MATERNAL N-3 FATTY ACID NUTRITION AND EARLY INFANT OUTCOMES

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ABSTRACT

Research indicates maternal docosahexaenoic acid (DHA, 22:6n-3) status during pregnancy is positively associated with infant neurodevelopment. Dietary deficiency, if present, usually occurs at the lower end of the intake distribution. Both DHA intake and the current intakes of other n-3 and n-6 fatty acids may affect risk of deficiency. Regardless, the maternal dietary intakes, and biochemical markers, or infant developmental scores indicative of maternal DHA deficiency are not defined. The objectives of this research are to determine the distribution of DHA intakes, the relationship between dietary DHA and n-6 fatty acid intakes and maternal red blood cell (RBC) phospholipid DHA, and whether or not maternal DHA status low enough to increase risk of poor infant development occurs in our community. This study was a prospective, randomized intervention study involving supplementation of healthy women from 16 weeks gestation to delivery of their infant with 400 mg/d DHA or placebo. Maternal dietary intakes and blood lipid DHA were measured at 16 and 36 weeks gestation, and infant visual acuity was assessed at 60 d of age. The results show DHA intake was skewed, and maternal dietary n-6 fatty acids were inversely, while DHA intake was positively related to levels of maternal RBC EPG DHA, $P<0.05$. Infant gender and maternal DHA supplementation were significantly related to infant visual acuity. More infant girls in the placebo than DHA supplementation group had a visual acuity below the group average for infant girls, $P<0.05$. Maternal RBC EPG 22:4n-6 at 36 weeks gestation was inversely related to infant visual acuity at 60 d of age, $P<0.05$. In conclusion the results suggest that DHA deficiency is present among pregnant women in our community and that maternal dietary n-6 fatty acid intake may be an important modifier of maternal DHA status.
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Dr. Sheila M. Innis is the principal investigator who conceived, designed and implemented this study. Russell W. Friesen undertook all of the dietary and analysis, and organized blood collections. The authors jointly conceived and wrote the manuscript. This paper also forms part of the graduate thesis degree requirement for Russell W. Friesen. None of the authors had any conflict of interests.

MANUSCRIPT 2

Dr. Sheila M Innis was the principal investigator that conceived, designed and implemented this study. Russell W. Friesen contributed throughout the entire study by compiling and analyzing the data, and at all stages of the manuscript preparation and data interpretation. None of the authors had any conflict of interest. The study design and statistical analyses were conducted with assistance of Ruth A Milner.
CHAPTER 1: INTRODUCTION

This thesis concerns the role of the n-3 fatty acid docosahexaenoic acid (DHA; 22:6n-3) during pregnancy and infant central nervous system (CNS) development. Humans lack the ability to synthesize n-3 fatty acids, thus all of the n-3 fatty acids accrued by the developing fetus must be derived from the maternal diet and via the placenta transfer and fetal circulation delivered tissues (1). DHA is present in tissues as a fatty acid acylated in glycerol-phospholipids, has a highly specific tissue and phospholipid distribution, and is particularly abundant in membrane lipids of the brain and retina (2, 3). In animals, dietary deficiency of n-3 fatty acids results in decreased DHA with a compensatory increase in n-6 fatty acids in the brain and retina, reduced visual function, and decreased performance on tasks of learning (4, 5). Epidemiological research has provided some evidence to suggest increased risk of poor visual and neural development among children of women with low DHA or intakes of fish during pregnancy and lactation (6-9), and intervention studies to increase DHA intakes among pregnant and lactating women have found early and long-term benefits on several tests of CNS-developmental test scores in children (10-13). Several expert groups have made recommendations to include fish, the major dietary source of DHA in the diet, or have made specific recommendations for DHA intakes during pregnancy and lactation (14-18). Regardless, it is still unknown whether or not maternal DHA intakes or status during pregnancy are so low among some women as to increase risk of poor infant CNS development. Furthermore, neither the dietary intakes nor biochemical markers of DHA deficiency are defined (18). The research described in this thesis was designed to address these objectives: to describe the distribution and sources of dietary n-6 and n-3 fatty acid intakes among pregnant women; to describe the relationships between dietary n-6 and n-
3 fatty acid intakes and maternal DHA status in gestation; and to describe an approach to identify whether or not DHA deficiency is present among pregnant women in our community by comparing the distribution of visual acuity scores at 60-d of age among infants born to women taking 400 mg/d DHA during pregnancy compared to women taking a placebo. Finally, the data collected was used to explore possible biochemical markers of poor DHA status during pregnancy to provide relevant data useful to consider the dietary recommended intakes for DHA during pregnancy.

1.1. Polyunsaturated Fatty Acids

1.1.1. Essential fatty acids

Dietary essential nutrients are nutrients that cannot be synthesized by humans but are needed to support growth, normal cell function and freedom from disease (18). There are two families of essential fatty acids, for which the parent 18-carbon polyunsaturated fatty acids are linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) (18). Fatty acids have a carboxyl end (COOH), a methylene carbon chain (CH\(_n\)) and a methyl end (CH\(_3\)), and are distinct in their structure and functions based on their degree of unsaturation, and the position of the first methylene-interrupted double bond from the methyl (n) end in unsaturated fatty acids (18). Humans lack the delta-12 and delta-15 desaturase enzymes necessary to insert a double bond at the sixth and third carbon from the methyl end of a fatty acid chain necessary for the synthesis of LA and ALA (18). LA is the parent fatty acid of the n-6 fatty acid family, which includes arachidonic acid (ARA, 20:4n-6), docosatetraenoic acid (DTA, 22:4n-6) and docosapentaenoic acid (DPA, 22:5n-6). ALA is the parent fatty acid of the n-3 family of fatty acids, which includes eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPAn-3, 22:5n-3) and DHA (Figure 1-1).
The essentiality of LA was first recognized in rats fed diets almost completely devoid of fat, which resulted in deficiency symptoms that included scaly skin, growth retardation, reproductive failure and histological abnormalities (19). Providing LA in the diet reversed these symptoms (19). Subsequent studies in infants and children fed skimmed cows’ milk and patients receiving parenteral nutrition without a source of fat (20-23), demonstrated clinical signs of a deficiency that was associated with a lack of LA (18). The importance of ALA was identified when altered electroretinograph (ERG) recordings and behaviour was found in animals fed n-3 fatty acid deficient diets (24-26). Further, a variety of changes in learning behaviors in animals fed ALA deficient diets have been reported (4). The biological functions of the n-6 and n-3 fatty acids include acting as structural components of cell membranes, metabolism to eicosanoids, docosanoids and resolvins, and direct and indirect influence on gene expression and on circulating lipid levels (27-33).

1.1.2. Metabolism

Fatty acids, including the n-6 and n-3 families of fatty acids are also used as substrates for beta-oxidation mainly for the provision of energy, and LA and ALA are also desaturated and elongated to their longer chain fatty acid metabolic products, most notably ARA, EPA and DHA (4, 5, 18, 34, 36). Partitioning of fatty acids towards these cycles is likely to be dependent upon many factors including, but not limited to, energy balance, energy substrate availability, hormone regulation, and fatty acid chain length and unsaturation.

1.1.2.1. Oxidation

Fatty acid beta-oxidation occurs mainly in muscle tissue and proceeds through two distinct cellular pathways; peroxisomal and mitochondrial (34-37). Each cycle of beta-oxidation results in the formation of four molecules of adenosine triphosphate (ATP), the major
chemical energy carrier, and an acetyl CoA that can be used for further ATP production via the Krebs cycle (34).

In vivo radioisotope tracer studies of fatty acid oxidation, measured in rats, have shown that 18:3n-3 is oxidized more rapidly than 18:1n-9, 18:2n-6, 18:0 or longer chain fatty acids (38). Similar findings were reported in radioisotope tracer studies in humans in which the oxidation of fatty acids was in the order of 18:3n-3>18:2n6>18:1n-9> 16:0>18:0 (39). The rapid oxidation of 18:3n-3 may be explained by a higher affinity for 18:3n-3 of the enzymes facilitating entry into the mitochondria. However, whether a specific acyl CoA synthetase preferentially binds 18:3n-3 and facilitates transfer into the mitochondria has not been described. Regardless, these findings suggest the possibility of limited ALA availability for desaturation and elongation to DHA.

1.1.2.2. Desaturation and elongation

Although mammals lack the delta-12 and delta-15 desaturase enzymes required for the synthesis of LA and ALA (18), LA and ALA derived from the diet can be further metabolized by a common pathway of desaturation and elongation to their longer chain metabolites including the n-6 fatty acids ARA, DTA and DPA, and the n-3 fatty acids EPA and DHA, respectively (4, 5, 18). Fatty acid desaturation and elongation are considered to occur sequentially via delta-6 desaturation, elongation and delta-5 desaturation on the endoplasmic reticulum (ER), and via this pathway LA and ALA are thought to be converted to ARA and EPA, respectively (4, 5, 18). The generally accepted pathway for DHA synthesis is further metabolism of EPA to tetracosahexaenoic acid (THA, 24:6n-3), followed by transport to peroxisomes for 1 cycle of peroxisomal beta-oxidation to generate DHA, after which DHA is shuttled back to the ER for incorporation into phospholipids (5, 40-43).
Synthesis of the n-6 fatty acid DPA from LA and ARA is believed to occur through the same pathway as for the synthesis of DHA (Figure 1-2, 4, 5,).

Because LA and ALA are thought to use the same desaturation and elongation enzymes, concern has been raised that high intakes of LA will competitively inhibit EPA and DHA synthesis from ALA (44, 45). Consistent with this, reducing the intake of LA, or increasing the intake of ALA increased blood levels of EPA, however, neither strategy has been shown to significantly increase blood lipid levels of DHA over the ranges of dietary LA and ALA intake studied thus far (46-51).

Several studies have shown that term and preterm infants and adults can desaturate and elongate ALA to DHA (52-54). Relevant to the studies in this thesis, the ability to desaturate and elongate n-6 and n-3 fatty acids is thought to increase during pregnancy, possibly through the action of estrogen (55, 56), and increases in plasma and RBC membrane phospholipid DHA concentrations during gestation have been reported (57-59). However, available evidence suggests that increasing intakes of ALA during gestation does not lead to a significant increase in blood lipid DHA in either the mother or in her newborn infant (51). Higher dietary intakes of preformed DHA on the other hand, do increase plasma and RBC phospholipid levels of DHA in adults and infants (60-64), and higher intakes of DHA by women during pregnancy also leads to increased levels of DHA in the cord blood lipids of newborn infants (1, 65-69). Thus, it may be desirable, or necessary, to bypass the ALA desaturation and elongation pathway, and provide a dietary supply of DHA when attempting to increase DHA status during pregnancy.
1.1.3. Sources

Linoleic acid and ALA are found in a wide range of plants commonly consumed in human diets such as nuts, seeds, vegetables, legumes, grains, and fruits. However, most plant products are relatively low in total fat content, and thus have a low quantity of LA and ALA (70). On the other hand, many oils commercially produced from plant seeds such as soybean and canola are rich sources of LA and ALA (70, 71). Plant cells, unlike mammalian cells, however, lack the desaturase and elongase enzymes required for LA and ALA metabolism to ARA, and EPA and DHA, respectively. Therefore, the only dietary sources of ARA, EPA and DHA are animal tissue lipids. Arachidonic acid is present in the diet mainly in meats and poultry, eggs, and some fatty fish (18, 71, 72), whereas the richest dietary source of EPA and DHA are fish and seafood (71, 73). More specifically, cold-water fatty fish including salmon, herring, mackerel, anchovies and sardines contain relatively high levels of EPA and DHA (18, 72, 74). However, the concentration of these fatty acids is known to vary widely among different fish species (74). For example, lean white fish such as Atlantic cod provides about 120 mg DHA/100g edible product, whereas a fatty fish such as Atlantic and Pacific salmon provides about 1100 mg DHA/100g (72, 74). This variability in species exemplifies the importance of determining the fish species consumed when estimating dietary DHA intakes. Other sources of DHA and EPA include some meats, liver, and eggs (70, 75), and these may be important dietary sources of n-3 fatty acids for individuals not consuming fish. Because fish and seafood are the richest dietary sources of DHA, it is possible that dietary ARA intakes, or the dietary balance of n-6/n-3 fatty acids, will differ among individuals with different intakes of fish. A summary of the LA and ALA and ARA, EPA and DHA content in the total fat of commonly consumed foodstuffs is provided in Table 1-1.
Triglycerides from certain marine single cell algae such as *Cryptocodinium cohnii* are also rich in DHA (70), and are generally recognized as safe (GRAS) for human consumption (76). Triglycerides from *Cryptocodinium cohnii* rich in DHA may provide a more practical source of DHA than modifying food consumption patterns when attempting to increase DHA intakes. Moreover, advice to eat fish to increase DHA intakes will lead to changes in the intake of other nutrients, and may not be acceptable to some.

1.2. Recommendations and Intakes

1.2.1. Recommendations

Nutrient intake recommendations for macronutrients including fatty acids for healthy individuals in North America are reported in the Institute of Medicine/National Academy of Science (IOM) 2002 Dietary Reference Intakes (DRI). Dietary requirement was defined in the IOM 2002 DRI

“as the lowest continuing intake level of a nutrient that, for a specific indicator of adequacy, will maintain a defined level of nutriture in an individual (18).”

Requirements are usually based on the evaluation of all relevant information from animal studies, human feeding studies, observational studies, and randomized clinical trials revealing a certain level of a nutrient intake that reduces the incidence of disease (18). An estimated average requirement (EAR) is then established, which is the intake of a specific nutrient that meets the requirements of 50% of the population by life-stage and gender. For nutrients for which the distribution of requirement is normal, two SD are added to the EAR to provide a recommended daily allowance (RDA), which is the intake of a specific nutrient
expected to meet the requirements for 97.5% of the population by life-stage and gender (18). Recommendations for the n-6 and n-3 fatty acids LA and ALA, on the other hand, were defined as Adequate Intakes (AI), and for these fatty acids are the median intakes for individuals by life-stage and gender, derived from national dietary intake surveys in the U.S. (18). An AI value was published for LA and ALA because the macronutrient panel considered that insufficient information was available for healthy individuals in which to establish requirements for either the n-6 or n-3 fatty acids (18). Thus, in groups with diets similar to the U.S., 50% of individuals will have an intake below the AI, and interpretation is limited to whether or not their diet is similar to, or different from the average U.S. diet. The AI for LA and ALA for men, women and pregnant women is provided in Table 1-2 (18). These intakes were considered acceptable intake goals for LA and ALA because the IOM 2002 DRI macronutrient panel considered that intakes much lower than the recommended AI occur in the sampled population without signs that can be attributed to deficiency symptoms (18).

In the IOM 2002 DRI, an Acceptable Macronutrient Distribution Range (AMDR) was established and was defined as a range

“…of macronutrient intakes that are associated with reduced risk of chronic disease, while providing recommended intakes of other essential nutrients (18),”

and are expressed as a percentage of total energy intake. LA and ALA also contribute to total energy; and thus an AMDR was developed for each of these two fatty acids. The lower
boundary for the intake of LA was set at the AI, or 7% total energy (18). The upper boundary for LA is set at 10% of energy because 1) individual LA intakes rarely exceed 10% total energy, 2) there is a lack of evidence on the safety of LA intakes above 10% total energy, and 3) it is thought that high intakes of LA may create a pro-oxidant state that may predispose individuals to chronic disease (18). The lower boundary of the AMDR for ALA is set at the AI, 0.6%, while the upper boundary corresponds to the highest ALA intake from foods consumed by individuals in the U.S., or 1.2% total energy (18). The IOM 2002 DRI also states that up to 10% of the AMDR for n-3 fatty acids can be consumed as EPA and DHA, based on the U.S. intake data, which indicates that EPA and DHA represent 10% of current total n-3 fatty acid intakes (18).

The Tolerable Upper Limit (UL) is defined as

“…the highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population (18).”

A UL was not set for n-6 polyunsaturated fatty acids because of the lack of a defined intake level at which an adverse reaction occurs. However, concern was raised over intakes greater than 10% of energy because insufficient information exists on intakes above this amount (18). Although EPA and DHA have been shown to have immuno-suppressive properties and high intakes may result in prolonged bleeding times and hemorrhagic stroke, no UL recommendations were set for the n-3 fatty acids by the IOM (18). Recommendations for upper intakes however, have been made by The Food and Drug Administration (FDA),
which recommends that the intake of EPA plus DHA not to exceed 3 g/d from both food sources and supplements, based on a review of scientific research on the association and affects of EPA and DHA on coronary heart disease (77). However, it is to note, that the endpoints considered by the FDA were not the same as those considered in by the IOM 2002 DRI macronutrient panel.

Several national and international health organizations and expert groups have also made recommendations for n-3 fatty acids. Among these, the World Health Organization (WHO) has recommended that men and women consume approximately 1 to 2% of total energy as n-3 fatty acids (14). The International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommended 0.7% of energy ALA, 500 mg/d EPA+DHA or more, and at least 300 mg/d DHA for pregnant women (17). In Canada, Health and Welfare Canada in 1990 recommended that pregnant women 19 to 24 years and 25-49 years of age should consume a minimum of 1.36 g/day and 1.26 g/day of n-3 polyunsaturated fatty acids, respectively, during the 2nd and 3rd trimester with a minimum intake of 300 mg/day DHA for pregnant and lactating women (16).

Epidemiological studies to show an association between low fish intakes during pregnancy and an increased risk of poor infant and child neuro-development (6-8), and decreased incidence of coronary heart disease (78, 79), have also prompted some health organizations and expert groups to make recommendations for fish consumption. The FAO/WHO recommended the consumption of 1-2 servings of fish per wk, which would provide approximately 200-500 mg EPA+DHA depending on the fish (14), while the Institute of Medicine recommended children up to 12 years of age, females who are or may become pregnant, and women who are breastfeeding, consume two 3 oz servings of seafood
Canada’s Food Guide for Healthy Eating recommends that all people consume two “Food Guide Servings” of fish per wk, and choose fish higher in fat content, such as salmon, mackerel, or herring (80). The American Dietetic Association and the Dietitians of Canada (ADA/DC) position paper published in 2007 encouraged the consumption of two 3 oz servings of fish per wk, particularly fish and seafood with higher EPA and DHA concentrations (81). Further, the Heart and Stroke Foundation recommended consuming n-3 fatty acids from such sources as fatty fish, flaxseed, canola oil, soybean oil, and nuts (82), while the American Heart Association (AHA) recommended people consume fish, and more specifically fatty fish, two times per wk to provide an intake of approximately 1 g/d n-3 fatty acids (83).

