused to answer ☐
Does not know ☐

Q.052 How much skin was exposed?

_____

Refused to answer ☐
Does not know ☐

Q.053 On a typical day, how many minutes per day were you exposed to direct sunlight?

_____

Refused to answer ☐
Does not know ☐

THIS SURVEY IS FINISHED - THANK YOU.

6.2 Appendix 2

Dietary Assessment

VITAMINS

1. Have you ever regularly taken **multi-vitamins**?

- ○ **Never**
- ○ Have in the **Past only**
  - **a) For how many years did you take them in the past?**
    ○ 1 year or less ○ 2-4 years ○ 5-9 years ○ 10 or more years
- ○ **Currently** take them
  - **a) If you currently take multi-vitamins, how many do you take per week?**
    ○ 2 or less ○ 3-5 ○ 6-9 ○ 10 or more
  - **b) If you are currently taking multi-vitamins, for how many years have you been taking them?**
    ○ 1 year or less ○ 2-4 years ○ 5-9 years ○ 10 or more years
  - **c) If you are currently taking them, what brand do you usually use?**
    (Specify exact brand and type)

2. <u>**Not counting multi-vitamins**</u>, have you ever taken any of the following specific vitamins or minerals?

Vitamin A

- ○ **Never** taken
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

→ **Dose per day ?**

- ○ less than 8,000 UI
- ○ 8,000 to 12,000 UI
- ○ 12,001 to 22,000 UI
- ○ 22,001 UI or more
- ○ Don't know

→ **How long?**

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Beta Carotene

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

→ **Dose per day ?**

- ○ less than 8,000 UI
- ○ 8,000 to 12,000 UI
- ○ 12,001 to 22,000 UI
- ○ 22,001 UI or more
- ○ Don't know

→ **How long?**

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Vitamin B_6

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

→ **Dose per day ?**

- ○ less than 10 mg
- ○ 10 - 39 mg
- ○ 40 - 79 mg
- ○ 80 mg or more
- ○ Don't know

→ **How long?**

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Vitamin C

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

→ **Dose per day ?**

- ○ less than 400 mg
- ○ 400 - 700 mg
- ○ 701 - 1,250 mg
- ○ 1,251 mg or more
- ○ Don't know

→ **How long?**

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Vitamin E

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

- ○ less than 100 UI
- ○ 100 - 250 UI
- ○ 251 - 500 UI
- ○ 501 UI or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Selenium

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

- ○ less than 80 mcg
- ○ 80 - 130 mcg
- ○ 131 - 250 mcg
- ○ 251 mcg or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Iron

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

mg of elemental iron (325 mg Ferrous Sulphate = 65 mg elemental iron)

- ○ less than 10 mg
- ○ 10 - 39 mg
- ○ 40 - 80 mg
- ○ 81 mg or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Zinc

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

- ○ less than 25 mg
- ○ 25 - 74 mg
- ○ 75 - 100 mg
- ○ 101 mg or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Calcium or Dolomite (Include Tums®)

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

mg elemental calcium (1 Tums® = 500 mg calcium carbonate = 200 mg elementary calcium)

- ○ less than 400 mg
- ○ 401 - 900 mg
- ○ 901 - 1,300 mg
- ○ 1,301 mg or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

Fish Oil (Omega 3 fatty acids)

- ○ **Never**
- ○ Taken in the **past only**
- ○ Yes, **currently** take it

Dose per day ?

- ○ less than 25 mg
- ○ 26 - 74 mg
- ○ 75 - 100 mg
- ○ 101 mg or more
- ○ Don't know

How long?

- ○ 0-1 year
- ○ 2-4 years
- ○ 5-9 years
- ○ 10 years or more

What <u>other</u> supplements are you taking <u>currently</u> on a regular basis (at least once per week)?

- ○ None
- ○ Metamucil®
- ○ Cod liver oil
- ○ Brewer's yeast
- ○ Vitamin D
- ○ Folic acid or folate (B_9)
- ○ Potassium
- ○ Magnesium
- ○ Niacin
- ○ Other supplements (specify)

_____

DAIRY FOODS

In the following section, please describe how often on average you have used the amount specified in the past year. Please indicate your average total use, taking the portion size into account. For example, if you use ½ a glass of milk twice a week, mark 1 glass per week to represent your average total intake.

