The Complex Relationship Between Omega-3 Fatty Acids and Early Neurodevelopment

by

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Abstract

Docosahexaenoic acid (DHA, 22:6ω-3) is an omega-3 fatty acid that is an important component of neural lipids accumulating in neural tissue during development. Decreased brain DHA is accompanied by increased 22:4ω-6 and 22:5ω-6, which has been shown to lead to deficits in neural function. However, dietary and other variables may impact DHA status and the potential for early deficiency to have lasting adverse effects on neurodevelopment remains unclear. Therefore, the effect of prenatal DHA and children's DHA intake and status on neurodevelopment was examined.

Pregnant women, n=271, were enrolled at 16 wk gestation and randomized to 400 mg/day DHA or placebo until delivery. Infant neurodevelopment was assessed at multiple time-points until 18 mo. Children returned for follow-up between 5-6 y (n=98). An additional group of 187 children (5-6 y) was enrolled at the same time to increase the sample size. Venous blood was collected, diet was assessed by food frequency questionnaire, and neurodevelopment was assessed.

Infants from the placebo group were less likely to achieve high neurodevelopmental test scores up to 18 mo than infants from the DHA group (OR=2.23-3.22, $P<0.05$), suggesting that fetal DHA inadequacy occurred in our population. No differences were detected in children (5-6 y, n=98) from the placebo and DHA groups achieving a high neurodevelopment test score ($P>0.05$). However, child DHA intake and status (n=98) were related to the mother's intake and status during pregnancy. For all children (n=285), DHA intake was positively associated with erythrocyte DHA. Child DHA status was associated with neurodevelopment test scores, but only short-term memory was associated with dietary DHA.

These results suggest that DHA low enough to constrain infant neurodevelopment to 18 mo does occur among pregnant women in Vancouver, but the long-term effects remain unclear. We also provide evidence that DHA status is related to cognitive performance in young children. However, the association of maternal and child DHA intake and status limits the interpretation of whether DHA before or after birth is important. Finally, the variability in erythrocyte DHA was high, raising questions about the relationship between DHA intake and other fatty acids, DHA status, and neural function.

Preface

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

The research in Chapter 2 has been published, (Mulder KA, King DJ, Innis SM. Omega-3 fatty acid deficiency in infants before birth identified using a randomized trial of maternal DHA supplementation during pregnancy. PLoS One. 2014, 9:e83764). My supervisor Dr. Sheila Innis designed the research program of which this study was a part, and obtained funding from the Canadian Institute of Health Research (CIHR). Several other published studies include data from this project (e.g. Stephens TV, *et al*. 2014; Wu BT, *et al.* 2013; Novak EM, *et al.* 2012; Xie L and Innis SM, 2009; Friesen RW, *et al.* 2009, 2010). Staff and graduate students administered the study procedures, with enrollment of pregnant women, infant neurodevelopmental testing, and blood sample analyses. I was primarily responsible for all data analysis and interpretation, and Dr. Innis and I wrote the manuscript together.

The research in Chapter 3 was designed by Dr. Sheila Innis, and also funded by CIHR research grants. A trained research assistant, with a graduate degree as a practitioner, conducted the child cognitive assessments, which were developed with assistance from expert faculty in child development. My major role was the dietary interviews with parents regarding their child's intake, as well as the analyses and interpretation of the dietary data. I was responsible for the assessment of the complex and large amount of dietary data, with assistance from dietetic students who I supervised. D. Janette King was the primary laboratory technician responsible for analysis of the blood samples, and who taught me how to perform the analyses of red blood cell fatty acids. I was also responsible for the data analysis with interpretation and preparation of the data and manuscript for Chapter 3 with supervision by Dr. Innis.

The research in Chapter 4 was also designed by Dr. Innis and funded by CIHR research grants, and used identical methods to those in Chapter 3, but included a cross-sectional cohort of children. In addition to dietary interviews and analysis, I had a major role in the recruitment of the cross-sectional children. I was also responsible for all data analysis and interpretation, leading to Chapter 4 preparation for this thesis with supervision by Dr. Innis.

Ethics approval was required for this research and was obtained from the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia

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List of Abbreviations

AI: adequate intake
ALA: alpha-linolenic acid
ALSPAC: Avon Longitudinal Study of Parents and Children
AMDR: acceptable macronutrient distribution range
ANCOVA: analysis of covariance
ANOVA: analysis of variance
ARA: arachidonic acid
BBB: blood brain barrier
Beery: Beery-Buktenica Developmental Test of Visual-Motor Integration
BMI: Body mass index (kg / m^2)
BSID: Bayley Scales of Infant Development
CCHS: Canadian Community Health Survey
CDI: Communicative Development Inventory
CFRI: Child and Family Research Institute
CNF: Canadian Nutrient File
CNS: Central nervous system
CVD: cardiovascular disease
D5D: delta-5 desaturase
D6D: delta-6 desaturase
DHA: docosahexaenoic acid
DRI: Dietary Reference Intakes
ELOVL: elongase
EPA: eicosapentaenoic acid
FADS: fatty acid desaturase
FADS1: fatty acid desaturase 1 (Δ5)
FADS2: fatty acid desaturase 2 (Δ6)
FAO: Food and Agriculture Organization of the United Nations
FFQ: food frequency questionnaire
GLC: gas-liquid chromatography
GM: grey matter
HDL: high-density lipoproteins
HPLC: high-performance liquid chromatography
IOM: Institute of Medicine
IQR: interquartile range
ISSFAL: International Society for the Study of Fatty Acids and Lipids
KABC: Kaufman Assessment Battery for Children
kcal: kilocalorie
LA: linoleic acid
LPC: lyso-phosphatidylcholine
LPL: lipoprotein lipase
M: myelin
MDI: Mental Development Index
mm: homozygous minor allele carriers