In summary, although an increasing body of evidence is accumulating to show that the n-3 fatty acids are important for human health, and numerous recommendations for dietary n-3 fatty acid intakes have been made, it is important to remember that current recommendations for n-6 and n-3 fatty acids are not based on scientific knowledge of actual physiological requirements, which may also differ for different end points such as CVD and mental health.

1.2.2. Intakes

Median dietary intakes for LA and ALA published in the IOM 2002 DRI were based on data collected from the Continuing Survey of Food Intakes by Individuals (CFSII, 1994-1996, 1998), which used 24-hour dietary questionnaires collected from over 20,000 individuals, including men and women of all ages, designed to gather information on the usual dietary habits of the U.S. population (18). The median intakes for LA and ALA for pregnant women were found to be 13 g/d and 1.4 g/d, respectively (18). Similar to the median intakes
reported in the IOM 2002 DRI, mean intakes of LA and ALA found in studies involving pregnant women in North America were 11.2-16.8 g/d and 1.5-1.7 g/d, respectively (75, 84, 85). Mean intakes among pregnant women in Europe were 9.3-13.3 g/d and 1.3-1.4 g/d, for LA and ALA, respectively (86, 87). Although the IOM 2002 DRI did not report intakes for ARA, the ranges for EPA and DHA intakes were 4 to 7 mg/d, and 52 to 93 mg/d, respectively (18). Several studies assessing dietary fatty acid intakes have reported mean intakes of ARA, EPA and DHA (75, 84-88). More specifically, mean DHA intakes of 82±115 mg/d and 160±246 mg/d have been reported among pregnant women in Canada (75, 84); 81±94 mg/d among African American pregnant women in the U.S. (85); and 280±190 mg/d, 140±224 mg/d, and 300±300 mg/d DHA among pregnant women in Europe (89-90). However, the large SD for DHA intakes reported in these studies suggests that individual intakes vary widely from the mean. Furthermore, it is not known whether the intake distribution for these fatty acids is normally distributed. Finally, because fish and not ruminant meats and dairy products, is naturally rich in DHA, it is unclear whether the balance of n-6/n-3 fatty acids differs among individuals with different intakes of fish, which may be important determinants of n-3 fatty acid recommendations if high n-6 fatty acids interfere with n-3 fatty acid metabolism (44, 45).

1.3. Functional Roles of n-3 Fatty Acids

Currently, the known functional roles of n-3 and n-6 fatty acids generally include structural roles within cell membranes, roles in regulation of gene expression, and as precursors for the synthesis of metabolically active compounds (27-33, 52, 89-100). Cell membranes, including RBC membranes, are composed of a phospholipid bilayer, where these phospholipids and the fatty acid moieties therein, play a role as structural components of cell membranes, while
creating the matrix in which membrane proteins function (28-29). The general structure of a phospholipid is a glycerol backbone, with a fatty acid esterified at the \( sn-1 \) and \( sn-2 \) position and an alcohol linked to the \( sn-3 \) position by a phosphodiester bond. The most abundant phospholipids, distinct by their alcohol moiety include phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) (Figure 1-3, 101). Further, although most phospholipids have a saturated fatty acid in the \( sn-1 \) position and an unsaturated fatty acid in the \( sn-2 \) position, the PE, PS and PC species in retinal outer segment membranes can have DHA at both the \( sn-1 \) and \( sn-2 \) position (5). Unsaturated lipids, such as DHA in the photoreceptor membrane, are thought to result in greater rhodopsin mobility within the membrane and an increase in retinal sensitivity (102-104), providing evidence to suggest that DHA plays an important structural role in membranes of the retina.

The n-3 and n-6 fatty acids are released from membrane phospholipids by phospholipases, and then can be further metabolized to eicosanoids, and in some cells to docosanoids and resolvins, which participate in many biological processes, including but not limited to playing a role in the pathology of inflammatory responses, and in the brain acting as secondary messengers, and providing neuroprotection (30, 31, 89-97). In the CNS, eicosanoids have been shown to serve as secondary messengers in the modulation of synaptic signal transduction pathways, which are thought to contribute to the development, differentiation, function, protection, and repair of the cells of the CNS (89, 90, 94). Once released into the cytosol by phospholipases, DHA can be further metabolized to docosanoids such as neuroprotectin D1 (NPD1), and resolvins such as resolvin D1 (RvD1) (93, 105, 106). Docosanoids, such as NPD1, were initially identified in isolated cells of the CNS and have
been shown to be potent inhibitors of oxidative stress (98-100); in retinal pigment epithelial cells under conditions of oxidative stress; and in the brain during experimental stroke in animals (107).

Polyunsaturated fatty acids and their metabolites also appear to have distinct capacities to modulate gene expression (32, 33, 98-100). Transcription factors identified as targets for fatty acid regulation of gene expression include peroxisome proliferator-activated receptors (PPAR), sterol regulatory element binding protein (SREBP), hepatic nuclear factor (HNF), retinoid X receptor (RXR), and liver X receptor (LXR) (32, 33), transcription factors that are involved in lipid and glucose metabolism, energy utilization, and inflammatory mediator synthesis (101). In addition, the n-3 fatty acid DHA has also been shown to regulate expression of genes associated with neurogenesis, neurotransmission and connectivity, as well as cell division, growth, and apoptosis (99, 100).

Although these functions serve as the basis for the current understanding of the roles in which n-3 and n-6 fatty acids play, their multiple functions in different tissues and cell types makes it exceedingly difficult to set target endpoints in which to develop dietary recommendations for humans.

1.4. N-3 Fatty Acids and the Central Nervous System

1.4.1. Development of the central nervous system

Critically important aspects of CNS structural development occur between conception and the first 2 years after birth (108). This period is considered to be a developmental window where the potential for adverse effects due to inadequate nutrition to have lasting consequences on neural functions is greatest (109). The manifestation of an insult, such as that resulting from dietary inadequacy during development, will depend not only on the
duration and severity of the dietary insult but also on the stage of development at which the insult occurs (109). Structural development of the brain has eight interrelated but distinguishable events that include: neuronal induction, neuroblast proliferation, neuronal migration, neuronal selective aggregation, neuronal differentiation and formation of specific patterns of connection, neuronal death, selective synapse elimination, and myelination (110). By six months of gestation, neurogenesis and migration within the cortex is largely complete (108). Importantly, the cortex is responsible for integration of sensory inputs including such processes as learning, speech, hearing and sight, and visual recognition processes (108).

The eye begins to develop approximately 22 d after conception, while the visual system begins to myelinate around 40 wk gestation and is complete within a few months after birth (111). Myelination is the addition of layers of phospholipids, cerebrosides, cholesterol, as well as other compounds surrounding the axons of neurons, producing an insulating sheath responsible for the speed at which impulses propagate along the myelinated fibre (3). When light falls on the retina, a conformational change occurs in the retinal pigment rhodopsin, resulting in the photoreceptors becoming hyperpolarized, and sending a signal to the horizontal and bipolar cells (112). Bipolar cells make synaptic contact with ganglion cells, ganglion cells and axons join and exit the retina at the optic nerve, which carries the signal to the lateral geniculate nucleus and primary visual cortex for processing (112). Because the final step in the structural development of the visual system is largely complete before birth (113), it is conceivable that an infant’s performance on tests of visual development may be affected by the supply of key nutrients during the prenatal period.
1.4.2. DHA in the central nervous system

Approximately 90% of fat present at birth is deposited in the last 10 wk of pregnancy (114). An infant born full term, 37-42 weeks gestation, has a brain weight of about 350 g (115), approximately 35 grams of which is lipid (5). Available estimates suggest that the fetus accumulates approximately 67 mg/d n-3 fatty acids, mostly DHA, in the last trimester of gestation, some of which is accrued in CNS tissue (116, 117). In brain grey matter and the rod and cone outer segments of the retina, the PE and PS contain high proportions of DHA (2, 5), relative to PE and PS in most other tissues. Moreover, as discussed earlier, the photoreceptor membrane PE, PS and PC may have DHA acylated at both the sn-1 and sn-2 positions (5). This unusual enrichment of DHA in the brain and retina suggest that DHA plays an important functional role in these cells.

Fatty acids are released from the placenta and are transported via the umbilical vein to the fetal liver (1). Alpha-fetoprotein the major plasma transport protein before birth, and has higher proportions of DHA than adult albumin (4). The brain takes up DHA from plasma, where it is transported mainly bound to transport proteins (4). Alternatively, in vitro studies have shown that astrocytes, and cerebral endothelial cells of the CNS can synthesize DHA from ALA (118-120), although whether or not these cells have the ability to contribute appreciable amounts of DHA to the brain remains uncertain.

In animals feeding fed a diet deficient in n-3 fatty acids in gestation reduces DHA accretion in the fetal brain (121), as well as in other tissues (122, 123). Autopsy studies in humans have reported lower DHA in the brain of infants who were fed formula without DHA than in infants who were breast-fed (124), suggesting that brain DHA accretion in the human infant is also influenced by dietary DHA supply. Moreover, the proportion of DHA in
plasma triglycerides and RBC phospholipids fatty acids in newborn infants at birth has also been shown to correlate with the levels of DHA in maternal blood lipids (1, 10, 68, 87, 125-128), and increasing DHA intakes during pregnancy result in an increase in both maternal and fetal cord blood RBC phospholipids and plasma levels of DHA (10, 67-69, 87). This information suggests that the maternal DHA status in gestation does influence the transfer of DHA on the infant before birth.

The ability of the CNS to accumulate a large amount of DHA and the unusually high concentrations of DHA in specific phospholipids in retinal rod and cone outer segments has led some to suggest that DHA may play important functional roles specific to visual processes (5). Thus, the positive relationship between maternal and infant DHA status suggests the importance of an adequate maternal prenatal DHA dietary supply to support optimal fetal CNS development.

1.4.3. DHA and Functional Development of the Central Nervous System

Several investigators have used electroretinography (ERG) to assess changes in retinal function in n-3 fatty acid deficient animals (24-26, 129). An ERG measures the physiological response evoked from the retina by brief flashes of light. Benolken et al. (1973) was the first to show changes in the amplitude of ERG recordings in essential fatty acid deficient rats (24), providing evidence that a lack of polyunsaturated fatty acids may lead to relevant changes in CNS functions. Among animals fed n-3 fatty acid deficient diets, subsequent research showed poorer performance in maze tasks, habituation, exploratory activity in novel environments, and brightness and olfactory-based discrimination learning than in animals fed n-3 fatty acid sufficient diets (4, 130-132). Altered ERG recordings have also been reported in non-human primates fed an n-3 fatty acid deficient diet when compared to recordings of
animals fed diets containing ALA or DHA, however, no differences in functional performance were detected between the groups provided ALA and DHA as sources of n-3 fatty acids (133). Further, infant monkeys of mothers fed n-3 fatty acid deficient diets during the prenatal period, but fed n-3 fatty acid sufficient diet after birth still had 25% poorer visual acuity at 4 wks of age and 50% poorer visual acuity at 8 and 12 wks of age than infants of mothers fed a diet with ALA during gestation (134). Together, these results provide evidence to suggest that the n-3 fatty acid supply before and after birth is important for the functional development of the CNS, particularly visual sensitivity, in animals and non-human primates.

Current epidemiological research also suggests increased risk of poor visual and neural development among children of women with self-reported low intakes of fish, during pregnancy and lactation (6-8), providing evidence for the importance of n-3 fatty acids during the prenatal period. Hibbeln et al. (2007) reported that low maternal low n-3 fatty acid intakes from seafood had children at higher risk for having low verbal IQ scores compared to children of mothers with higher n-3 fatty acid intakes from seafood during gestation (6). In the same population Williams et al. (2001) reported that at 3.5 years of age those children born to mothers who ate oily fish during pregnancy were more likely to achieve higher-grade stereopsis than children of mothers who did not eat oily fish during pregnancy (8). Oken et al. (2005) reported higher maternal fish intakes were associated with higher infant cognition when measured by visual recognition memory (VRM) testing at 6 months of age (7). Together, these studies provide evidence that maternal fish consumption during pregnancy may be important for CNS development and function in children (6-8). Further, Innis et al. (2001) found that strictly breast-fed children in the highest tertile of RBC PE DHA had
significantly higher visual acuity scores than children in the lowest tertile of RBC PE DHA at 2 and 12 months of age (9). However, observational studies are limited because they cannot conclude cause and effect, and other dietary factors associated with diets high in fish could be important.

Currently there are several intervention studies providing information on the effects of DHA supplementation during pregnancy and lactation on infant and child neural development (10, 11, 12, 87, 135-137), in which supplementation has been reported to result in both positive and no effects. Dunstan et al. (2006) studied the effects of fish oil supplementation containing 2.2 g/day DHA and 1.1 g/day EPA (n=33) or an olive oil placebo containing 2.7 g/d oleic acid (18:1n-9, n=39). In the results, eye and hand coordination was significantly higher in the fish oil compared to placebo group in 2 ½ year old children (10). Helland et al. (2003) described the results of a double-blind, randomized study. The results show that children of women taking cod liver oil supplements providing 1.2 g/d DHA and 0.8 g/d EPA had higher scores on the Mental Processing Composite of the Kaufman Assessment Battery for Children (K-ABC) than children of mothers taking the corn oil placebo providing 4.8 g/day LA and 0.1 g/day LA at 4 years of age (12). On the other hand, Malcolm et al. (2003) found that supplementation with 200 mg/d DHA provided as a fish oil supplement did not result in significant improvements in mean ERG implicit times, amplitudes, or stimulus-response function, when compared to infants in the placebo group (135).

Several methodological factors, however, are important and have not been addressed in previously reported studies. First, the biochemical cut-offs for circulating DHA or dietary intakes indicative of inadequate maternal DHA status to support optimal infant development
are not known, and thus it was not known whether or not the women were at risk of DHA inadequacy. Second, although increasing DHA intake has been shown to result in increased plasma DHA ([Figure 1-4a], 138), increasing DHA intake is not expected to have benefit in individuals with a DHA status above their physiological need, suggesting that supplementing women with an adequate intake would not result in a difference in developmental scores of their infants ([Figure 1-4b], 138). Moreover, infant development has a distribution in which the developmental potential of individual infants is unknown. Thus, infants with low developmental potential and high DHA, or high developmental potential and low DHA, cannot be distinguished by currently used tests ([Figure 1-4c], 138). Lastly, because development has a distribution and because infant development is not static, nutrient deficiency would result in a slowing of development. Therefore, DHA inadequacy would be evident at the higher end of the distribution curve and may not result in an overall significant difference in mean developmental scores ([Figure 1-4d], 138).

In conclusion, although many studies have investigated the relationship between maternal DHA intake or status, and CNS development in infants, it is not known whether DHA intake or status is so low among some pregnant women as to prevent optimal infant CNS development, and neither the intake or biochemical cut-offs that identify individuals at risk are defined. Regardless, studies to show that changes from the normal trajectory of brain development have the potential to lead to lasting effects on neural function (139-140), and studies to show the importance of DHA in the CNS place particular emphasis on the need to elucidate n-3 fatty acid recommendations during pregnancy to avoid potential deficits in infant CNS development.
1.5. Purpose

The purpose of this thesis is to determine whether the dietary DHA intakes or status among Canadian pregnant women in our community is so low as to pose risk of insufficient maternal-infant DHA transfer to support infant CNS development. The overall goal is to provide information necessary to consider the need to establish dietary recommended intakes for pregnant women to minimize risk of insufficient n-3 fatty acids to support early human development.
1.6. Objectives

1) To determine the distribution of DHA and other n-3 and n-6 fatty acid intakes and major food sources of these fatty acids among pregnant women in Vancouver following their usual diet.

2) To determine the relationship between the intakes of fish, DHA and n-6 fatty acids and RBC EPG and PC DHA status among pregnant women.

3) To determine whether the DHA status of some women is so low in pregnancy as to increase risk of poor early infant neuro-development when based on measures of infant visual acuity at 60-d of age.

4) To explore possible maternal biochemical markers of n-3 and n-6 fatty acid status that identify women at risk of inadequate DHA status during pregnancy based on measures of infant visual acuity at 60-d of age.
1.7. Specific Aims

1) To describe the distribution of n-3 and n-6 fatty acids and fish intakes among a group of health pregnant women in Vancouver.

2) To determine the dietary ARA/n-3 fatty acid balance among women grouped by their intakes of fish.

3) To determine if DHA and n-6 fatty acid intakes among pregnant women are associated with their RBC EPG and PC levels of DHA.

4) To determine if maternal supplementation with DHA from 16 weeks gestation until infant delivery will reduce the risk of low infant CNS development when assessed through measures of infant visual acuity at 60 d of age.

5) To explore the maternal biochemical markers of fatty acid status associated with low infant visual acuity scores at 60 days of age.
1.8. Hypothesis

For the purpose of this research the null hypotheses are:

1) Intakes of DHA among pregnant women in our population are normally distributed.

2) The dietary n-6/n-3 fatty acid balance does not differ among pregnant women, regardless of their fish intake.

3) The DHA to n-6 fatty acid balance will have no affect on the RBC EPG or PC levels of DHA in pregnant women.

4) The distribution of visual acuity scores of infants at 60-days of age born to mothers assigned to take 400 mg/d DHA or a placebo from 16 wk gestation to infant birth will not differ.
1.9. Tables

Table 1-1  The n-3 and n-6 polyunsaturated fatty acid content of common oils and foods.