3. For each food listed, fill in the circle indicating your average total use of the amount specified during the past year.

Skim milk (8 oz. glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

1% or 2% milk (8 oz. glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

Whole milk (8 oz. glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

Cream, e.g., in coffee, whipped or sour cream (1 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 or more tbs. per day

Non-dairy coffee whitener (tsp.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 or more tbs. per day

Frozen Yogurt, sherbet or non-fat ice cream (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Ice cream (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Flavored yogurt, without sweetener (Aspartame, NutraSweet) (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Yogurt, plain or with sweetener (Aspartame, NutraSweet) (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

What type of yogurt do you usually eat?

- ○ None
- ○ Regular
- ○ Low fat
- ○ Nonfat

Cottage or ricotta cheese (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Cream cheese (1 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Other cheese, e.g. American, Cheddar, plain or part of a dish (1 slice or 1 oz. serving)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

What type of cheese do you usually eat?

- ○ None
- ○ Regular
- ○ Low fat or light
- ○ Nonfat

Butter (tbs.) added to food or bread; exclude use in cooking

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Margarine (tbs.) added to food or bread (exclude use in cooking)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

What type of margarine do you usually use? (do not include "spray" type margarine)

- ○ None

Form?

- ○ Stick
- ○ Tub
- ○ Squeeze (liquid)

Type ?

- ○ Regular
- ○ Light spread
- ○ Extra light spread
- ○ Nonfat

What specific **brand** and **type** (e.g. Becel Leger®)

FRUITS

4. Please fill in your average total use, during the past year, of each specified food.

Please try to average your seasonal use of foods over the entire year. For example, if a food such as cantaloupe is eaten 4 times a week during the 3 months that it is in season, then average total use would be once per week over the year.

Raisins or grapes (1 oz. or small pack)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Prunes (7 prunes or ½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day

Bananas (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ One per day
- ○ 2 or more per day

Cantaloupe (¼ melon)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2-3 times per day
- ○ 4 or more servings per day

Avocado (½ fruit or ½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Applesauce (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ One or more per day

Fresh apples or pears (1)

- ○ Never
- ○ Less than one per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ One per day
- ○ 2-3 per day
- ○ 4 or more per day

Apple juice or cider (small glass or 4 oz.)

- ○ Never
- ○ Less than one per month
- ○ 1-3 glasses per month
- ○ One glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ One glass per day
- ○ 2 or more glasses per day

Oranges (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ One per day
- ○ 2-3 per day
- ○ 4 or more per day

Orange juice (small glass or 4 oz.)

- ○ Never
- ○ Less than one per month
- ○ 1-3 glasses per month
- ○ One glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ One glass per day
- ○ 2 or more glasses per day

Grapefruit (½)

- ○ Never
- ○ Less than one per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2-3 times per day
- ○ 4 or more times per day

Grapefruit juice (small glass or 4 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ One glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ One glass per day
- ○ 2 or more glasses per day

Other fruit juice (small glass or 4 oz.)

- ○ Never
- ○ Less than one per month
- ○ 1-3 glasses per month
- ○ One glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ One glass per day
- ○ 2 or more glasses per day

Strawberries, fresh, frozen or canned (½ cup)

- ○ Never
- ○ Less than one per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once or more per day

Blueberries, fresh, frozen or canned (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once or more per day

Peaches, apricots or plums (1 fresh or ½ cup canned)

- ○ Never
- ○ Less than one per month
- ○ 1-3 per month
- ○ Once per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 or more per day

In summary, how many servings of fruit do you usually eat, not counting juices?

- ○ Never
- ○ Less than one per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4 or more per day

VEGETABLES

5. Please fill in your average total use, during the past year, of each specified food.

Tomatoes (1)

- ○ Never
- ○ Less than one per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ One or more per day

Tomato juice (small glass or 4 oz.)