<table>
<thead>
<tr>
<th>Foods</th>
<th>n-3 fatty acids</th>
<th>n-6 fatty acids</th>
<th>g fat/100g portion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DHA</td>
<td>EPA</td>
<td>ALA</td>
</tr>
<tr>
<td>Flax oil</td>
<td>-</td>
<td>-</td>
<td>55.3</td>
</tr>
<tr>
<td>Canola oil</td>
<td>-</td>
<td>-</td>
<td>7.85</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>-</td>
<td>-</td>
<td>7.24</td>
</tr>
<tr>
<td>Olive oil</td>
<td>-</td>
<td>-</td>
<td>0.86</td>
</tr>
<tr>
<td>Milk, 2%</td>
<td>0.01</td>
<td>0.06</td>
<td>0.52</td>
</tr>
<tr>
<td>Cheese</td>
<td>0.01</td>
<td>0.06</td>
<td>0.63</td>
</tr>
<tr>
<td>Egg</td>
<td>0.85</td>
<td>0.02</td>
<td>0.36</td>
</tr>
<tr>
<td>Beef, roast</td>
<td>0.09</td>
<td>0.37</td>
<td>1.08</td>
</tr>
<tr>
<td>Hotdog</td>
<td>0.02</td>
<td>0.1</td>
<td>0.46</td>
</tr>
<tr>
<td>Ham</td>
<td>0.18</td>
<td>0.06</td>
<td>0.71</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.73</td>
<td>0.42</td>
<td>1.03</td>
</tr>
<tr>
<td>Lamb</td>
<td>0.48</td>
<td>1.51</td>
<td>2.97</td>
</tr>
<tr>
<td>Salmon</td>
<td>10</td>
<td>4.87</td>
<td>2.6</td>
</tr>
<tr>
<td>Tuna, canned</td>
<td>31.4</td>
<td>5.88</td>
<td>0.23</td>
</tr>
<tr>
<td>Cod</td>
<td>28.2</td>
<td>14.7</td>
<td>0.27</td>
</tr>
<tr>
<td>Shrimp</td>
<td>19.2</td>
<td>23.2</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Foods were analyzed in our laboratory and for all meats excluded skin and visible fat, represents cooked portions. Values are g/100g fatty acids unless otherwise stated.
Table 1-2  Dietary recommended intakes for linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3) by life stage and gender published by the National Academy of Sciences U.S in 2002.

<table>
<thead>
<tr>
<th>Life Stage</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6 m</td>
<td>4.4</td>
<td>4.4</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>7-12 m</td>
<td>4.6</td>
<td>4.6</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1-3 y</td>
<td>7</td>
<td>7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>4-8 y</td>
<td>10</td>
<td>10</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>9-13 y</td>
<td>12</td>
<td>10</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>14-18 y</td>
<td>16</td>
<td>11</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>19 - 50 y</td>
<td>17</td>
<td>12</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>&gt; 50 y</td>
<td>14</td>
<td>11</td>
<td>1.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>13</td>
<td></td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Lactation</td>
<td>13</td>
<td></td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

Intakes given are adequate intakes (AI) in g/day, and are equivalent to the median intake for individuals by life-stage and gender, derived from national dietary intake surveys in the U.S. (18). The AI for infants is the mean intake of infants fed human milk estimated from the average amount of the given fatty acid in human milk and the average intake of breast milk of infants fed breast milk (18).
1.10. Figures

Figure 1-1  Schematic of some n-6 and n-3 fatty acids.
Figure 1-2  Schematic of the desaturation and elongation pathway of dietary 18:2n-6 and 18:3n-3. The desaturation enzymes include the delta-6 desaturase and the delta-5 desaturase (5).
Figure 1-3  Schematic of common phospholipids.
Figure 1-4  Schematic to illustrate the potential effects of DHA supplementation on the distribution of visual acuity scores among a population of infants of mothers with both adequate and deficient diets (138).
1.11. Bibliography


8. Williams C, Birch EE, Emmett PM, Northstone K, Avon Longitudinal Study of Pregnancy and Childhood Study Team. Stereoacuity at age 3.5 y in children born full-term is


70. Sanders TA. Essential fatty acid requirements of vegetarians in pregnancy, lactation, and infancy. Am J Clin Nutr 1999;70:555S-9S.


81. Kris-Etherton PM, Innis S, American Dietetic A, Dietitians of C. Position of the
American Dietetic Association and Dietitians of Canada: dietary fatty acids. J Am Diet Assoc
2007;107:1599-611.

82. The Heart and Stroke Foundation.
2007.

83. American Heart Association.

84. Denomme J, Stark KD, Holub BJ. Directly quantitated dietary (n-3) fatty acid intakes of
pregnant Canadian women are lower than current dietary recommendations. J Nutr

from African-American women at gestation, delivery, and postpartum. J Lipid Res

86. De Vriese SR, De Henauw S, De Backer G, Dhont M, Christophe AB. Estimation of
dietary fat intake of Belgian pregnant women. Comparison of two methods. Ann Nutr Metab


120. Innis SM, Dyer RA. Brain astrocyte synthesis of docosahexaenoic acid from n-3 fatty acids is limited at the elongation of docosapentaenoic acid. J Lipid Res 2002;43:1529-36.


122. Arbuckle LD, Innis SM. Docosahexaenoic acid is transferred through maternal diet to milk and to tissues of natural milk-fed piglets. J Nutr 1993;123:1668-75.


2.1. Introduction
The n-3 fatty acids are becoming increasingly recognized as important, particularly in relation to reducing the risk of cardiovascular disease, and some neurological and retinal disorders (1-4). In animals, dietary deficiency of n-3 fatty acids during pregnancy decreases maternal-to-fetal transfer of docosahexaenoic acid (DHA, 22:6n-3), decreases DHA in the fetal brain, impairs neurogenesis and neurotransmitter metabolism, and results in deficits in visual function and learning behavior (5,6). In humans, epidemiological studies have shown increased risk of poor visual and neural development among children of women with low intakes of fish, the major dietary source of DHA, during pregnancy and lactation (7-11). Further, recent interventions to increase DHA intakes among pregnant and lactating women have found early and long term benefits in visual, mental and motor skill development in children (12-17). Recent epidemiological studies have also provided evidence that higher DHA and fish intakes are associated with decreased risk of low cognitive performance and cognitive decline in older individuals (18-21).

The n-3 fatty acids are present in the diet as alpha-linolenic acid (ALA, 18:3n-3), and its metabolites eicosapentaenoic acid (EPA, 20:5n-3) and DHA. ALA is obtained mainly from vegetable oils, and provides about 90% of the n-3 fatty acids in Western diets (22). EPA and DHA are formed from ALA in mammalian but not plant cells, although in humans desaturation of ALA is limited beyond EPA and endogenous synthesis of DHA appears to be

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1 A version of this chapter has been submitted for publication. Friesen RW, Innis SM (2008). Low fish intakes are associated with high dietary n-6 arachidonic to n-3 eicosapentaenoic and docosahexaenoic acid balance in pregnant women. Am J Clin Nutr.
very low (23-25). The n-6 linoleic acid (LA, 18:2n-6) is also obtained from vegetable oils, is desaturated to arachidonic acid (ARA, 20:4n-6) in mammals, and is present in the diet mainly in meats and poultry, rather than fish. ARA is released from tissue phospholipids and further metabolized to eicosanoids with multiple functions including neurotransmission, and pro- and anti-inflammatory actions (26-28). Notably, whereas n-3 fatty acids have been associated with decreased risk (1-4), several studies have suggested that higher dietary ARA, n-6/n-3 fatty acid ratios and meat consumption, the major dietary source of ARA, are associated with increased risk of cardiovascular, immunological and neurological diseases (29-35). The current dietary reference intakes for n-3 and n-6 fatty acids from the Institute of Medicine are Adequate Intakes (AI), which are the median n-3 and n-6 fatty acid intakes estimated from dietary surveys in the U.S. (22). Thus, in groups with diets similar to the U.S., 50% of individuals will have an intake below the AI, and interpretation is limited to whether their diet is similar to, or different from the average U.S. diet. The dietary requirement for n-3 fatty acids to prevent deficiency of DHA in membrane lipids was not defined (22). Furthermore, because fish, but not meat is naturally rich in DHA, DHA intakes are likely to vary among individuals, with the possibility that dietary ARA intakes and balance of n-6/n-3 fatty acids may also differ among individuals with different intakes of fish.

Our objective in the present study was to determine the distribution of fish and DHA intakes, and address whether the dietary ARA and ARA/n-3 fatty acid ratio differs among pregnant women with different intakes of fish. Because red blood cell (RBC) phospholipid fatty acids are used as biomarkers of tissue fatty acids (10,11,13,16,17,36-38), we also addressed the relationship between dietary DHA, ARA and LA and the RBC DHA as a potential index of those dietary patterns that may increase risk of low DHA status.
2.2. Subjects and Methods

2.2.1. Subjects and design
The present study involved \(n=140\) pregnant women, 20-40 y of age, 36 wk gestation with no known maternal or fetal pregnancy complications and were apart of larger study designed to determine whether DHA supplementation. All of the women were registered to deliver one full-term infant, with no anticipated complications at the British Columbia’s Women’s and Children’s Hospital. Potentially eligible subjects were identified from the hospital registrations. Each subject was seen during an appointment at the hospital at 36 wk of gestation. Women consuming a vegan diet, or with any known metabolic disease, including diabetes, immune disorders, or communicable diseases were not enrolled. Women with insufficient English language skills to complete the informed signed consent or in-person dietary interviews were also ineligible to participate. Our intent, therefore, is not to provide representative data on the n-6 and n-3 fatty acid intakes of the population, but to describe the distribution of DHA intakes and potential differences in dietary n-6/n-3 fatty acid ratios among women with different intakes of fish. The protocol and procedures were approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia and the British Columbia’s Children’s and Women’s Hospital. All subjects provided written informed consent prior to participation.

2.2.2. Subject characteristics and dietary assessments
Socio-demographic data, including age, parity, and highest level of education, family income and ethnic background were collected from each subject by questionnaire (Appendix A). Dietary assessment was conducted using an interview-administered food frequency questionnaire designed to collect information on fat and fatty acid intakes (Appendix B, 39).
We collected information on the frequency with which each food was eaten, portion size, brand name or place of purchase, methods of preparation, types of margarine, fats and oils, and all types of fish and seafood. Information from the dietary records was entered into a nutrient database (FOOD PROCESSOR 11; ESHA Research, Salem, OR) containing the Canadian Nutrient File, and updated with the fatty acid composition of foods, including meats, wild, farmed and canned fish, shellfish, eggs, dairy products, margarines, shortenings, and oils analyzed in our laboratory.

To address the distribution of fatty acid intakes, and the importance of different foods to the total intake of DHA, the women were grouped by their intake of fish (all fish and seafood) as \( \leq 100 \text{ g} \), 101-200 g, 201-300 g and \( \geq 301 \text{ g/wk} \). The upper limit of \( \geq 301 \text{ g/wk} \) was chosen because only 7/140 of the women in the present study consumed more than 400 g fish/wk. To determine the contribution of different foods to the total intake of DHA, as well as EPA and ARA, we grouped foods into 6 categories: poultry, ruminant meats, non-ruminant meats, eggs, dairy milks and milk products, and fish, then determined the intake of DHA, EPA and ARA from each food group for each subject. Based on the responses in the food records, we defined 6 categories of fish: fresh and frozen salmon, lean white fish, canned tuna, canned salmon, crustaceans and shellfish, and other fish, then determined the intake of DHA from each fish category for each subject. Foods of vegetable origin were not included because, unless supplemented, DHA, EPA and ARA are present exclusively in animal tissue lipids. In all cases, a serving was designated as 100 g edible, cooked food.

2.2.3. Red blood cell fatty acids

Whereas plasma phospholipid ARA and DHA increase with increasing dietary intake (40,41), RBC phospholipid fatty acids are often used as a biomarker of DHA in tissues, such
as the brain, that cannot be assessed in studies with humans (10,11,13,16,17,36-38). RBC, like other cell phospholipids are heterogeneous, with the ethanolamine phosphoglycerides (EPG, includes both ethanolamine plasmalogen and phosphatidylethanolamine) preferentially distributed on the inner membrane bilayer and high in ARA and DHA, while phosphatidylcholine (PC) is mainly on the outer membrane surface, contains low levels of ARA and DHA (42,43), and also exchanges with PC in the plasma (44). Nutrients present in regulated tissue pools increase in individuals with a deficient diet when the dietary intake is increased, but reach a plateau at intakes above requirements. However, fatty acyl substrates compete for acylation to phospholipid sn-2 position, meaning that the balance of DHA and n-6 fatty acid substrates may also be important (45). Therefore, to address the relationship between the RBC EPG or PC DHA, and dietary DHA and n-6 fatty acids, fasting venous blood was collected from a subset of women who were not taking any supplemental fish oil or DHA at 36 wk gestation, n=78. The RBC were separated from plasma by centrifugation (2000 g, 15 min, 4°C), washed 2 times by re-suspension in normal saline and centrifugation, then frozen at -70°C until later analysis (10,40). Total lipids were extracted from the RBC, then the EPG and PC were separated from other lipids and the fatty acids separated and quantified by routine methods (Appendix C, 10,38,40,46).

2.2.4. Statistical analysis

We first used a Kolmogrov-Smirnov Test to test for Gaussian distribution and calculated the median, inter-quartile range, 5-95th range, mean and standard deviation of dietary fat and fatty acid intakes using descriptive statistics. Wilcoxon and Mann-Whitney U tests were used to determine potential differences in fatty acid intake among women grouped by fish intake. Tukey’s honestly significant difference was used to detect significant differences in
RBC EPG and PC fatty acids among women grouped by fish intake, when a significant effect of fish intake was found by ANOVA. Spearman Rho correlation coefficients were used to determine potentially significant relationships between dietary intake of n-6 and n-3 fatty acids and the RBC EPG and PC levels of n-6 and n-3 fatty acids. Pearson correlation coefficients were used to detect the range of dietary DHA intakes over which the relationship to the RBC EPG and PC DHA was linear. Multiple linear regression was used to determine best fit models to explain the relationships, if any between dietary DHA and n-6 fatty acids (LA, ARA) and the RBC EPG and PC DHA, and when significant relationships were found, the significance of each dietary fatty acid in the model was determined. All statistical analyses were performed using SPSS software (version 15.0), with the level of significance set at \( P<0.05 \).

2.3. Results

2.3.1. Subject characteristics

The mean ± SD age of women in the present study was 33.3 ± 0.4 y, 71% were Caucasian, 12% were Chinese, and 17% were from other ethnic groups. A total of 27% of the subjects had a high school education only or high school with some vocational training, and 73% had attended post-secondary education. Of the 140 women in the present study, \( n=17 \) consumed meat but no fish, \( n=123 \) consumed fish, and \( n=5 \) consumed no meat or poultry, but did consume eggs and dairy products.

2.3.2. Dietary fat and fatty acid intakes

The dietary intake of n-6 and n-3 fatty acids, but not total energy, total fat, or saturated or monounsaturated fatty acids were not normally distributed, with lower median than mean intakes of LA, ALA, ARA, EPA and DHA (Table 2-1, \( P<0.05 \)). Fat provided (mean ± SD)
34 ± 10% of total energy, with a 5th-95th range of intake of 24 - 44% total energy from fat. The mean ± SD intake of saturated, monounsaturated and polyunsaturated fatty acids was 11.0 ± 3.0, 12.5 ± 3.1, and 4.6 ± 2.3% of total energy, respectively. The median intake of LA was 4.0% dietary energy with a 25th-75th and 5th-95th range of intake of 2.9 - 5.5, and 2.0 - 9.5% total energy, respectively. The median intake of ALA was 0.5% of total energy, with a 5-95th range of intake of 0.2 - 1.4% total energy. The SD of the intakes of LA, ALA, ARA and EPA were all 50% or more of the mean intake, and for DHA the SD exceeded the mean intake (Table 2-1). Comparison of the median intake of 60 mg/d with the 5-95th range of intakes of 10 - 200 mg/d EPA, and the median intake of 100 mg/d with the 5-95th range of intakes of 10 - 390 mg/d DHA also shows that EPA and DHA intakes vary widely among individuals, with the mean intake skewed to higher values by a small number of individuals. The dietary ARA/EPA and ARA/DHA ratios were also skewed (P<0.001), with the median dietary ARA/DHA ratio about 50% lower than the mean, and a 5-95th range of dietary ARA/DHA ratios that varied over 200%.

2.3.3. Dietary sources of long chain n-6 and n-3 fatty acids and fish intakes

The contribution of different food groups to the median intakes of ARA, EPA and DHA are illustrated in Figure 2-1. Fish was the major dietary source of EPA and DHA, providing about 60% and 73% of EPA and DHA, respectively. Ruminant animal meats provided 9%, and milk and milk products provided 16% of EPA, while poultry contributed 17% of the intake of DHA. Poultry and ruminant animal meats contributed 43% of dietary ARA, and although dairy milks and milk products are relatively low in ARA, they were consumed frequently and contributed 35% of dietary ARA. Because fish vary widely in DHA, with fatty fish such as salmon and herring being richer sources of EPA and DHA than lean
whitefish (47,48), we also determined the contribution of different fish to the intake of DHA. In the present study, 62% of dietary DHA from fish was from fresh and frozen salmon, with a further 11.1% from canned salmon, 4.5% from canned tuna, and 6.6% from shrimp and other shellfish (Appendix D).

Regardless of the coastal location in which this study was conducted, 42% of the women consumed ≤100 g/wk fish, 26% consumed 101-200 g/wk fish, 14% consumed 201-300 g/wk fish, and only 18% consumed ≥301 g/wk fish. We found no significant differences in the intake of LA, ALA (Appendix E) or the dietary LA/ALA ratio in the women when grouped by fish intake (Figure 2-2, \(P>0.05\)). However, the intakes of EPA and DHA increased, and the dietary ARA/EPA and ARA/DHA ratios decreased with increasing fish intake (Figure 2-2, \(P<0.05\)). The median intakes of EPA were 20, 70, 125 and 150 mg/d and for DHA were 20, 110, 235 and 290 mg/d DHA for women who consumed ≤100, 101-200, 201-300 and ≥301 g/wk fish, respectively. The median dietary ARA/EPA ratios were 3.0:1, 1.1:1, 0.7:1 and 0.8:1, and the ARA/DHA ratios were 3.0:1, 0.7:1, 0.4:1 and 0.4:1 among women consuming ≤100, 101-200, 201-300 and ≥301 g/wk fish, respectively; with a higher dietary ARA/EPA and ARA/DHA ratio in the group consuming ≤100 g/wk fish than in the groups consuming >100 g/wk fish (Figure 2-2, \(P<0.001\)).

Because fish vary considerably in EPA and DHA (47,48), individuals consuming similar amounts fish may have different intakes of EPA and DHA. As shown in Figure 2-2, as fish intake increased the 25-75\(^{th}\) and 5-95\(^{th}\) range of EPA and DHA intake also increased, with considerable overlap in dietary intakes of EPA and DHA between women consuming 201-300 g and those consuming >301 g/wk fish.
2.3.4. Biochemical measures of long chain n-6 and n-3 fatty acid status

The fatty acid composition of the RBC EPG and PC is reported in Table 2-2. The mean levels of ARA, DTA and DHA in RBC EPG were 20.4, 6.77 and 8.77 g/100g fatty acids, respectively, and in RBC PC were 5.20, 0.29 and 1.99 g/100g fatty acids, respectively, emphasizing that phospholipid fatty acids depend on the phospholipid class, and that PC is relatively low in carbon chain 20 and 22 n-6 and n-3 fatty acids. Our results show that although the RBC EPG and PC levels of LA, ALA and ARA were not different (P>0.05), the levels of EPA and DHA in PC, and DTA, DPA and EPA in EPG differed among women with different intakes of fish (Table 2-2, P<0.05). Specifically, the RBC EPG levels of DTA and DPA, which are known to increase in tissues of animals fed an n-3 fatty acid deficient diet (5,6,47), were significantly higher in women consuming ≤100 compared to those consuming >201 g/wk fish (P<0.05). Women consuming 101-200 g/wk fish also had higher RBC EPG levels of 22:5n-6 than women consuming >201 g/wk fish (P<0.05). In contrast to EPG, 22:4n-6 and 22:5n-6 represented <0.3% of RBC PC fatty acids, and were not different among the women grouped by fish intake (P<0.05). The RBC EPG and PC levels of EPA, on the other hand, increased with increasing fish intake (P<0.05), with higher levels of EPA in the RBC PC and EPG of women consuming >301 g/wk fish than in women consuming ≤100 g/wk fish (P<0.05). The RBC PC, but not EPG, levels of DHA also increased with increasing fish intake (P<0.05), with lower levels of DHA in the RBC PC fatty acids of women consuming ≤100 g than in women consuming >101-200 or >301g/wk fish (P<0.05).

2.3.5. Dietary fatty acid determinants of docosahexaenoic acid status

Using Spearman Rho analysis we found no significant relationships between dietary intake of LA or ALA (Appendix E) or ARA (Figure 2-3) and RBC EPG or PC levels of the same
fatty acid \(P>0.05\). However, the dietary intake of EPA and DHA was positively related to RBC EPG levels of EPA (Rho=0.346, \(P<0.05\)) and DHA (Figure 2-3, Rho=0.240, \(P<0.05\), \(n=78\)). The dietary intake of DHA was also positively and significantly related to the level of DHA in the RBC PC fatty acids (Rho=0.302, \(P<0.05\)).

Biologically regulated pathways are non-linear and plateau when substrates and cofactors are adequate. Therefore, the range of DHA intakes below which dietary DHA is linearly related to RBC EPG DHA, could provide an index of the range of DHA intakes over which DHA is limiting for RBC EPG DHA incorporation and turnover. To address this, we used Pearson’s linear analysis with incremental intakes of 25 mg/d DHA to determine if an intake of DHA exists below and above which the RBC EPG levels of DHA are and are not linearly related, respectively, to DHA intake. Our results show that DHA intake was linearly related to the RBC EPG DHA at intakes of 0-150 mg/d DHA (r=0.27, \(P<0.05\), \(n=55\), with the linear relationship lost at higher intakes of DHA (Table 2-3). PC is preferentially located on the outer RBC membrane and exchanges with PC in plasma (42–44), which is a useful biomarker of DHA intake (40,41). In contrast to the EPG, the RBC PC DHA was linearly related to dietary DHA across all intakes in the present study (Table 2-3, r=0.269, \(P=0.017\), \(n=78\)).

However, as shown in Figure 2-3 and evident from the weak correlation coefficients, the RBC EPG levels of DHA varied widely, regardless of similar intakes of DHA and particularly among women with intakes of \(\leq 150\) mg/d DHA. LA, ARA and DHA are acylated at the phospholipid \(sn\)-2 position, raising the possibility that high n-6 fatty acid intakes could by competition, decrease phospholipid DHA. The regression planes in Figure 2-4 illustrate the strength and direction of effect of changes in dietary DHA and ARA or LA.
on the RBC EPG and PC levels of DHA in models derived from multiple linear regression. The models relating dietary DHA and ARA to DHA in the RBC EPG ($\beta = 9.55$, SE 0.58, 95% CI 8.39-10.7, $R = 0.304$, $P = 0.03$) and PC ($\beta = 1.77$, SE 0.21, 95% CI 1.54-2.38, $r = 0.297$, $P = 0.03$), and those for dietary DHA and LA with DHA in the RBC EPG ($\beta = 9.41$, SE 0.56, 95% CI 8.32-10.5, $R = 0.294$, $P = 0.03$) and PC ($\beta = 1.92$, SE 0.20, 95% CI 1.22-2.01, $R = 0.290$, $P = 0.037$) were all significant. The models showed a decrease in RBC EPG DHA with either increasing ARA ($R = -0.288$, $P = 0.019$), or LA ($R = -0.252$, $P = 0.025$) in the diet. For example, the regression equations in the model with dietary DHA and ARA predict a decrease in the RBC EPG DHA from 11.5 to 8.6 g/100 g fatty acids in individuals consuming 600 mg/d DHA when the intake of ARA is increased from 0 to 200 mg/d (Figure 2-4). The models for DHA in the RBC PC, in contrast, showed that changes in dietary ARA ($R = -0.137$, $P = 0.261$) or LA ($R = 0.107$, $P = 0.337$) did not contribute. Although changes in dietary DHA were significant when modeled with either dietary ARA ($R = 0.325$, $P = 0.009$) or LA ($R = 0.272$, $P = 0.02$). The regression planes thus show that the effect of dietary DHA and the DHA/n-6 fatty acid balance differs between EPG and PC, with the n-6 fatty acids reducing DHA in EPG, but having no significant effect on the increase in DHA in PC that occurs with higher DHA intakes.

2.4. Discussion

An increasing body of information suggests that the n-3 fatty acids are important in reducing the risk of some neurological, cardiovascular, and immune and inflammatory disorders, and that DHA is important for optimal neural and visual development (1-21). Although several expert groups have made recommendations for fish or DHA as part of a healthy diet (49-52), little specific information is available on those fatty acid intakes that pose risk of DHA.
deficiency, and neither the dietary intakes nor biomarkers indicative of deficiency are defined. However, in well-nourished populations adverse effects due to inadequate intakes of essential nutrients are more likely to occur in individuals at the lower end of the intake distribution. In the present study, we show that dietary intakes of DHA vary widely, even among a relatively homogenous group of Canadian women. Furthermore, the distribution of DHA and EPA intakes was skewed, with lower median than mean intakes. The mean±SD intake of DHA in the present study was 139±153 mg/d (n=140), while other studies with pregnant women in Canada have reported intakes of 82±115 mg/d and 160±246 mg/d DHA (39,53), with intakes of 81±94 mg/d among pregnant African American women (54), and 280±190 mg/d, and 300±300 mg/d DHA among pregnant women in Belgium and Holland, respectively (55,56). The high SD values (Table 2-1, 39,53-56) illustrate that DHA intakes vary widely within populations. Furthermore, in the present study, only 21% of the women consumed between 100-170 mg/d DHA, the range of intakes at ± 25% of the mean DHA intake, while 43% consumed <100 mg/d DHA. The 2002 Dietary Reference Intakes reported median LA and ALA intakes by life stage and gender among individuals in the US, designated AI (22). The median intake of 0.5% total energy from ALA, with 10% total intake of n-3 fatty acids from EPA plus DHA in our study (Table 2-1) is similar to the dietary surveys in the U.S. used to set the 2002 AI values (22).

Clinical studies often measure RBC fatty acids as a biomarker of DHA in tissues such as the brain (10,11,13,16,17,36-38). Regulated biological processes are non-linear, thus when dietary intakes are above requirements, further increases in intake are not expected to increase regulated metabolic pathways, although increases in storage or transport pools, such as plasma lipids, may occur. In the present study, we show a non-linear relationship between
dietary intake and the RBC EPG level of DHA (Rho 0.240, $P<0.05$, $n=78$), suggesting that the DHA status of some individuals in our study was limiting. Similarly, a saturable, curvilinear increase in DHA in the RBC phospholipids of infants fed human milk with increasing levels of DHA has been reported (16). Tissue EPG levels of 22:4n-6 and 22:5n-6 increase in animals fed a diet deficient in n-3 fatty acids (5,6,36,46), suggesting that the higher RBC EPG levels of 22:4n-6 and 22:5n-6 among women in our study with low intakes of fish could reflect inadequate intakes of n-3 fatty acid in some individuals. The linear relationship between DHA intake and the RBC EPG DHA at intakes up to 150 mg/day DHA ($r=0.268$, $P<0.05$, $n=55$), which was lost at intakes above 150 mg/d DHA may suggest dietary intakes below 150 mg/d DHA are limiting for RBC EPG DHA incorporation and turnover (Table 2-3). Hibbeln et al. reported a non-linear dose-response curve with no further benefit to child verbal IQ scores when the maternal n-3 fatty acid intake from seafood increased above 0.1% total energy (7). Using the DHA and EPA provided by salmon, which was the dietary source of DHA, an intake of 150 mg DHA is equivalent to about 220 mg EPA+DHA (48,49) and represented about 0.1% total energy from long chain n-3 fatty acids for the subjects in our study, remarkably consistent with Hibbeln et al (7) in suggesting low risk of deficiency at intakes of above 0.1% energy n-3 fatty acids from fish.

With respect to the n-6 fatty acids, we found no association between the dietary intake of LA or ARA and the levels of LA or ARA in the RBC EPG, suggesting that n-6 fatty acid intakes were not limiting among the women in our study. The median and 5th percentile of intake of n-6 fatty acids was 4.0% and 2.0% dietary energy, respectively (Table 2-1), and above the intake considered to meet requirements for n-6 fatty acids (6). However, several reports have raised concern that diets high in meat, ARA, or a high dietary n-6/n-3 fatty acid
ratio may contribute to risk of some diseases, particularly cardiovascular disease, diabetes type II, and immune and inflammatory and neurological disorders (29-35). Regardless, little specific information is available to address whether the concurrent intake of ARA is an important modifier of n-3 fatty acid requirements or status. Central contributions of our study are the results to show that dietary intakes of ARA and the dietary ARA/n-3 fatty acid balance varies widely, particularly among individuals with low intakes of fish. Further, we show that membrane lipid levels of DHA also vary, regardless of apparently similar intakes of DHA (Figure 2-3). The modeling of dietary intakes of DHA with the major n-6 fatty acids, LA and ARA show that DHA and the n-6 LA and ARA mutually and inversely interact to determine membrane EPG pools of DHA (Figure 2-4). Importantly, the inhibition of DHA accumulation in EPG with increasing dietary n-6 fatty acids shown in the multiple regression analyses was not apparent in PC, which showed a continuing increase in DHA with increasing DHA intake. These results suggest that diets high in LA derived mainly from polyunsaturated vegetable oil, or ARA derived mainly from meats and poultry may contribute to reduced levels of DHA in specific membrane lipid pools, such as EPG which are usually enriched in DHA. Our results also show that analyses of PC may provide an index of groups or individuals with higher DHA intakes, but caution that important interactive effects of other dietary fatty acids may not be evident.

Most of the recent emphasis on the dietary n-6/n-3 fatty acid balance has been with respect to competition between LA and ALA for desaturation to ARA, and EPA and DHA (25,35), rather than competition among fatty acids for acylation into membrane lipids. Consistent with our results, studies in animals have provided evidence that increasing dietary ARA reduces DHA in the brain (57), and formula with high amounts of LA result in lower
DHA in the RBC EPG of formula-fed infants (58). Conversely, increasing dietary DHA has been shown to decrease brain ARA in animals (57), and RBC ARA in breast-fed infants (16). Thus, although limited information is available to suggest that dietary fatty acids may influence membrane phospholipid fatty acids at the level of competition for phospholipid synthesis and fatty acid turnover, these studies suggest a significant negative effect of dietary n-6 fatty acids on phospholipid DHA status. More specifically we emphasize the negative affects of dietary ARA on RBC EPG DHA seems much more potent than LA on a per gram basis.

The present study has several weaknesses. Errors in estimating dietary intakes including missing fatty acid values could contribute to weakening of the relationships between dietary variables and measures of fatty acid status. In addition, although we demonstrate considerable variability in dietary fatty acid intakes, our study had low power with respect to subjects with low intakes of n-6 fatty acids and insufficient power to construct non-linear models. Several factors, other than dietary DHA and ARA not addressed by us may influence DHA incorporation into tissue lipids, including synthesis of DHA from ALA, and variables that influence phospholipid synthesis and turnover. Next, RBC are convenient, but are enucleated cells with anaerobic metabolism, and although RBC EPG DHA has been shown to correlate with DHA in brain EPG (36,57), extrapolation to other cells needs caution. Finally, although the role of DHA in the retina is becoming increasingly understood (59), the functional basis of the enrichment of DHA in EPG in cells, including RBC when compared with plasma lipids or PC is unclear. Thus, an importance weakness of our work is the absence of functional tests to show that differences in DHA, or n-6 fatty acid/DHA balance in the RBC EPG among our subjects is of any physiological importance.
In summary, the present study emphasizes that DHA intakes are skewed, and that
dietary ARA/n-3 fatty acid balance varies widely. We provide evidence to suggest the
possibility of unsaturated tissue DHA among some pregnant women in our community, and
show that dietary n-3 fatty acid requirements may differ depending on the concurrent intake
of ARA, and hence differ among individuals with different intakes of meats, and with choice
of vegetable oils.
2.5. Tables

**Table 2-1**  Dietary fat and n-3 and n-6 fatty acid intakes among pregnant women in their third trimester of pregnancy.¹

<table>
<thead>
<tr>
<th></th>
<th>Median</th>
<th>25-75&lt;sup&gt;th&lt;/sup&gt;</th>
<th>5-95&lt;sup&gt;th&lt;/sup&gt;</th>
<th>Mean±SD</th>
<th>Skewness Test&lt;sup&gt;2&lt;/sup&gt;</th>
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<tr>
<td>Energy, KJ</td>
<td>9868</td>
<td>8449-11133</td>
<td>6498-14290</td>
<td>10006±2261</td>
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<td>70.0-104</td>
<td>52.9-139.8</td>
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<td>Saturates, g</td>
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<td>15.4-48.4</td>
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<td>Monounsaturates, g</td>
<td>31.8</td>
<td>25.3-41.5</td>
<td>19.3-59.3</td>
<td>33.9±11.4</td>
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<td>Polyunsaturates, g</td>
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<td>6.8-31.3</td>
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<td>DHA, mg</td>
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<td>10-390</td>
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<td>EPA, mg</td>
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<td>10-200</td>
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<td>0.7-4.1</td>
<td>1.8±1.1</td>
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<td>47-206</td>
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<td>LA, g</td>
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<td>5.8-27.7</td>
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<td>LA:ALA</td>
<td>8</td>
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<td>4.1-12.8</td>
<td>8.1±3.2</td>
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<td>0.50-6.0</td>
<td>2.1±1.8</td>
<td>&lt;0.001</td>
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<tr>
<td>ARA/DHA</td>
<td>0.8</td>
<td>0.40-2.5</td>
<td>0.30-8.0</td>
<td>1.9±2.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹ Results shown are medians, ranges and mean±SD at 36 wk gestation; DHA, docosahexaenoic acid, DHA (22:6n-3); EPA, eicosapentaenoic acid, (20:5n-3); ALA, alpha linoleic acid, (18:3n-3); ARA, arachidonic acid (20:4n-6); LA, linoleic acid, (18:2n-6).

² Skewness was assessed using Kolmogorov-Smirnov test, values of <0.05 have non-normal distributions.
Table 2-2  Major n-6 and n-3 fatty acids in RBC ethanolamine phosphoglyceride (EPG) and phosphatidylcholine (PC) of Canadian pregnant women grouped by fish intake.