- ○ Never
- ○ Less than one per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2 or more glasses per day

Tomato Sauce (½ cup) e.g. spaghetti sauce

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5 or more per week

Salsa or taco sauce (¼ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Tofu or soybeans (3-4 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

String beans (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5 or more servings per week

Broccoli (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Cabbage or cole slaw (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Cauliflower (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Brussels sprouts (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Carrots, raw (½ carrot or 2-4 sticks)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Carrots, cooked or juice (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ Once per day
- ○ 2 or more servings per day

Corn (1 cob or ½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ Once per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 or more per day

Peas or lima beans (½ cup fresh, frozen or canned)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Mixed vegetables (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Beans or lentils (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Dark orange squash (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Eggplant, zucchini or other summer squash (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Yams or sweet potatoes (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Spinach, cooked (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Spinach, raw (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Kale, mustard, or chard greens (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Iceberg or head lettuce (serving)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Romaine or leaf lettuce (portion)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ Once per day
- ○ 2 or more servings per day

Celery (½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Green peppers (3 slices or ¼ pepper)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Onions (1 slice)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

In summary, how many servings of vegetables do you usually eat, not counting salad or potatoes?

- ○ None
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4-5 per day
- ○ 6+ per day

EGGS, MEAT & FISH

6. Please fill in your average total use, during the past year, of each specified food.

Egg whites only (¼ cup or 1 egg)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Eggs whole, with yolk (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Bacon (2 slices)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Chicken or turkey sandwich

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5 or more per week

Chicken or turkey, with skin (4-6 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Chicken or turkey, without skin (4-6 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ Once per day
- ○ 2 or more servings per day

Beef or pork hotdogs (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ One per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ One per day
- ○ 2 or more servings per day

Chicken or turkey hotdogs (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ One per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ One per day
- ○ 2 or more servings per day

Salami, bologna, or other processed meat sandwiches

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Processed meats, e.g. sausage, kielbasa, etc. (2 oz. or 2 links)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Hamburger, lean or extra lean (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Hamburger, regular (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 servings per week
- ○ 1 or more servings per day

Beef, pork or lamb as a sandwich or mixed dish, e.g. Stew, casserole

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Pork as a main dish e.g. ham or chops (4-6 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Beef or lamb as a main dish e.g. steak or roast (4-6 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Liver: beef, calf or pork (4 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2 or more servings per week

Liver: chicken or turkey (1 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2 or more servings per week

Canned tuna fish (3-4 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Breaded fish cakes, pieces, or fish sticks (1 serving, store bought)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Shrimp, lobster, scallops or clams as a main dish (1 serving)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Dark meat fish e.g. mackerel, salmon, sardines, blue fish or swordfish (3-5 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

Other fish e.g. cod, haddock or halibut (3-5 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 or more servings per day

CEREALS, BREADS & STARCHES

7. Please fill in your average total use, during the past year, of each specified food.

Cold breakfast cereal (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2-3 cups per day
- ○ 4 or more cups per day

Cooked oatmeal/oat bran (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2-3 cups per day
- ○ 4 or more cups per day

Other cooked breakfast cereal (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2-3 cups per day
- ○ 4 or more cups per day

What brand and type of cold breakfast cereal do you usually eat?

Specify brand & type (e.g. Kellogg's Rice Krispies) →

- ○ Don't eat cold breakfast cereal

White bread (slice), or pita

- ○ Never
- ○ Less than once per month
- ○ 1-3 slices per month
- ○ 1 slice per week
- ○ 2-4 slices per week
- ○ 5-6 slices per week
- ○ 1 slices per day
- ○ 2-3 slices per day
- ○ 4-5 slices per day
- ○ 6 or more slices per day

Dark bread (slice), including wheat pita bread

- ○ Never
- ○ Less than once per month
- ○ 1-3 slices per month
- ○ 1 slice per week
- ○ 2-4 slices per week
- ○ 5-6 slices per week
- ○ 1 slices per day
- ○ 2-3 slices per day
- ○ 4-5 slices per day
- ○ 6 or more slices per day

Bagels, English muffins, or rolls (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more per day

Muffin (regular) or biscuits (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2 or more per day

Brown rice (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2 or more cups per day

White rice (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2 or more cups per day

Pasta e.g. spaghetti, noodles, etc. (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2 or more cups per day

Tortillas (1)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2-3 cups per day
- ○ 4 or more cups per day

Other grains e.g. bulgur, kasha, couscous, etc. (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2 or more cups per day

Pancakes or waffles (3 pieces)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 serving per day
- ○ 2 or more servings per day

French fried potatoes (small order or ½ cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 serving per day

Potatoes, baked, boiled or mashed (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ 1 serving per day
- ○ 2 or more servings per day

Potato chips or corn chips (small bag or 1 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2 or more servings per day

Crackers, Triscuits®, Wheat Thins (5)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2-3 times per day
- ○ 4 or more servings per day

Pizza (2 slices)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

BEVERAGES

CARBONATED BEVERAGES—Consider the serving size as one 12 oz. glass, bottle or can for these carbonated beverages.