<table>
<thead>
<tr>
<th>g/100g fatty acid</th>
<th>All Subjects (n=140)</th>
<th>&lt;100g (n=35)</th>
<th>101-200g (n=20)</th>
<th>201-300g (n=13)</th>
<th>&gt;301g (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC EPG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>5.81±0.13</td>
<td>5.74±0.20</td>
<td>5.69±0.23</td>
<td>6.21±0.30</td>
<td>5.75±0.62</td>
</tr>
<tr>
<td>ARA</td>
<td>20.4±0.25</td>
<td>20.6±0.42</td>
<td>20.9±0.55</td>
<td>19.2±0.37</td>
<td>19.5±0.47</td>
</tr>
<tr>
<td>DTA,n-6²</td>
<td>6.77±0.18</td>
<td>7.40±0.28a</td>
<td>6.72±0.38a</td>
<td>5.94±0.24b</td>
<td>5.88±0.28b</td>
</tr>
<tr>
<td>DPA,n-6²</td>
<td>1.02±0.04</td>
<td>1.19±0.06a</td>
<td>0.98±0.05b</td>
<td>0.85±0.05b</td>
<td>0.78±0.07b</td>
</tr>
<tr>
<td>ALA</td>
<td>0.33±0.01</td>
<td>0.34±0.02</td>
<td>0.30±0.02</td>
<td>0.36±0.01</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>EPA²</td>
<td>0.78±0.03</td>
<td>0.68±0.03a</td>
<td>0.82±0.06a</td>
<td>0.80±0.05a</td>
<td>1.00±0.12b</td>
</tr>
<tr>
<td>DPA,n-3</td>
<td>3.86±0.09</td>
<td>3.92±0.12</td>
<td>3.87±0.18</td>
<td>3.62±0.20</td>
<td>3.91±0.34</td>
</tr>
<tr>
<td>DHA</td>
<td>8.77±0.24</td>
<td>8.36±0.31</td>
<td>9.40±0.58</td>
<td>8.48±0.38</td>
<td>9.19±0.76</td>
</tr>
<tr>
<td>RBC PC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA</td>
<td>21.1±0.27</td>
<td>20.8±0.46</td>
<td>21.3±0.44</td>
<td>21.8±0.56</td>
<td>20.9±0.85</td>
</tr>
<tr>
<td>ARA</td>
<td>5.20±0.13</td>
<td>5.19±0.22</td>
<td>5.37±0.23</td>
<td>5.00±0.26</td>
<td>5.22±0.35</td>
</tr>
<tr>
<td>DTA,n-6</td>
<td>0.29±0.01</td>
<td>0.30±0.02</td>
<td>0.31±0.02</td>
<td>0.26±0.02</td>
<td>0.28±0.03</td>
</tr>
<tr>
<td>DPA, n-6</td>
<td>0.18±0.01</td>
<td>0.20±0.02</td>
<td>0.21±0.02</td>
<td>0.13±0.02</td>
<td>0.17±0.03</td>
</tr>
<tr>
<td>ALA</td>
<td>0.38±0.01</td>
<td>0.39±0.02</td>
<td>0.35±0.02</td>
<td>0.36±0.03</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>EPA²</td>
<td>0.30±0.02</td>
<td>0.26±0.01a</td>
<td>0.32±0.03ab</td>
<td>0.27±0.03a</td>
<td>0.44±0.09b</td>
</tr>
<tr>
<td>DPA, n-3</td>
<td>0.33±0.01</td>
<td>0.30±0.02</td>
<td>0.36±0.02</td>
<td>0.31±0.03</td>
<td>0.37±0.04</td>
</tr>
<tr>
<td>DHA²</td>
<td>1.99±0.09</td>
<td>1.67±0.11a</td>
<td>2.25±0.15b</td>
<td>1.96±0.16a</td>
<td>2.53±0.29b</td>
</tr>
</tbody>
</table>

¹Values shown are mean ± SEM g/100g fatty acid; LA, linoleic acid (18:2n-6); ALA, alpha linoleic acid (18:3n-3); ARA, arachidonic acid (20:4n-6); DTA n-6, docosatetraenoic acid, 22:4n-6; DPA n-6, docosapentaenoic acid, 22:5n-6; EPA, eicosapentaenoic acid, 20:5n-3; DHA, docosahexaenoic acid, 22:6n-3.

²Indicates fish intake was associated with a significant difference the level of RBC EPG or PC fatty acid indicated. Values with a different superscript are significantly different by Tukey’s Multiple Comparison test (P<0.05).
Table 2-3: Relationship between dietary intake of docosahexaenoic acid (DHA) and RBC ethanolamine phosphoglyceride (EPG) and phosphatidylcholine (PC) DHA among women in their third trimester of pregnancy.1

<table>
<thead>
<tr>
<th>DHA intake (mg/d (n))</th>
<th>RBC EPG DHA</th>
<th>RBC PC DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>≤50 (27)</td>
<td>0.031</td>
<td>0.879</td>
</tr>
<tr>
<td>≤75 (33)</td>
<td>0.046</td>
<td>0.797</td>
</tr>
<tr>
<td>≤100 (45)</td>
<td>0.12</td>
<td>0.431</td>
</tr>
<tr>
<td>≤125 (47)</td>
<td>0.26</td>
<td>0.078</td>
</tr>
<tr>
<td>≤150 (55)</td>
<td>0.268</td>
<td>0.048*</td>
</tr>
<tr>
<td>≤175 (57)</td>
<td>0.195</td>
<td>0.146</td>
</tr>
<tr>
<td>≤200 (61)</td>
<td>0.094</td>
<td>0.471</td>
</tr>
<tr>
<td>≤225 (62)</td>
<td>0.142</td>
<td>0.273</td>
</tr>
<tr>
<td>≤250 (66)</td>
<td>0.179</td>
<td>0.15</td>
</tr>
<tr>
<td>≤300 (71)</td>
<td>0.124</td>
<td>0.305</td>
</tr>
<tr>
<td>All (78)</td>
<td>0.154</td>
<td>0.179</td>
</tr>
</tbody>
</table>

1Correlation coefficients for the relationship between dietary DHA, docosahexaenoic acid (22:6n-3) intake and RBC EPG and PC DHA

* Indicates significance by Pearson Correlation (two-tailed probability). P-values <0.05 are considered significant.
2.6. Figures

Figure 2-1  Percent contribution of different food groups to n-6 and n-3 long chain polyunsaturated fatty acid intake.
Figure 2-2  Box plots showing median and the 25-75th range and minimum and maximum non-outlier observations of n-6 and n-3 polyunsaturated fatty acids intakes based on fish consumption.
Rho = 0.240*

Rho = 0.302*

Rho = 0.346*

Rho = 0.153
Figure 2-3  Scatter plots showing relationship between the dietary intake of n-6 and n-3 polyunsaturated fatty acids and the same fatty acid in the RBC EPG and PC among Canadian pregnant women.
Figure 2-4: Regression planes to illustrate the best fit model between the RBC EPG and PC levels of DHA and dietary intakes of DHA and ARA or LA among Canadian pregnant women.
2.7. Bibliography


3.0 CHAPTER 3: ESSENTIAL N-3 FATTY ACIDS AMONG PREGNANT WOMEN AND EARLY VISUAL ACUITY MATURATION IN TERM INFANTS

3.1. Introduction

The importance of the n-3 fatty acid docosahexaenoic acid (DHA) is one of the most intensely studied areas relating nutrition to central nervous system (CNS) development, and is also emerging as important to several neurological problems in adults (1-10). Inadequate DHA during early development results in decreased DHA in the brain and retina, impairs neurogenesis and visual function, and results in long-term deficits in neurotransmitter metabolism and visual function in animals (1,11-14). Intervention studies to show that dietary DHA increases visual, mental and motor skill development in some preterm and term infants fed formula provides evidence that DHA is also important in early human development (reviewed in 2-5). More recently, it has become clear that maternal to fetal transfer of DHA during gestation is influenced by the maternal circulating and dietary intake of DHA (15-19). Thus, attention has turned to consider whether low DHA intakes among pregnant women may contribute to poor infant CNS development (20-27). However, the extent of DHA deficiency, if present, biochemical cut-offs for circulating DHA, dietary intakes, or infant visual or other developmental scores indicative of inadequate maternal DHA status to support optimal infant development are not known.

Because n-3 fatty acids are essential nutrients, all of the DHA accumulated by the fetus must be derived by placental transfer and must originate from the maternal diet, either as DHA or as a precursor n-3 fatty acid (1,15). Alpha linolenic acid (ALA, 18:3n-3), not DHA is currently considered the essential dietary n-3 fatty acid because humans lack a Δ-15

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desaturase, but can desaturate ALA via eicosapentaenoic acid (EPA, 20:5n-3) to DHA (1). However, stable isotope tracer studies have shown that conversion of ALA to DHA is low in humans (28,29) and interventions to increase ALA intake during pregnancy do not increase DHA in either maternal or fetal circulating lipids (30). On the other hand, observational and intervention studies provide consistent evidence that maternal dietary and circulating DHA is an important determinant of fetal blood levels of DHA (14-18). Although the estimated average intakes of DHA among pregnant women in North America is 40-120 mg/d (31-33), individual intakes vary widely from 20 to over 500 mg/d among women, excluding those following vegan diets (31).

We seek to determine whether poor DHA status sufficient to decrease infant CNS development occurs among pregnant women. However, although circulating levels of DHA increase with increasing DHA intake (34,35), enhanced DHA intake is not expected to have benefit in individuals with a DHA status above their physiological need (Figure 1-4, pg 31). Neither the DHA status that meets the needs for CNS function, nor who or how many individuals are able to benefit from enhanced DHA nutrition is known. Adding complexity, infant development has a distribution in which the developmental potential of individual infants is unknown (Figure 1-4, pg 31). In this report we describe an intervention with 400mg/d DHA from 16 wk gestation designed to determine if DHA deficiency is present among pregnant women. The purpose of DHA intervention is to develop a distribution of infant developmental scores for infants of mothers with a DHA intake considered to be above requirements against which to compare the development of infants of women not given DHA. We illustrate our approach to identifying DHA deficiency using the measures of infant visual acuity at 60 d of age and show why deficiency sufficient to delay development can
occur in a group without apparent changes in mean test scores. Our results also suggest that increased carbon chain (C) 22 n-6 fatty acids, particularly 22:4n-6 may be a sensitive indicator of poor maternal n-3 fatty acid status.

3.2. Subjects and Methods

3.2.1. Subjects and design

This is a prospective study designed to determine if DHA status is so low among some pregnant women as to pose risk of low infant CNS development. This study was not designed to demonstrate efficacy of DHA supplements in increasing infant development, which requires a different design. Healthy pregnant women were randomly assigned to 400mg/d DHA or a soybean/corn oil placebo from 16 wk of gestation until delivery of their infant. The reason for using a placebo group and randomization is to minimize group bias. In this study, as in some studies with infant formulas, the supplements were not labeled with individual codes corresponding to subject codes, but with a product code. To avoid randomization to only two groups known by those conducting randomization to differ, we used 4 groups; two identical DHA and two identical placebo groups. On enrolment, each subject was assigned a unique, computer generated, random code held in sealed opaque envelopes. Individual subject codes, not supplement codes, were used on data sheets and blood samples, and were analyzed blinded. The research staff conducting biochemical analyses, compiling data and conducting statistical analyses had no contact with the study subjects (double-double blind).

Eligible participants were 14-16 wk gestation, not taking any lipid supplement, had no complications likely to impact maternal or fetal metabolism, or fetal development, and were expected to deliver one full-term infant. Maternal blood and dietary intake was
collected at 16 and 36 wk gestation, and infant visual acuity was determined at 60 d of age. Because our purpose is to relate biochemical indices of maternal DHA status to infant development, subjects who did not complete all assessments, or whose infants were premature, low birth weight or had any complications likely to interfere with growth or development are not included in this study. All procedures were reviewed and approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of B.C. and the B.C. Children’s and Women’s Hospital. All subjects provided written informed consent before participating in this study.

3.2.2. Supplements

The supplements were provided as two 500 mg/d capsules, each one providing 200 mg DHA from the single cell organism Cryptecodinium cohnii, or a corn-soybean oil blend providing 265 mg linoleic acid (LA, 18:2n-6) and 40 mg ALA, both containing an orange flavour to assist in further blinding (Appendix F, Martek Biosciences, Columbia, MD). Each subject was asked to take two capsules/d with meals. The capsules provided about 9 Kcal/d and negligible LA and ALA compared to that in the usual diet. No dietary instructions were given, except not to take any lipid or fatty acid supplement other than that provided as part of this study. We chose a supplement of 400 mg/d DHA (equivalent to about 400 g/wk fatty fish such as salmon) because available estimates suggest the fetus accumulates about 70 mg/d DHA during the last trimester of gestation (36) and the International Society for the Study of Lipids and Fatty Acids (ISSFAL) has suggested pregnant women consume 300 mg/d DHA (37). Thus, we considered that a DHA supplement of 400 mg/d in addition to the usual diet should result in low risk of DHA deficiency.
3.2.3. Red blood cell fatty acids

Maternal venous blood was collected from each subject at 16 and 36 wk gestation in the outpatient laboratory of the B.C.’s Children’s Hospital, labelled with the subject code and immediately transferred to our Nutrition Research lab in the adjacent Child and Family Research Institute. The RBC were separated from plasma then stored at -70°C as described previously (18,38,39). We chose the RBC ethanolamine phosphoglycerides (EPG) fatty acids as a measure of maternal DHA status to avoid problems of analysis of fatty acids in plasma total lipids or phospholipids that arise due to inter and intra-individual differences in plasma lipoproteins, which are known to differ in fatty acid composition with stage of gestation and with short-term changes in dietary fat intake (40,41). The RBC EPG fatty acids were analyzed because EPG is preferentially distributed on the inner surface of the RBC membrane bilayer and contains higher levels of DHA and the C 22 n-6 fatty acids docosatetraenoic acid (22:4n-6) and docosapentaenoic acid (22:5n-6) than phosphatidylcholine, which is predominately on outer membrane bilayer of the RBC and exchanges with plasma phosphatidylcholine (42,43). The RBC lipids were extracted, EPG separated from other lipids, then the fatty acid composition analyzed by GLC as described in detail by us (Appendix C, 18,38,39,43).

3.2.4. Socio-demographic, dietary and anthropometric data collection

Socio-demographic data including the mother’s highest level of education attained, family income, ethnicity, age, height, pregnancy weight gain, and obstetric history were recorded (Appendix A). The TONI III, a test of non-verbal intelligence (3rd edit., Pro-Ed., Austin, TX), was given to each woman as a measure of IQ. Dietary intake for the preceding 4 wk was recorded at 16 and 36 wk gestation using an interview administered food frequency
questionnaire validated for estimation of fatty acid intakes (30). We collected specific information on all sources of fat, including product brand names, and types of fish and seafoods. Infant weight and length was measured at birth, then at 30 and 60 d of age, and type of milk feeding was recorded (38,39,43).

3.2.5. Infant visual acuity assessments

Visual acuity was assessed using the Teller Acuity Card Procedure (Vistech Inc., Dayton, OH) at 60 ± 3 d of age using a test distance of 38 cm (39,44). Looking acuity was determined as the finest grating to which the infant showed a reliable and consistent fixation response, based on the infant’s looking behaviour. Visual acuity was determined at 60 d postnatal because meta-analyses of studies with preterm and term infants have shown that the behavioural tests of visual acuity used in the present study acuity is a sensitive and robust measure of differences in visual acuity due to differences in DHA status when given at 60 d postnatal (45,46).

3.2.6. Statistical analysis

Base-line differences in subject characteristics between the groups were compared using Student’s $t$ test or $\chi^2$ for categorical variables. Visual acuity scores were converted to cycles/degree, and a log$_{10}$ transformation applied to the data for statistical analyses. The results for visual acuity are presented as means (cycles/degree) and SD (octaves), where one cycle/degree is equivalent to 0.301 octaves, as is convention (39,44). Changes in maternal fatty acid status due to stage of gestation (16 or 36 wk) or DHA intervention (DHA or placebo group) were determined using 2 factor ANOVA, with paired $t$ tests and Fischer’s Least Significant Difference Test to detect significant effects of stage of gestation or DHA intervention, respectively, where appropriate. Visual acuity assessed with the acuity card
procedure is a discontinuous variable (pass/fail), and visual acuity is higher in infant girls than boys (44). Therefore, distribution curves were constructed to examine the frequency with which infant boys and girls in the two groups achieved a visual acuity score of ≤1.6, 2.4, 3.2 or ≥ 4.8 cycles/degree. In multiple logistic regression, maternal DHA intervention, infant gender, birth weight, birth length, gestation length, breast-feeding duration, and maternal ethnicity accounted for 86% of the variability in infant visual acuity scores, but of these variables only maternal DHA intervention and infant gender were significant. Fischer’s exact test was used to determine whether infants in the placebo group were more likely than those in the DHA group to have a visual acuity score below the mean, after controlling for gender. Spearman rank correlation coefficients were calculated to determine the relationships between the maternal RBC EPG levels of DHA, 22:4n-6 and 22:5n-6 and visual acuity in the infant girls and boys. The maternal dietary fatty acid intakes in the placebo and DHA intervention group were compared using the Mann-Whitney U test. All analyses were done with SPSS for Windows (version 15; SPSS Inc., Chicago).

3.3. Results

3.3.1. Subject characteristics

A total of 135 women, n=67 in the placebo and n=68 in the DHA intervention group, completed this study to 60 d postnatal. There were no significant differences in maternal age, ethnicity, education, family income, or any other socio-demographic variable between women assigned to the DHA and placebo groups (Table 3-1), although our study population is one of predominately Caucasian (73% of the subjects), well-educated (76% had attended university) mature women with a mean age of 33.2 y at enrolment. All the infants were born after term gestation (37 - 42 wk gestation), as defined by the inclusion criteria. There were no
statistically significant differences in infant birth weight or length between the two groups, \( P>0.05 \). At 60 d of age, 50/67 infants in the placebo group and 42/68 infant in the DHA intervention group were exclusively breast-fed, an advantage potentially favoring the placebo group. All the infants fed with formula were given a term infant formula containing DHA and the n-6 fatty acid arachidonic acid (ARA, 20:4n-6). Despite randomization, 50% of infants in the placebo and 30% of infants in the DHA group were boys.

3.3.2. Maternal dietary n-6 and n-3 fatty acid intakes

We found no statistically significant differences in the maternal dietary fatty acid intakes at 16 and 36 wk gestation (data not shown). In Table 3-2 we report the mean, inter-quartile ranges and ranges of saturated, monounsaturated, n-6 and n-3 fatty acid intakes at 36 wk gestation. The median intake of linoleic acid (LA, 18:2n-6) was 5.0% total energy and ALA represented 0.66% total dietary energy (n=134). The median intakes of ARA, EPA and DHA were 90, 70 and 110 mg/d, respectively. However, we draw attention to the large differences in fatty acid intake among individuals, which included a range of 16 to 760 mg/d DHA. There were no statistically significant differences in the intakes of total fat, saturated fat, monounsaturated fat, LA, ALA, EPA or DHA between the placebo and DHA intervention group. However, the DHA group had a significantly higher median intake of ARA than the placebo group, \( P<0.05 \) (Table 3-2).