8. Please fill in your average total use, during the past year, of each specified food.

LOW-CALORIE (Sugar-free types)

Low-calorie cola e.g. Diet Coke® with caffeine (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Low-calorie caffeine-free soda (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Other low-calorie carbonated beverage e.g. diet 7-Up, diet ginger ale (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

REGULAR TYPES (not sugar-free)

Coke®, Pepsi® or other cola with sugar (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Caffeine-Free Coke®, Pepsi® or other cola with sugar (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Other carbonated beverage with sugar e.g. 7-Up® (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

OTHER BEVERAGES

Hawaiian Punch®, lemonade or other non-carbonated fruit drinks (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

Beer, regular (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Light beer e.g., Bud Light® (1 glass, bottle, can)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cans per month
- ○ 1 can per week
- ○ 2-4 cans per week
- ○ 5-6 cans per week
- ○ 1 can per day
- ○ 2-3 cans per day
- ○ 4 or more cans per day

Red wine (4 oz. glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

White wine (4 oz. glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4 or more glasses per day

Liquor e.g. whisky, gin (1 drink or 1 oz. shot)

- ○ Never
- ○ Less than once per month
- ○ 1-3 drinks per month
- ○ 1 drink per week
- ○ 2-4 drinks per week
- ○ 5-6 drinks per week
- ○ 1 drink per day
- ○ 2-3 drinks per day
- ○ 4 or more drinks per day

Plain water, bottled or tap including mineral water and soda water (1 cup or glass)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4-5 glasses per day
- ○ 6 or more glasses per day

Herbal tea (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4-5 glasses per day
- ○ 6 or more glasses per day

Tea (1 cup), Not herbal teas

- ○ Never
- ○ Less than once per month
- ○ 1-3 drinks per month
- ○ 1 drink per week
- ○ 2-4 drinks per week
- ○ 5-6 drinks per week
- ○ 1 drink per day
- ○ 2-3 drinks per day
- ○ 4-5 glasses per day
- ○ 6 or more glasses per day

Decaffeinated coffee (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4-5 glasses per day
- ○ 6 or more glasses per day

Coffee with caffeine (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 glasses per month
- ○ 1 glass per week
- ○ 2-4 glasses per week
- ○ 5-6 glasses per week
- ○ 1 glass per day
- ○ 2-3 glasses per day
- ○ 4-5 glasses per day
- ○ 6 or more glasses per day

SWEETS, BAKED GOODS & MISCELLANEOUS

9. Please fill in your average total use, during the past year, of each specified food.

Pure chocolate candy bar or packet e.g. Hershey's®, M&M's®

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4 or more servings per day

Other mixed candy bars e.g. Snickers®, Reeses®, Milky Way®

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2-3 times per day
- ○ 4 or more servings per day

Candy without chocolate e.g. 1 pack mints, Lifesavers®

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2-3 per day
- ○ 4 or more servings per day

Jams, jellies, preserves, syrup or honey (1 tbs.)

- Never
- Less than once per month
- 1-3 tbs. per month
- 1 tbs. per week
- 2-4 tbs. per week
- 5-6 tbs. per week
- 1 tbs. per day
- 2-3 tbs. per day
- 4 or more tbs. per day

Peanut butter (1 tbs.)

- Never
- Less than once per month
- 1-3 tbs. per month
- 1 tbs. per week
- 2-4 tbs. per week
- 5-6 tbs. per week
- 1 tbs. per day
- 2-3 tbs. per day
- 4 or more tbs. per day

Popcorn (1 cup)

- Never
- Less than once per month
- 1-3 cups per month
- One cup per week
- 2-4 cups per week
- 5-6 cups per week
- One cup per day
- 2 or more cups per day

Pretzels (1 oz. or small bag)

- Never
- Less than once per month
- 1-3 times per month
- Once per week
- 2-4 times per week
- 5-6 times per week
- Once per day
- 2-3 times per day
- 4 or more times per day

Cookies, home baked (1)

- Never
- Less than once per month
- 1-3 cookies per month
- 1 cookie per week
- 2-4 cookies per week
- 5-6 cookies per week
- 1 cookie per day
- 2-3 cookies per day
- 4 or more cookies per day

Cookies, ready made (1)

- Never
- Less than once per month
- 1-3 cookies per month
- 1 cookie per week
- 2-4 cookies per week
- 5-6 cookies per week
- 1 cookie per day
- 2-3 cookies per day
- 4 or more cookies per day

Brownies (1)

- Never
- Less than once per month
- 1-3 times per month
- Once per week
- 2-4 times per week
- 5-6 times per week
- Once per day
- 2 or more per day

Doughnuts (1)

- Never
- Less than once per month
- 1-3 times per month
- Once per week
- 2-4 times per week
- 5-6 times per week
- Once per day
- 2-3 per day
- 4 or more per day

Cake, home baked (slice)

- Never
- Less than once per month
- 1-3 slices per month
- One slice per week
- 2-4 slices per week
- 5-6 slices per week
- 1 or more slices per day

Cake, ready made (slice)

- Never
- Less than once per month
- 1-3 slices per month
- 1 slice per week
- 2-4 slices per week
- 5-6 slices per week
- 1 or more slices per day

Pie, homemade (slice)

- Never
- Less than once per month
- 1-3 slices per month
- 1 slice per week
- 2-4 slices per week
- 5-6 slices per week
- 1 or more slices per day

Pie, ready made (slice)

- Never
- Less than once per month
- 1-3 slices per month
- 1 slice per week
- 2-4 slices per week
- 5-6 slices per week
- 1 or more slices per day

Sweet roll, coffee cake or other pastry, home baked (1 serving)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Sweet roll, coffee cake or other pastry, ready made (1 serving)

- ○ Never
- ○ Less than once per month
- ○ 1-3 times per month
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Once per day
- ○ 2 or more servings per day

Peanuts (small packet or 1 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ One per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ One per day
- ○ 2 or more servings per day

Other nuts (small packet or 1 oz.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2 or more per day

Oat bran, added to food (1 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Other bran, added to food (1 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Wheat germ (1 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Chowder or cream soup (1 cup)

- ○ Never
- ○ Less than once per month
- ○ 1-3 cups per month
- ○ 1 cup per week
- ○ 2-4 cups per week
- ○ 5-6 cups per week
- ○ 1 cup per day
- ○ 2 cups or more per day

Ketchup or red chili sauce (1 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Salt added at table (1 shake)

- ○ Never
- ○ Less than once per month
- ○ 1-3 shakes per month
- ○ 1 shake per week
- ○ 2-4 shakes per week
- ○ 5-6 shakes per week
- ○ 1 shake per day
- ○ 2-3 shakes per day
- ○ 4-5 shakes per day
- ○ 6 shakes or more per day

How many teaspoons of sugar Do you add to your beverages or food each day?

Teaspoons

NutraSweet® or Equal® (1 packet) NOT Sweet 'N Low®

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4-5 per day
- ○ 6 packets or more per day

Garlic (1 clove or 4 shakes)

- ○ Never
- ○ Less than once per month
- ○ 1-3 per month
- ○ 1 per week
- ○ 2-4 per week
- ○ 5-6 per week
- ○ 1 per day
- ○ 2-3 per day
- ○ 4-5 per day
- ○ 6 or more per day

Low-fat or fat-free mayonnaise (2 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Regular mayonnaise (2 tbs.)