3.3.3. Biochemical measures of fatty acid status

Although dietary assessments can provide useful information on nutrient intakes of groups, they have limited precision for calculating the intakes of individuals. For this reason, we used biochemical measures of the RBC EPG fatty acids to assess the maternal DHA status. In Figure 3-1 we report the changes in RBC EPG n-6 and n-3 fatty acids between 16 and 36
wk gestation and effect of intervention to enhance maternal DHA intake. There were no statistically significant differences in the RBC EPG levels of n-6 or n-3 fatty acids between women randomized to the DHA and placebo groups at 16 wk gestation, with exception of ALA which was lower in the DHA than placebo group at both 16 and 36 wk gestation, $P<0.05$. The maternal RBC EPG levels of DHA increased significantly, and EPA and ARA decreased between 16 and 36 wk gestation in both groups, $P<0.05$. However, while the RBC EPG levels of EPA were not different between the placebo and DHA intervention groups at 36 wk gestation, the levels of DHA were 32% higher in the DHA intervention than placebo group (mean ± SEM, 11.7±0.29 and 8.87 ± 0.27 g/100 g fatty acids, respectively, $P<0.001$). Women in the DHA intervention group, but not women in the placebo group also had a significantly lower RBC EPG 22:4n-6, 22:5n-6 and 22:5n-3 at 36 compared to 16 wk gestation (Figure 3-1). In contrast, women in the placebo group had higher levels of 22:5n-6 in their RBC EPG at 36 than 16 wk gestation, $P<0.05$. Together, the effect of DHA intervention during gestation was to increase the maternal RBC EPG levels of DHA, but decrease ARA, 22:4n-6, 22:5n-6 and 22:5n-3 in the DHA intervention when compared to placebo group at 36 wk gestation, $P<0.05$.

3.3.4. Infant visual acuity

In the absence of n-3 fatty acid deficiency, intervention to enhance maternal DHA status can have no effect on the distribution of infant visual acuity scores. We report the frequency of visual acuity scores of ≤1.6, 2.4, 3.2 or ≥4.6 cycles/degree for infant boys and girls in the placebo and DHA intervention groups in (Figure 3-2). The mean (SD) in cycles/degrees (octaves) visual acuity of the infants (n=134) was 2.51 (0.37), with a visual acuity of 2.65 (0.50) and 2.35 (0.63) cycles/degree (octaves) for the girls (n=74) and boys (n=60),
respectively, \( P=0.08 \). The visual acuity of infants in the placebo (\( n=67 \)) and DHA intervention (\( n=68 \)) groups were 2.42 (0.50) and 2.60 (0.63) cycles/degree, respectively, \( P=0.30 \). The visual acuity of girls in the placebo (\( n=33 \)) and DHA intervention (\( n=42 \)) group was 2.46 (0.39) and 2.81 (0.57) cycles/degree (octaves), \( P=0.10 \), and 2.39 (0.6) and 2.30 (0.68) cycles/degree (octaves) for boys in the placebo (\( n=34 \)) and DHA intervention (\( n=26 \)), respectively, \( P<0.73 \).

In multivariate analysis, only infant gender (girls > boys, \( \beta = 0.660 \), SE 0.93, odds ratio 1.93) and study group (DHA > placebo, \( \beta = 1.215 \), SE 1.642, odds ratio 3.37) were related to infant visual acuity. Maternal smoking and alcohol were not important variables related to infant visual acuity in the present study. All of the infants were born at term gestation, and at 60 d of age were breast-fed or fed an infant formula containing DHA and ARA. Neither birth characteristics nor breast-feeding or breast-feeding duration were related to infant visual acuity at 60 d of age in our study.

Important to the present study, infant visual acuity has a distribution in which the potential visual acuity of individuals is unknown. As illustration, a theoretical distribution of visual acuities for \( n=150 \) infants could be, for the cycles/degree of 1.6, \( n=30 \); 2.4, \( n=50 \); 3.2, \( n=50 \); 4.8, \( n=20 \), to give a mean of 2.67 cycles/degrees (SD 0.48 octaves). Assuming DHA deficiency does not limit development in infants with a visual acuity of 4.8 cycles/degree, then an increase by one acuity level in 10% infants at all other acuity levels would change the distribution for the cycles/degree of 1.6, \( n=27 \); 2.4, \( n=48 \); 3.2, \( n=50 \); 4.8, \( n=25 \) to give a mean of 2.75 cycles/degree (SD 0.50 octaves), \( P=0.433 \). Regardless of improved outcome in 8.7% of infants, which would have important consequences at a population level, statistically significant differences in visual acuity are not apparent by comparing the group means.
However, if DHA status is so low among some pregnant women as to limit infant
development, then deficiency will be apparent by comparison of the distribution of test
scores for infants of mothers following their usual diet to that of infants born to mothers at
low risk of inadequate DHA. The frequency with which girls and boys in the placebo and
DHA intervention groups achieved visual acuity thresholds of \( \leq 1.6, 2.4, 3.2 \) or \( \geq 4.8 \)
cycles/degree reported in Figure 3-2 show that infants in the placebo group were more likely
to have a lower visual acuity than infants in the DHA group, odds ratio 3.37, from the
multivariate analysis. The proportion of infants with acuity scores above the mean for their
gender was higher in the DHA than placebo group for girls, \( P=0.048 \), although not for boys,
\( P=0.322 \).

Next, we used Spearman rank correlation analyses to determine the strength of the
relationships between the maternal RBC EPG levels of DHA, 22:4n-6 and 22:5n-6 and visual
acuity for infant girls and boys (Figure 3-3). We do not combine results for the placebo and
DHA intervention groups because no functional benefit is expected at intakes above need
(Figure 1-4, pg 31). Results for boys and girls were analyzed separately because gender
influences visual acuity, odds ratio 1.93 from the multivariate analyses. The maternal RBC
EPG levels of 22:4n-6 were inversely related to infant visual acuity at 60 d of age in girls,
Rho = -0.37, \( P<0.05 \) and boys, Rho = -0.48, \( P<0.01 \). The inverse relationship between the
maternal RBC EPG levels of 22:5n-6 and infant visual acuity did not reach statistical
significance in either girls or boys, Rho = -0.24, Rho = -0.21, respectively, the maternal RBC
EPG DHA showed no statistically significant relationship to infant visual acuity, Rho = 0.10
and 0.07, for girls and boys, respectively.
3.4. Discussion

Although it is well-accepted that DHA is critically important in the CNS, human requirements for n-3 fatty acids remain uncertain. Central to current questions over n-3 fatty acid nutrition is whether DHA is so low among some individuals as to impair neural development in infants and children, or increase the risk of neurological problems in adults (1-10). However, neither biochemical nor dietary indices of inadequate DHA to support CNS functions are defined. Furthermore, CNS development and function is influenced by many variables other than DHA. Adding complexity, infant development is a distribution in which an individual’s potential is unknown. Thus, infants with low developmental potential and high DHA or high developmental potential and low DHA cannot be distinguished by currently used tests (Figure 1-4, pg 31). The present report describes a novel approach using a randomized intervention to establish a group of infants for whom maternal DHA deficiency during gestation is considered unlikely against which to compare the distribution of development of infants of mothers following their usual diet. The presence of inadequate DHA in our population is suggested by comparison of the distribution of visual acuities which shows that more infants of women following their diet have lower visual acuities than in the group of infants of mothers in the DHA intervention group, odds ratio 3.37 (β=0.60, SE=1.93).

Several studies have considered the effects of supplementation of pregnant or lactating women with DHA from fish oils, DHA-enriched foods or triglycerides (20-26). In their study, Helland et al (20) supplemented pregnant women with 1183 mg EPA plus 803 mg DHA/d in gestation, but found no significant difference in electroencephalogram tests of infant neural development or novelty preference when compared to a placebo group using
Student $t$ or $\chi^2$ tests, although infants with more mature electroencephalogram patterns had high plasma DHA at birth. Similarly, Malcolm et al (22) found no difference in visual evoked potentials between infants of mothers assigned to 200mg/d DHA or a placebo during gestation, but visual evoked potential peak latencies were significantly related to the infant RBC DHA at birth. We also found no significant difference in the mean visual acuity between the group of infants of mothers assigned to 400mg/d DHA and the group of infants of mothers in the placebo group, regardless of a 32% higher maternal RBC EPG level of DHA at 36 wk gestation in the DHA intervention than placebo group. However, we illustrate that mean scores among groups for functional tests is not expected to reveal the presence of deficiency because the CNS development is a distribution, individual potential is unknown, and the study population is not limited to individuals known to be deficient prior to intervention.

We also considered information on DHA accretion in fetal tissues to provide supporting evidence that dietary n-3 fatty acid intakes are limiting in our population. Autopsy analyses have suggested that about 70 mg/d n-3 fatty acids, mostly DHA is accumulated in fetal tissue during the last trimester of gestation (36). At term gestation, the fetus represents about 25% of the total weight gain in pregnancy, but data on DHA accretion in the placenta or maternal pregnancy-associated tissues is not available. Women in the present study had a median intake of 110 mg/d DHA, and 40% of the women consumed less than 70 mg/d DHA. ALA and EPA can be desaturated and elongated to DHA, but stable isotope tracer and dietary intervention studies show that conversion of ALA to DHA is limited, particularly at the level of EPA to DHA (28-30,48). The median intakes of ALA and EPA in our study were 1480 and 70 mg/d, respectively, which assuming 0.5% conversion to
DHA (28,29), could provide a potential mean total DHA from dietary n-3 fatty acids of 118 mg/d. Using a value of 9% conversion of dietary ALA to DHA in pregnant women (49), then the estimated potential median DHA derived from dietary n-3 fatty acids for the women in the present study could be 250 mg/d. Regardless of the limitations of the latter approach, it is clear that many women in our study consumed less than the recommended 300 mg/d DHA (37), from which we suggest that risk of insufficient n-3 fatty acid nutrition seems likely. Consistent with this, recent epidemiological studies have shown a significant positive relationship between seafood consumption in pregnant women and verbal communication skills in their infants at 6 and 18 mo of age (27).

The extent to which the n-3 fatty acid intakes of women in our study are representative of a broader population is important. Our study involved predominately well-educated, mature Caucasian women, which has advantages in reducing the effects of environment on infant development. In previous studies we estimated dietary intakes of 150 mg/d DHA, with a range of 20-520 mg/d among pregnant women (31). Studies in central Canada, the United States, the Netherlands and Norway have reported mean intakes of 82, 81, 140 and 200 mg/d DHA, respectively (20,32,33,50). Pregnant women in the present study had a median intake of 110 mg/d DHA, suggesting that the dietary patterns with respect to sources of n-3 fatty acids are similar to those of other groups of pregnant women.

Although an increase in desaturation of n-6 fatty acids leading to increased 22:5n-6 is a characteristic finding in animals fed an n-3 fatty acid deficient diet (51), metabolism of fatty acids beyond the Δ-5 desaturase is limited in humans (28-30,48,49). In the present study, the mean maternal RBC EPG levels of 22:5n-6 and DHA were both higher at 36 than 16 wk gestation for the group of women in placebo group. However, the inter-individual
differences in 22:5n-6 and DHA were wide and 30% of the women in the placebo group showed no increase in their RBC EPG DHA between 16 and 36 wk gestation. Interestingly, even though we found no significant difference in the mean RBC EPG 22:4n-6 at 16 compared to 36 wks gestation in the placebo group, the maternal RBC EPG 22:4n-6 was significantly inversely related to visual acuity in the infant girls, Rho -0.37, \( P<0.05 \), and boys, Rho -0.48, \( P<0.01 \). Previous studies by us also reported a positive, rather than inverse relationship between DHA and 22:5n-6 in RBC lipids of preschool children, even though their intakes of n-3 fatty acids were very low (51). More specific studies will be needed to assess whether 22:4n-6 in RBC or plasma lipids can provide a useful biomarker of DHA deficiency in humans.

In summary, the present study used a novel approach to address whether poor DHA status sufficient to delay infant development occurs among pregnant women in our population. We show an increased risk of low visual acuity among 60 d-old infants of mothers following their usual diet when compared to infants of women considered at low risk of inadequate DHA due to DHA supplementation. The low dietary n-3 fatty acid intakes provide supporting evidence that current dietary practices place women of inadequate DHA during pregnancy. However, the present study does not address n-3 fatty acid requirements, which requires identifying the asymptote in function(s) above which no further benefit occurs, regardless of increasing DHA status. Whether subtle delays in early visual acuity maturation can be recovered later on is also not addressed. However, studies in animals subjected to n-3 fatty acid deficiency during intrauterine development have found persistent deficits in retina electroretinogram responses and brain monoamine neurotransmission, regardless of restitution of an n-3 fatty acid adequate diet (11,14). Some evidence for a
positive relationship between maternal DHA intake during gestation and mental skills in young children has also been published (21,25,27). We suggest the need for further studies to address dietary n-3 fatty acid requirements for pregnancy and lactation, specific stages of the life cycle involving anabolism and transfer of nutrients to the developing infant.
3.5. Tables

**Table 3-1  Subject characteristics^1**

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=67)</th>
<th>DHA intervention (n=68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Age, y</td>
<td>33.6±0.40</td>
<td>32.9±0.49</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>56</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>31</td>
</tr>
<tr>
<td>&gt;2</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Ethnicity, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian, Other^2</td>
<td>76,24</td>
<td>69,31</td>
</tr>
<tr>
<td>Income^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle or higher, %</td>
<td>78</td>
<td>77</td>
</tr>
<tr>
<td>Vegetarian^2, n</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Smoker^2, n</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Education, ^3 %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High, moderate, low</td>
<td>73,22,5</td>
<td>78,16,6</td>
</tr>
<tr>
<td>Toni III^3</td>
<td>68.3±3.55</td>
<td>60.3±3.83</td>
</tr>
<tr>
<td>Infant gender, boy/girl</td>
<td>51/49</td>
<td>30/62</td>
</tr>
<tr>
<td>Weight, g</td>
<td>3562±59.7</td>
<td>3472±47.1</td>
</tr>
<tr>
<td>Length, cm</td>
<td>52.0±0.27</td>
<td>51.7±0.29</td>
</tr>
<tr>
<td>Infant diet at 60 d^4, %</td>
<td>75,27,2</td>
<td>61,30,9</td>
</tr>
<tr>
<td>Breast-fed, mixed, formula</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1 Results are means ± SEM, percent, or n as indicated; there were no statistically significant differences between the DHA supplement and placebo group, P<0.05.

^2 Others included mostly Chinese and other Asians, n=26, with n=10 subjects of other ethnic backgrounds. No woman followed a vegan diet, or smoked > 10 cigarettes/d.

^3 We classified university, college and <high school as high, moderate and low education, and an income of ≥$50,000/y as middle or higher. The Toni-III is a non-verbal intelligence test.

^4 For practical purposes, infants consuming <2x250 mL formula/wk or <2 breast feeds/wk were considered breast-fed or formula-fed, respectively, all others were considered as mixed.
### Table 3-2 Dietary fat and fatty acid intake of pregnant women randomized to a placebo or DHA supplement

<table>
<thead>
<tr>
<th></th>
<th>All Subjects (n=135)</th>
<th>Placebo (n=67)</th>
<th>DHA (n=68)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fat, g/d</td>
<td>85.6 (32.4)</td>
<td>86.1 (33.3)</td>
<td>83.3 (30.3)</td>
</tr>
<tr>
<td></td>
<td>30.5 - 221</td>
<td>30 - 221</td>
<td>51.5 - 161</td>
</tr>
<tr>
<td>Saturates, g/d</td>
<td>27.3 (13.6)</td>
<td>27.4 (16.3)</td>
<td>26.3 (12.1)</td>
</tr>
<tr>
<td></td>
<td>9.5 - 78.3</td>
<td>9.7 - 78.3</td>
<td>11.6 - 68.9</td>
</tr>
<tr>
<td>Monounsaturates, g/d</td>
<td>32.4 (15.2)</td>
<td>33.3 (16.5)</td>
<td>31.5 (13.9)</td>
</tr>
<tr>
<td></td>
<td>9.9 - 90.6</td>
<td>10 – 91</td>
<td>57.5 - 16</td>
</tr>
<tr>
<td>Linoleic acid, g/d</td>
<td>13.5 (7.45)</td>
<td>12.1±7.4</td>
<td>11.5 (6.9)</td>
</tr>
<tr>
<td></td>
<td>2.52 - 43</td>
<td>2.5 – 43</td>
<td>4.4 - 31</td>
</tr>
<tr>
<td>Alpha-Linolenic acid, g/d</td>
<td>1.48 (0.95)</td>
<td>1.60 (0.99)</td>
<td>1.35 (0.96)</td>
</tr>
<tr>
<td></td>
<td>0.46 - 9.21</td>
<td>0.46 - 9.2</td>
<td>0.63 - 5.2</td>
</tr>
<tr>
<td>Arachidonic acid, mg/d</td>
<td>90 (50)</td>
<td>80 (50)</td>
<td>100 (60)²</td>
</tr>
<tr>
<td></td>
<td>20 - 360</td>
<td>20 - 150</td>
<td>20 – 360</td>
</tr>
<tr>
<td>Eicosapentanoic acid, mg/d</td>
<td>70 (80)</td>
<td>60 (90)</td>
<td>70 (120)</td>
</tr>
<tr>
<td></td>
<td>10 - 280</td>
<td>10 - 280</td>
<td>10 – 270</td>
</tr>
<tr>
<td>Docosahexanoic acid, mg/d</td>
<td>110 (190)</td>
<td>100 (160)</td>
<td>130 (200)</td>
</tr>
<tr>
<td></td>
<td>10 - 760</td>
<td>10 - 510</td>
<td>10 – 760</td>
</tr>
</tbody>
</table>

¹ Results shown are medians (interquartile ranges) and ranges, exclusive of supplements at 36 wk gestation.

² The intake of arachidonic acid (ARA,20:4) in the DHA intervention group was significantly higher than in the placebo group, Mann Whitney U, $P=0.01$. 
3.6. Figures

![Figures](image-url)
Figure 3-1  Box plots showing median, 25-75th range and minimum and maximum non-outlier observation ranges of maternal RBC EPG n-3 and n-6 fatty acids at 16 and 36 wks gestation. * Indicates significance between groups and # indicates significant difference over time.
Figure 3-2  Distribution of visual acuities at 60 d of age among boys and girls born to women assigned to DHA or a placebo from 16 wks of gestation until delivery.
Figure 3-3  Relationships between the maternal RBC EPG long chain n-6 and n-3 fatty acids. RBC EPG 22:4n-6, 22:5n-6 and 22:6n-3 (DHA) at 36 wk gestation and infant visual acuity at 60 d of age.
3.8. Bibliography


47. Makrides M, Neumann MA, Gibson RA. Perinatal characteristics may influence the outcome of visual acuity. Lipids 2001;36:897-900.