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Salad dressing (2 tbs.) →

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

Type of salad dressing:

- ○ Non fat
- ○ Low fat
- ○ Olive oil dressing
- ○ Regular

Olive oil added to food or bread (1 tbs.); exclude use in cooking

- ○ Never
- ○ Less than once per month
- ○ 1-3 tbs. per month
- ○ 1 tbs. per week
- ○ 2-4 tbs. per week
- ○ 5-6 tbs. per week
- ○ 1 tbs. per day
- ○ 2 tbs. or more per day

10. How much of the visible fat on your beef, pork or lamb do you remove before eating?

- ○ Don't eat meat
- ○ Remove all visible fat
- ○ Remove most
- ○ Remove small part of fat
- ○ Remove none

11. What kind of fat is usually used for frying and sautéing at home?

- ○ Don't fry
- ○ Butter
- ○ Margarine
- ○ Olive oil
- ○ Vegetable oil
- ○ Vegetable shortening
- ○ Lard/bacon fat
- ○ Pam type spray

12. What kind of fat is usually used for baking at home?

- ○ Don't bake
- ○ Butter
- ○ Margarine
- ○ Olive oil
- ○ Vegetable oil
- ○ Vegetable shortening
- ○ Lard/bacon fat
- ○ Pam type spray

13. How often do you eat food fried, stir-fried in oil or sautéed at home?

- ○ Never
- ○ Less than once a week
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Daily

14. How often do you eat deep fried food away from home or as take out (e.g. french fries, fried chicken, fish, clams, shrimp, etc.)?

- ○ Never
- ○ Less than once a week
- ○ Once per week
- ○ 2-4 times per week
- ○ 5-6 times per week
- ○ Daily

15. **What type of cooking oil is usually used at home (e.g. Crisco, Mazola, etc)?**

(Specify brand and type)

16. **Are there any other foods not mentioned above that you usually eat <u>at least once per week</u>?**

Other foods that you usually eat at least once per week	Usual serving size	Servings per week
(a)		
(b)		
(c)		

17. Do you currently follow a special diet?

- ○ No
- ○ Yes → ○ Physician prescribed
  ○ Self prescribed

a) If yes, for how many years?

(Number of years on diet)

b) If yes, what kind of diet do you follow?

- ○ Weight reduction (low calorie)
- ○ Low cholesterol
- ○ Low sodium
- ○ Diabetic
- ○ Low fat
- ○ Low triglyceride
- ○ Ulcer
- ○ High potassium

(Specify type of diet)

- ○ Other →

18. How has your use of the following foods and beverages changed over the PAST TEN YEARS:

Whole milk	**Butter**	**Margarine**
○ Use has decreased	○ Use has decreased	○ Use has decreased
○ Use about the same	○ Use about the same	○ Use about the same
○ Use has increased	○ Use has increased	○ Use has increased
Eggs	**Fish**	**Red meat**
○ Use has decreased	○ Use has decreased	○ Use has decreased
○ Use about the same	○ Use about the same	○ Use about the same
○ Use has increased	○ Use has increased	○ Use has increased
Fruits	**Vegetables**	**Whole wheat bread**
○ Use has decreased	○ Use has decreased	○ Use has decreased
○ Use about the same	○ Use about the same	○ Use about the same
○ Use has increased	○ Use has increased	○ Use has increased
Whole grains	**Sugar**	**Alcohol**
○ Use has decreased	○ Use has decreased	○ Use has decreased
○ Use about the same	○ Use about the same	○ Use about the same
○ Use has increased	○ Use has increased	○ Use has increased

Date:
Questionnaire by:

Vitamin D dose-response study to establish dietary requirements in infants.
Subject code:_____

24-Hour Recall Form - Mother

Subject code:_____ Visit number: 1st ☐ 3mo ☐ 6mo ☐ 9mo ☐ 12mo ☐

Date of birth:_____/_____/_____
DA MO YEAR

Age:_____years

Sex: F

Interviewer name:_____

Date of interview:_____/_____/_____
DA MO YEAR

Day of interview:_____

Time started:_____ AM / PM

Time ended:_____ AM / PM

Interview conducted: In person / By telephone

Vitamin D dose-response study to establish dietary requirements in infants.
Subject code:_____

Step 1	Step 2	Step 3	Step 4						Step 5
Quick List (write down all foods named by participant)	**Food/Drink and Additions** (items identified in quick list)	**Time** (record when item was consumed)	**Mode of Preparation**-if applicable						**Description of Food/Drink and Ingredient Amount** (how much and type of item that was consumed)
			raw	Baked	fried	boiled	microwave	barbeque	
	1.	a p							
	2.	a p							
	3.	a p							
	4.	a p							
	5.	a p							
	6.	a p							
	7.	a p							
	8.	a p							
	9.	a p							
	10.	a p							
	11.	a p							
	12.	a p							
	13.	a p							