4.0 CHAPTER 4: GENERAL DISCUSSION

4.1. Discussion

Epidemiologic and intervention studies have provided evidence to show that the prenatal supply of DHA is important for infant neuro-development (1-10). Although considerable information is available to suggest that the maternal dietary intake of DHA and blood lipid DHA status influences infant neuro-developmental outcomes, it is not known whether or not current intakes of DHA are so low among pregnant women in Canada as to limit infant CNS development. Furthermore, the dietary or biochemical markers of n-3 fatty acid deficiency are not as yet defined (11). Therefore, the specific aims addressed by this thesis were 1) to describe the distribution of n-3 and n-6 fatty acid and fish intakes among pregnant women in our population, 2) to address whether or not the dietary n-6/n-3 fatty acid balance differs among women with different intakes of fish, 3) to assess the relationship between the intake of fish, DHA and other n-3 and n-6 fatty acids and maternal RBC EPG and PC fatty acids, 4) to develop a distribution curve of visual acuity scores for infants born to mothers considered likely to have intakes of DHA above requirement against which to compare the distribution of infant visual acuity scores for infants born to mothers following their regular diet, and 5) to explore the dietary intakes and biochemical markers indicative of DHA deficiency during pregnancy. The overall goal was to provide functionally relevant data towards establishing dietary recommendations to minimize risk of n-3 fatty acid deficiency during pregnancy.

4.1.1. Dietary intakes and sources of polyunsaturated fatty acids

Because adverse effects due to inadequate intakes of essential nutrients are more likely to occur in individuals at the lower end of the intake distribution, plotting the distribution of intakes is an essential first step towards evaluating those who may be at risk of adverse
outcomes due to inadequate intake. To address the size and shape of the distribution of n-6 and n-3 fatty acid intakes, the median, inter-quartile range, 5-95th percentile range, and mean and standard deviation of intakes were calculated from food frequency questionnaire data collected from women in their third trimester of pregnancy. The results of these studies show large variations in the intakes of the 20 and 22 carbon n-6 and n-3 fatty acids, particularly DHA, which was evident from the wide interquartile and 5-95th percentile ranges that varied over 9.5- and 39- fold. Furthermore, the standard deviation of DHA intake exceeded the mean. A large variation in individual intakes for DHA is also apparent in other studies involving pregnant women in both North America and Europe (5, 12-15), suggesting wide inter-individual differences in dietary intakes of DHA occur in our population as well as others. A test for Gaussian distribution showed that n-6 and n-3 fatty acid intakes, including DHA, were not normally distributed, but were skewed to higher values by a small number of individuals with higher intakes.

Because only a few foods, such as ruminant and poultry meats, are rich dietary sources of DHA, and fish and shellfish was found to account for over 70% of total DHA intake (Figure 2-1), women were then grouped by incremental intakes equivalent to 100 g/wk fish to enable evaluation of the dietary patterns of women with different fish intakes. The results from Chapter 2 show that 42% of the women consumed ≤100 g/wk fish, while only 18% consumed more than 300 g/wk fish, further supporting the findings of a non-normal distribution for DHA intake. This approach also aids in showing that a significant proportion of our population may be at risk of inadequate intakes of long chain n-3 fatty acids. Importantly, these results also show that it is difficult to have a diet rich in DHA when not consuming fish. In addition, the results presented in Chapter 2 add to the current state of
knowledge by showing that intakes of n-6 and n-3 fatty acids, and more specifically DHA, vary widely among a relatively homogenous population. Further, the approach used in these studies to evaluate intakes and sources of n-6 and n-3 fatty acids may have important future implications for identifying at risk populations.

4.1.2. Relationship between dietary fatty acid intake and RBC phospholipid fatty acids in pregnant women

An understanding that biologically regulated pathways are non-linear and plateau when substrates and cofactors are adequate is essential to determining dietary and biochemical markers of deficiency, or alternatively, the dietary intake that minimizes risk of deficiency. Novel to these studies performed in Chapter 2, linear regression analysis was performed with incremental intakes of 25 mg/d DHA to determine if an intake of DHA exists below and above which DHA intakes are and are not linearly related to RBC EPG and PC DHA status, respectively. This method was used as an exploratory approach to provide an index of the range of DHA intakes over which DHA may be limiting for RBC EPG and PC incorporation and turnover. PC however, is mainly found on the outer RBC membrane surface (16, 17), and exchanges with PC in the plasma (18). The results presented in Chapter 2 to show a significant linear relationship between RBC PC DHA over the entire range of DHA intake among all subjects not taking supplemental DHA, n=78, P<0.05, suggests that RBC PC DHA may not be an accurate measure of DHA status. Conversely, EPG is preferentially distributed on the inner membrane bilayer and is considered a better indicator of DHA in tissues. In the present studies, DHA intake was significantly linearly related to RBC EPG levels of DHA at intakes up to 150 mg/day DHA. A linear relationship between DHA intake and the RBE EPG DHA was not found at intakes above 150 mg/d DHA, suggesting that DHA was not
limiting for RBC EPG incorporation and turnover, suggesting possible adequate intake at intakes above 150 mg/d DHA. Notably, however, the r-value for the relationship between DHA intake and RBC EPG DHA for intakes below 150 mg/d was low, R=0.268, \( P=0.048 \), suggesting wide variability in RBC EPG DHA status among women with seemingly similar intakes, and the possibility that other factors may be involved in determining the expression of DHA in EPG phospholipids. However, it is also important to note that in the present studies the number of subjects was low. Limitations in the estimation of dietary DHA intake may have contributed to the low r-value for the relationship between DHA intake and biochemical measures of DHA status.

4.1.3. The n-6 and n-3 fatty acid intakes among individuals with varying intakes of fish

Although the intakes of fish and n-3 fatty acids have been associated with decreased risk of CVD and several other human health related diseases (19-22), several recent studies have suggested that high meat, the major dietary source of ARA, and ARA and n-6/n-3 fatty acid intakes are associated with increased risk of several immune and inflammatory disorders and diseases such as metabolic syndrome, coronary artery disease, type 2 diabetes, and some neurological diseases (23-27). This led to the question of whether or not women with different intakes of fish also have different intakes of other n-6 and n-3 fatty acids. The results in Chapter 2 show that although women who consumed \( \leq 100 \text{ g/wk} \) fish had significantly lower intakes of ARA than women who consumed \( \geq 201 \text{ g/wk} \) fish, their dietary ARA/DHA ratios were significantly higher than those consuming \( \geq 101 \text{ g/wk} \) fish, \( P<0.05 \). Further, the 5-95th percentile range for ARA and ARA/DHA intakes was relatively wide for women with intakes of \(<100 \text{ g/wk} \) fish, suggesting that individual intakes of ARA and
ARA/DHA ratio varied widely among women with low intakes of fish. To the best of our knowledge, this is the first study to examine trends in dietary n-6 and n-3 fatty acid intakes among individuals grouped by intakes of fish. These results also demonstrate that even among women with seemingly similar intakes of DHA, heterogeneity exists in their intakes of ARA and dietary ARA to DHA fatty acid balance. The extent to which differences in n-6 fatty acid intake contribute to the wide variation in RBC EPG DHA status, possibly explained by fatty acid competition for esterification into phospholipids, seemed an important question. Therefore, further studies were conducted to address the question of whether or not dietary n-6 fatty acids or the n-6/DHA balance contributed to the differences in RBC membrane DHA. This would also mean that the intake of n-6 fatty acids would need to be considered in developing dietary DHA recommendations.

4.1.4. Dietary n-6 fatty acids and maternal DHA status

As discussed in Chapter 1, conversion of LA and ALA via the desaturation and elongation pathway to longer chain n-6 and n-3 polyunsaturated fatty acids appears to be inhibited by high amounts of substrate, as well as by the products of the reaction (11). Moreover, n-6 and n-3 fatty acyl substrates compete for incorporation to the \( sn-2 \) position of phospholipids (28). Thus, it is possible that an abundance of dietary n-6 fatty acids may inhibit conversion of ALA and EPA to DHA, and displace DHA by competition for incorporation to the \( sn-2 \) position of phospholipids. Although higher dietary intakes of ARA have been shown to decrease DHA in membrane lipids of the brain in animals (29), surprisingly little information is available on the relationship between dietary n-6 fatty acids intakes and DHA status in humans. Therefore, in the present thesis multivariate linear regression analysis was used to derive models to test if co-relationships exist between dietary DHA and dietary n-6 fatty acid
and the RBC EPG and PC DHA. The results presented in Chapter 2 show that in the best-fit model to explain the relationship between dietary DHA and either LA or ARA, and RBC PC DHA status, only the dietary intake of DHA was significantly related to the RBC PC levels of DHA. On the other hand, the results for RBC EPE DHA show that dietary DHA and LA or ARA interacted in significant, but opposite ways, to influence membrane EPG pools of DHA. Although both LA and ARA were negatively associated with the RBC EPG DHA, only small increases in dietary ARA intake were needed to decrease the RBC EPG levels of DHA, whereas relatively large changes in LA intake were needed to produce the same effect on lowering DHA. To the best of our knowledge, these are the first studies to investigate the interaction between dietary DHA and n-6 fatty acids on RBC membrane phospholipid DHA status. The results provide some evidence to suggest that the wide variation in RBC EPG levels of DHA among women with similar intakes of DHA, may involve the concurrent differences in the intake of n-6 fatty acids. These results therefore, highlight the possible need to consider the modifying effects of n-6 fatty acids when addressing the relationship between dietary n-3 fatty acids and n-3 fatty acid status.

4.1.5. Maternal DHA and infant visual acuity

Although it has been well established that DHA is accumulated in the CNS, and several studies have provided evidence that DHA supply plays an important role in neural development, neural function, and neuroprotection in animals and humans (1-5, 10, 30-38), it is not yet clear if DHA intake or status is so low among some individuals as to pose risk of adverse health outcomes, related to CNS development and function. The results in Chapter 3 found no significant differences in mean infant visual acuity scores between the group of infants of mothers assigned to take 400 mg/d DHA and the group of infants of mothers in the
placebo group (39). The results from multivariate analysis however, show that of the variables tested only DHA intervention and infant gender were significantly related to infant visual acuity at 60-d of age, and thus the results for infant boys and girls were analyzed separately. Although no differences in visual acuity scores were found among boys in the placebo and DHA intervention groups at 60-d, the results showed that significantly more infant girls in the placebo group had visual acuities below the median visual acuity score for all girls, than among infant girls of mothers provided supplemental DHA, \( P<0.05 \) (39). The lack of significant effect in the group of infant boys may be explained by the slower development of visual acuity in the infant boys than infant girls.

4.1.6. Maternal n-6 and n-3 fatty acid status and infant visual acuity

The final aim of this thesis was to explore biochemical markers that may be used to aid in identifying groups of women at risk for inadequate DHA status in gestation. As discussed in Chapter 1, available estimates suggest that the fetus accumulates approximately 67 mg/d n-3 fatty acids, mostly DHA, in the last trimester of gestation (40, 41), suggesting maternal dietary intakes of greater than 67 mg/d n-3 fatty acids are required to support both the mother and the developing fetus. The results from Chapter 2 provide some support for this suggestion through the results to suggest that DHA is limiting for RBC EPG incorporation and turnover at intakes below 150 mg/d DHA. However, as discussed earlier, caution is warranted due to the low correlation coefficient relatively low sample number and wide variability in DHA and n-6 fatty acid intake among the women in the present study. Moreover, the results to show that dietary LA and ARA were significantly inversely related to the RBC membrane EPG DHA status among women not taking DHA supplements provides evidence to suggest that dietary n-6 as well as n-3 fatty acids are significant
determinants of DHA status. On the other hand, for women in the DHA supplement group
the n-6 fatty acids DTA and DPA were both significantly lower at 36 wk gestation than in the
same women at baseline (16 wk gestation), and significantly lower than women in the
placebo group at 36 wk gestation (39). Previous studies have shown that animals fed an n-3
fatty acid deficient diet have significantly higher levels of 22-carbon n-6 fatty acids DTA and
DPA in tissues including the brain and retina when compared to animals fed an n-3 fatty acid
adequate diet (31, 42-47). Moreover, although poorer performance on visual, behavioural,
and learning tasks has been shown in animals fed n-3 fatty acid deficient diets (31, 48-50),
the results from Chapter 3 show that maternal RBC EPG DTA was significantly inversely
related to visual acuity in both infant girls and boys of mothers in the placebo group.
However, no such association could be found among infants of mothers in the DHA
supplement group (39), which is consistent with the absence of deficiency in this group.
Thus, these results raise the possibility that elevated maternal RBC EPG levels of DTA might
provide a sensitive indicator of poor infant CNS development (39). However future
investigations are needed to confirm this relationship.

4.1.7. Conclusions

In summary, the results presented in Chapter 2 of this thesis show that the distribution of
DHA intakes among pregnant women are skewed, and that dietary ARA/n-3 fatty acid
balance varies widely among a relatively homogenous population of women in their third
trimester of pregnancy. The results presented in Chapter 2 also provide some evidence to
suggest the possibility of unsaturated membrane lipid DHA among some women. However,
although current evidence suggests that the intake of preformed DHA is an important
determinant of blood and tissue lipid DHA, the results raise an important question of whether
or not n-6 fatty acid intakes significantly also impact DHA status. The results presented in Chapter 3 show an increased risk of low visual acuity among 60 d-old infants of mothers following their usual diet when compared to infants of women assigned to take a supplement of 400 mg/d DHA. These findings provide functionally relevant data to suggest that inadequate n-3 fatty acid nutrition does occur among pregnant women in our population. Together, the overall significance of these studies provide supporting evidence that current dietary practices among some women in our population place them at risk of inadequate n-3 fatty acid status during pregnancy to support optimal CNS maturation in their infants. These studies emphasize the need for future public health strategies aimed at improving the n-3 fatty acid status of pregnant women in Canada.

4.2. Strengths and Limitations

4.2.1. Strengths

Scientifically sound research studies, grounded in the principles for assessing dietary requirement, provide the foundation for deriving nutrient requirements and recommendations. The strength of this thesis in adding to the body of knowledge available to aid in determining n-3 fatty acid requirements was the particular attention paid to the distribution of DHA intakes and the foods providing DHA to the diet. This approach was used to help identify possible at risk populations and provide insight into the dietary patterns placing them at risk. Secondly, several important methodological considerations important for reducing errors in estimating DHA intakes were addressed in this thesis. Because fish and seafood are the major dietary sources of DHA, but are less common and sometimes infrequently consumed, a FFQ designed to collect food consumption habits over a longer time period was used for estimating usual intakes of DHA. Further, once a FFQ is complete,
converting frequency estimates to nutrient values requires an appropriate nutrient database (51). All foods and portion sizes were entered into a nutrient database (FOOD PROCESSOR 11; ESHA Research, Salem, OR), containing the Canadian Nutrient File with the addition of over 500 locally consumed foods analyzed by this laboratory, to aid in nutrient intake estimation accuracy. Thirdly, in this thesis RBC membrane EPG and PC fatty acids were used as a measure of maternal DHA status to avoid changes in plasma fatty acid pools due to short-term changes in dietary fat intakes (52, 53). Separating the RBC EPG and PC phospholipids classes also revealed a linear relationship between dietary DHA intake and RBC PC levels of DHA, but a non-linear relationship or curvilinear relationship between dietary DHA intake and RBC EPG level of DHA. The latter non-linear relationship aided in identifying the asymptote where for some women dietary DHA intake was sufficient for RBC membrane EPG synthesis and turnover during pregnancy. Finally, the results from the sociodemographic data collected from each of the participants show that the women were predominantly Caucasian, well-educated, and had a family income in the highest defined tertile, suggesting that these women may have had greater access to better prenatal care, more nutritious foods, and may have been able to provide a more enriched learning environment for their infants (39). However this relatively homogenous study population has advantages in reducing the effects of environmental variables on infant development, making confounding less likely. Together these methodological considerations applied in this thesis add to the strength of the results.

4.2.2. Limitations

There are also several limitations to these studies that need to be addressed. First, potential inaccuracies in nutrient intake estimation, which include under- or over-reporting intakes by
the subjects due to social desirability, or inaccuracies due to imprecise subject recall (51).
Secondly, although the study subjects were asked to take two supplements per d providing
400 mg/d DHA or placebo and not take any other lipid supplements, compliance could not be
accurately determined. The subjects were asked to return unused supplements at the end of
the intervention period and provide information on the regularity of taking the supplements,
but accuracy is unknown. Not taking, or irregularity in taking the supplements, would
impact the accuracy of evaluating any effects of the supplementation on the biochemical
measures of DHA status, although this study was not designed as an efficacy trial. However,
failure to comply with supplementation may have contributed to the large overlap in RBC
EPG DHA status among women in the DHA supplement and placebo group. Thirdly,
research on CNS development has established that infant boys and girls develop at different
rates with girls outperforming boys on communication elements, on tests of hearing and
speech, eye-hand co-ordination, contrast sensitivity function assessed with forced choice
preferential looking methods, stereopsis, and binocular rivalry (54-58). Similarly, the results
of the present studies show that although the proportion of infant girls with acuity scores
above the mean visual acuity score for infant girls was higher in the DHA than placebo
group, no changes in the distribution among infant boys was detected. Because only one
time point and one measure of infant CNS development was assessed, whether differences in
CNS development among infant boys could be detected if testing was performed at a later
age, or if an alternative methods of CNS testing was used, is not known. Lastly, although
there were no statistically significant differences in the RBC EPG levels of n-6 or n-3 fatty
acids between women randomized to the DHA and placebo groups at baseline (16 wk
gestation), with the exception of ALA which was lower in the DHA than placebo group at
both 16 and 36 wk gestation, DHA supplementation resulted in not only an overall significant increase in maternal RBC EPG DHA, but also a significantly lower maternal RBC EPG ARA, DTA, and DPA when compared to placebo group at 36 wk gestation, \( P<0.05 \) (39). Further, an inverse relationship was found between maternal RBC EPG DTA and visual acuity in both boys and girls in the placebo group. Based on these results, it cannot be concluded whether or not the benefits in infant CNS function were a direct result of increased DHA status and maternal fetal transfer, or if strategies to reduce maternal 22-carbon n-6 fatty acid status by some other method would produce similar results.

4.3. Potential Implications

This thesis focused on the dietary determinants of DHA status during pregnancy and whether or not low DHA status during pregnancy pose risk of poor infant neural development when measured by infant visual acuity at 60-d of age. The results show that in the absence of fish it is difficult to obtain sufficient DHA from meats and dairy products, and that poor DHA status within a population is not obvious from estimated average intakes. Moreover, the relationship between DHA intake and DHA status was curvilinear, suggesting that the DHA status of some women was adequate above intakes of 150 mg/d DHA. Potential implications could include a simple survey at early prenatal visits to assess DHA intake to provide the mother with information on the contributing food sources of DHA to her diet and provide a rough estimate of her current DHA intake. Finally, 400 mg/d DHA supplementation significantly shifted the distribution of infant visual acuity scores, suggesting inadequate n-3 fatty acid nutrition in our population. However, these studies stress the incomplete knowledge on whether or not the concurrent intakes of DHA and the n-6 fatty acids LA and
ARA contribute to maternal and infant DHA status which may then have important implications on the infant’s functional development.

4.4. Future Directions

1) Future research should be directed to elucidate whether other factors found in fish, for example, choline and EPA, contribute to optimal infant neuro-developmental outcomes (59, 60).

2) Address the possible importance of high n-6 fatty acid and genetic variation in fatty acid desaturase genes in modifying maternal DHA status (29, 61, 62), thus contributing to n-3 fatty acid requirements.

3) Address whether or not early differences in infant visual acuity perpetuate, or are associated with, other developmental difference in the children at older ages.

4) Future research is needed to address the safe upper limits for the n-3 and n-6 fatty acids.
4.5. Bibliography


62. Xie L, Innis SM. Genetic variants of the FADS1 FADS2 gene cluster are associated with altered (n-6) and (n-3) essential fatty acids in plasma and erythrocyte phospholipids in women during pregnancy and in breast milk during lactation. J Nutr 2008;138:2222-8.

APPENDICES

Appendix A

Socio-Demographic and Health Questionnaire

Date: ID #: 

Some of these questions are personal but very important to our study. Please answer questions as accurately as possible. Remember, your answers will be kept strictly confidential. Your name is not attached to any of these sheets.

Socio-Demographic Information

1. **Your** date of birth day (    ) month (    ) year (    )

2. **Your** country of birth _________________

3. **Your** Ethnicity

   - [ ] White
   - [ ] First Nations
   - [ ] Black
   - [ ] Chinese
   - [ ] East Indian
   - [ ] Other Asian (please specify) _________________

4. The infant’s Paternal Ethnicity

   - [ ] White
   - [ ] First Nations
   - [ ] Black
   - [ ] Chinese
   - [ ] East Indian
   - [ ] Other Asian (please specify) _________________

Other (please specify) _________________ Other (please specify) _________________
5. Did you complete high school?
   - [ ] yes
   - [ ] no

6. If you attended college, how many years did you complete?
   a. less than one year
   b. one year
   c. two years
   d. more than two years

7. If you attended university, how many years did you complete?
   a. less than one year
   b. one year
   c. two years
   d. three years
   e. four years
   f. more than four years

8. What is your usual occupation? ______________________________________

9. What is the total income in your family? (i.e. you and your partner’s income)
   - [ ] less than $20,000
   - [ ] $20,000 - $50,000
   - [ ] more than $50,000

10. How many people are supported by the family income? ________________
Health Information

The next sets of questions ask about your personal health and lifestyle habits. These questions are very important to our clinical study. I would again like to remind you that all of the information given is strictly confidential.

1. What is your height? ______ feet ______ inches

2. How much did you weigh before you became pregnant? _____ lbs.

3. Is this your first pregnancy? □ yes □ no

   If no, how many times have you been pregnant? _________

4. What is your due date? ____/____/________

5. Have you ever had a miscarriage? □ yes □ no

   If yes, how many times have you miscarried? ______

6. Have you ever had preterm labour? □ yes □ no

7. How many live births have you had? __________

8. How many baby boys have you had? __________

9. Have you ever had twins or triplets? □ yes □ no

10. Have you smoked cigarettes during this pregnancy?

    □ yes □ no

    If yes, what is the average number of cigarettes you smoke per week? _________

11. Have you consumed alcoholic beverages during this pregnancy?

    □ yes □ no

    If yes, what is the average number of drinks per week? _________
(1 drink = 1.5 oz of spirits, whiskey, etc., OR 12 oz of beer, OR 5 oz of wine)

12. During this pregnancy have you taken any vitamin and/or mineral supplements? (Please include all supplements – eg. herbal supplements, fish oil supplements)

☐ yes ☐ no

1. Supplement name ____________________________
   How often do you take them? _________________
   When did you start taking them? _________________
   Why did you start taking them? _________________
   ____________________________________________________________________________

2. Supplement name ____________________________
   How often do you take them? _________________
   When did you start taking them? _________________
   Why did you start taking them? _________________
   ____________________________________________________________________________

3. Supplement name ____________________________
   How often do you take them? _________________
   When did you start taking them? _________________
   Why did you start taking them? _________________
   ____________________________________________________________________________

(If more than three supplements were used, please continue on the back of the page.)
1. During this pregnancy did you follow any particular diet?

☐ yes  ☐ no

If yes, what diet did you follow?

4. Lacto-ovo vegetarian (eats all milk and milk products and eggs)
5. Semi-vegetarian (eats all milk and milk products, eggs, poultry and fish)
6. Vegan (avoids ALL animal products)
7. Other, please specify: ____________________________________________

2. During this pregnancy have you taken any medications? (include current use)

☐ yes  ☐ no

If yes:

1. Medication: _______________  dose: ____________
   Condition: _______________  time period: _______

2. Medication: _______________  dose: ____________
   Condition: _______________  time period: _______

3. Medication: _______________  dose: ____________
   Condition: _______________  time period: _______

(If more than three medications were taken, please continue on the back of the page.)

Thank you for taking the time to complete this questionnaire.
Your cooperation is greatly appreciated!
<table>
<thead>
<tr>
<th>Item Name</th>
<th>Amount</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dairy Products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Milk</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- cow (homo, 2%, 1%, skim)</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>- goat, rice, soy, chocolate</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>2) Hard cheese</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- cheddar, mozzarella</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>3) Soft cheese</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- brie, cottage cheese</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>4) Yogurt</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>-</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>5) Ice cream/</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>Frozen Yogurt</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>6) Eggs</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>-</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>7) Other dairy products</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- cream cheese, sour cream</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td><strong>Table/Cooking Fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Margarine/Butter</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- used for spreading</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>2) Margarine/Butter</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- used in cooking</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>3) Cooking oil (specify)</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>- olive, canola, etc.</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>4) Salad dressings</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>-</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>5) Mayonnaise</td>
<td>□ oz/g</td>
<td>□ Day</td>
</tr>
<tr>
<td>Miracle Whip</td>
<td>□ cup/ml</td>
<td>□ Week</td>
</tr>
<tr>
<td>-</td>
<td>□ piece</td>
<td>□ Month</td>
</tr>
<tr>
<td>Item Name</td>
<td>Amount</td>
<td>Frequency</td>
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<td>---------------------------------</td>
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</tr>
<tr>
<td>6) Peanut or Nut Butter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- tahini, almond butter</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Snack items</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Chocolate bars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chocolates, Candy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Potato chips</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nacho chips</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Popcorn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- microwave, movie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Party snacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- pretzels, nuts &amp; bolts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) Crackers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Granola/Cereal bars</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7) Nuts/Seeds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- almonds, cashews, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8) Other snack items</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Breads, Cereals and Baked Goods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Bread (specify)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Buns/Rolls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(including hamburger &amp; hot dog buns)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Bagel/English Muffin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Item Name</td>
<td>Amount</td>
<td>Frequency</td>
</tr>
<tr>
<td>-----------------------------------------</td>
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<td>-----------</td>
</tr>
<tr>
<td>4) Cereal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- oatmeal, Raisin Bran, Cheerios, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) Pancakes/Waffles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Donut, Pastry, Danish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7) Cake, Squares, Pie, Muffins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8) Cookies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9) Other Baked Goods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9) Rice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10) Pasta</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Combination Foods/Meals</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Sauces (no meat)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. tomato, cheese, pesto, hollandaise, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Sauces with meat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. spaghetti sauce</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Prepared pasta/rice dish (no meat)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. Kraft Dinner, Lipton’s Sidekicks, etc.</td>
<td></td>
<td></td>
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<tr>
<td>3) Prepared dish with meat</td>
<td></td>
<td></td>
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<tr>
<td>- eg. Lasagna, Meat pies, Sheppard’s pie</td>
<td></td>
<td></td>
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<tr>
<td>Item Name</td>
<td>Amount</td>
<td>Frequency</td>
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<tr>
<td>-----------------------------------------------</td>
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<td>-------------</td>
</tr>
<tr>
<td>5) Taco, Burrito, Quesadilla, Pizza</td>
<td></td>
<td></td>
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<tr>
<td>Meats and Poultry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Poultry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- chicken or turkey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Fried chicken</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. KFC, chicken nuggets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Beef</td>
<td></td>
<td></td>
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<tr>
<td>- roast, steak, cubes, ground</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) Hamburgers</td>
<td></td>
<td></td>
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<tr>
<td>5) Pork</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Lamb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7) Hot dogs and sausages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8) Deli meats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. pepperoni, bologna, bacon, lunch meats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) Salmon (fresh or frozen)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2) Other fish (fresh or frozen)</td>
<td></td>
<td></td>
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<tr>
<td>- please specify</td>
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<td></td>
</tr>
<tr>
<td>3) Battered or breaded fish</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- eg. Fish ’n chips, fish burger, etc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Item Name</td>
<td>Amount</td>
<td>Frequency</td>
</tr>
<tr>
<td>---------------------------------</td>
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<td>-----------</td>
</tr>
<tr>
<td>4) Canned tuna</td>
<td>☐ oz/g</td>
<td>☐ Day</td>
</tr>
<tr>
<td></td>
<td>☐ cup/ml</td>
<td>☐ Week</td>
</tr>
<tr>
<td></td>
<td>☐ piece</td>
<td>☐ Month</td>
</tr>
<tr>
<td>5) Canned salmon</td>
<td>☐ oz/g</td>
<td>☐ Day</td>
</tr>
<tr>
<td></td>
<td>☐ cup/ml</td>
<td>☐ Week</td>
</tr>
<tr>
<td></td>
<td>☐ piece</td>
<td>☐ Month</td>
</tr>
<tr>
<td>6) Other canned fish</td>
<td>☐ oz/g</td>
<td>☐ Day</td>
</tr>
<tr>
<td>- please specify</td>
<td>☐ cup/ml</td>
<td>☐ Week</td>
</tr>
<tr>
<td></td>
<td>☐ piece</td>
<td>☐ Month</td>
</tr>
<tr>
<td>7) Sushi (specify type)</td>
<td>☐ oz/g</td>
<td>☐ Day</td>
</tr>
<tr>
<td></td>
<td>☐ cup/ml</td>
<td>☐ Week</td>
</tr>
<tr>
<td></td>
<td>☐ piece</td>
<td>☐ Month</td>
</tr>
<tr>
<td>8) Shellfish (specify)</td>
<td>☐ oz/g</td>
<td>☐ Day</td>
</tr>
<tr>
<td>- eg. crab, lobster, shrimp, mussel, oysters</td>
<td>☐ cup/ml</td>
<td>☐ Week</td>
</tr>
<tr>
<td></td>
<td>☐ piece</td>
<td>☐ Month</td>
</tr>
</tbody>
</table>

**Vegetables and Fruits**

1) Potatoes
- boiled, mashed, roasted

2) Fries
- eg. French fries, hash brown

3) Raw vegetables
- eg. lettuce, tomatoes, etc.

4) Green vegetables, cooked
- eg. broccoli, etc.

5) Orange vegetables, cooked
- eg. carrots, squash, etc.

6) Beans, Legumes
- eg. baked beans, lentils, kidney beans, etc.

7) Other vegetables (specify)
<table>
<thead>
<tr>
<th>Item Name</th>
<th>Amount</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>8) Fruits</td>
<td>☐ serving</td>
<td>☐ Day ☐ Week</td>
</tr>
</tbody>
</table>

- List the 3 fruits you eat most often:

<table>
<thead>
<tr>
<th>Beverages</th>
<th>Amount</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Soft Drinks (regular)</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- eg. Coca Cola, Sprite, etc.</td>
<td>☐ cup/ml ☐ piece</td>
<td></td>
</tr>
<tr>
<td>2) Soft Drinks (diet)</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- ☐ cup/ml ☐ piece</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) Sweetened juices</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- eg. Snapples, Kool-Aids, etc.</td>
<td>☐ cup/ml ☐ piece</td>
<td></td>
</tr>
<tr>
<td>4) Fruit juices, pure</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- ☐ cup/ml ☐ piece</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) Vegetable juice</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- ☐ cup/ml ☐ piece</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) Other Beverages</td>
<td>☐ oz/g</td>
<td>☐ Day ☐ Week ☐ Month</td>
</tr>
<tr>
<td>- ☐ cup/ml ☐ piece</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix C

Lipid Extraction of RBC's (63)

1. Thaw RBC samples and aliquot 0.5 ml. into 16 x 150 screw-cap (s/c) tubes (work on ice).

2. Add 2.2 ml. Isopropyl Alcohol to each sample. Individually cap tube each and vortex.

3. Add 1.4 ml. of Chloroform to each tube, and individually cap and vortex (try and vortex any dried sample off the sides of the tube) for a further 1 to 2 minutes. Cap tubes and vortex.

4. Centrifuge at 2000 rpm for 5 minutes and remove supernatants to 16 x 150 tubes, trying to remove as much solvent as possible.

5. Add 2 ml. Methanol and 4 ml Chloroform to each extraction (ie. s/c) tube, vortex and centrifuge at 2000 rpm for 5 minutes. Remove supernatants, adding them to the ones previously removed.

6. Centrifuge tubes containing supernatants for 10 minutes at 2000 rpm.

7. Remove supernatants to 16 x 100 tubes. Dry samples under nitrogen. Add 3 mL of Chloroform/Methanol (2:1) and vortex.

8. Equilibrate TLC tank using solvent system (about 3 hours). Proportions are:

   CHLOROFORM   50
   METHANOL     30
   GLACIAL ACETIC ACID  8
   WATER        4

9. Activate pre-run, 'scored' (~25 mm. lanes) TLC plates for 30 minutes in a 120 degree oven.

10. When plates are ready, spot PC and PE standards in one lane on each plate (approx.
10 ul.) and spot samples -- reconstituted in 50 ul. Chloroform /Methaonol on 25 mm lanes. Allow plate to dry for 5 minutes after last sample spotted, then place plate in TLC tank for approximately 90 minutes.

11. Once plate(s) is run, allow to air dry until solvent is evaporated, about 20 minutes. Spray plate with 2, 7'-dichlorofluorescein to visualize phospholipids. Read under long wave UV light. If plate is still wet, bands will be difficult or impossible to see – just allow the plate more drying time.

12. Once bands are visualized and identified by marking silica with pencil, plates can be stored in the cold room to minimize auto-oxidation. Scrape desired bands into screw cap tubes.

13. Add 2 ml hexane and 1 ml BF3 to each tube vortexing after each. Cap once complete.

14. Methylate at 100°C for 10 minutes. Cool in refrigerator before uncapping.

15. Add 3 ml saline and 6 ml pentane, vortexing after addition of each. Centrifuge at 2000 rpm for 5 minutes.

16. Remove supernatants to 16 x 150 tubes. "Backwash" these supernatants by adding 3 ml of saline to supernatants. Vortex and centrifuge.

17. Remove supernatants to 13 x 100 tubes, being careful not to take up any of the bottom phase. Dry down under N2 to ~2 ml. in volume. Vortex and finish drying.

18. Samples are ready for G.C. analysis. Minus 20 freezer is OK for short term storage, but for long term (more than a few days) store samples in -70 degree freezer.
Appendix D

Percent contribution of different fish and shellfish species to the total DHA intake from fish and shellfish.
Appendix E

Scatter plots showing relationship between the dietary intake of LA and ALA and the same fatty acid in the RBC EPG and PC among Canadian pregnant women.
Appendix F

Box plots showing median and the 25-75th range and minimum and maximum non-outlier observations of the LA and ALA intakes based on fish consumption.
Appendix G

Marktek Biosciences Corporation (Columbia, MD) fatty acid analysis of the DHA supplement and placebo capsules.

<table>
<thead>
<tr>
<th></th>
<th>DHA Supplement</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8:0</td>
<td>0</td>
<td>1.46</td>
</tr>
<tr>
<td>C10:0</td>
<td>2.05</td>
<td>0.92</td>
</tr>
<tr>
<td>C12:0</td>
<td>5.38</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C14:0</td>
<td>15.46</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C16:0</td>
<td>14.64</td>
<td>10.55</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.62</td>
<td>0</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.58</td>
<td>4.45</td>
</tr>
<tr>
<td>C18:1n-9</td>
<td>14.85</td>
<td>21.41</td>
</tr>
<tr>
<td>C18:1n-7</td>
<td>&lt;0.1</td>
<td>1.53</td>
</tr>
<tr>
<td>C18:2n-6</td>
<td>0.78</td>
<td>51.17</td>
</tr>
<tr>
<td>C18:3n-6</td>
<td>0</td>
<td>0.38</td>
</tr>
<tr>
<td>C18:3n-3</td>
<td>&lt;0.1</td>
<td>6.25</td>
</tr>
<tr>
<td>C20:0</td>
<td>&lt;0.1</td>
<td>0.41</td>
</tr>
<tr>
<td>C20:1n-9</td>
<td>0</td>
<td>0.32</td>
</tr>
<tr>
<td>C20:3n-6</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>C20:4n-6</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>C20:5n-3</td>
<td>&lt;0.1</td>
<td>0</td>
</tr>
<tr>
<td>C22:0</td>
<td>0</td>
<td>0.46</td>
</tr>
<tr>
<td>C22:5n-3</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td>C22:6n-3</td>
<td>41.54</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>C24:0</td>
<td>0</td>
<td>0.16</td>
</tr>
</tbody>
</table>

All values are g/100g fatty acid.