Mm: heterozygous major allele carriers
MM: homozygous major allele carriers
MUFA: monounsaturated fatty acids
n: number of participants
NAL: National Agricultural Library
NEFA: non-esterified fatty acids
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PI: phosphatidylinositol
PL: phospholipid
PPVT: Peabody Picture Vocabulary Test
PS: phosphatidylserine
PUFA: polyunsaturated fatty acids
r: Pearson's correlation coefficient
RBC: red blood cells
rho: Spearman's rank correlation coefficient
RT: Response Time
SD: standard deviation
SE: standard error
SFA: saturated fatty acids
***sn*:** stereo-specifically numbered
SNP: single nucleotide polymorphisms
SPH: sphingomyelin
TAG: triacylglycerols
TFA: total fatty acids
TMFA: total milk fatty acids
TONI-3: Test of Nonverbal Intelligence-3
TOVA: Test of Variables of Attention
WHO: World Health Organization
USDA: United States Department of Agriculture

Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Sheila Innis, for her wisdom, guidance, and most of all, her patience. Her infectious passion for research transformed my eight-week undergraduate research experience into a desire to understand nutrition research and pursue doctoral training. Dr. Innis' dedication to her students provided a stimulating academic environment, and I am very grateful for all of the opportunities that broadened my graduate experience.

I would also like to thank my supervisory committee members, Dr. Gwen Chapman, Dr. Rajavel Elango, and Dr. Tim Green, who provided invaluable expertise, support and mentorship throughout my studies.

This research would not have been possible without the support and assistance of the Innis Lab family, especially Roger Dyer, Janette King, Debbie Zibrik, Kelly Richardson, and Julie Matheson. I would also like to acknowledge Ruth Milner, Boris Kuzeljevic, and Dr. Roland Brant for their expertise and assistance with the statistical analysis of this work. Volunteers were essential to the completion of this project, and I would like to thank Alexa Ferdinands and Janet Mmbando for all of their help with entering the dietary data. In addition, I am grateful for the assistance and support of my fellow students, especially Melissa Glier, Sara Moukarzel, Alexandra Wiedeman, Lynda Soberanes, and Betina Rasmussen.

Support for this research was provided by a grant from the Canadian Institute of Health Research (CIHR), and I would also like to gratefully acknowledge funding by the CIHR Frederick Banding and Charles Best Canada Graduate Scholarship and the Child and Family Research Institute Graduate Studentship.

There are several other important people to whom I owe deep gratitude. First, to Becky Blair who started this journey by sparking my interest in nutrition and to follow in her footsteps, and also Dr. June Matthews for her inspiration to pursue research and ability to always know when I needed some heartfelt words of encouragement. To Liz Novak and Russ Friesen, who were there with me in the beginning of my research training and their continued friendship and support gave me the confidence to keep going. To the best friends a gal could ask for, especially Marisa Marciano, Stephanie Irvine, Kristi Serwa, Meher Shergill, Sarah Harvey, Ashley Hardill, and Taryn Deane. These lovely ladies have stuck with me through the last decade of schooling, providing a support system full of laughter, inspiration, the occasional bottle of wine, and a lifetime of memories. To Joris de Wit, for his love and constant encouragement through even the most challenging times, and especially for ensuring that I always had a healthy breakfast. Lastly, I want to thank my family, who have always supported and believed in me through all of my endeavors. My parents, for instilling a solid foundation to manage challenges and teaching me the importance of hard work, persistence, and independence, and my brother for always reminding me not to take myself too seriously.

Dedication

This dissertation is dedicated to the parents and children who participated in this study for their time, effort, and occasional tears. Without them, this work would not have been possible.

Chapter 1: Introduction

1.1 Background and Rationale

The omega (ω)-3 fatty acid docosahexaenoic acid (DHA, 22:6ω-3) is an important component of neural lipids, accumulating in neural tissue during development [1]. Experimental work has shown that DHA has critical roles in neural development, function, and protection [2-6]. Dietary deficiency of ω-3 fatty acids or high intakes of ω-6 fatty acids has been reported to lead to reduced brain DHA which is accompanied by increased 22:4ω-6 and 22:5ω-6, and deficits in neural function, including altered neurochemistry and behaviour, have been associated with low brain DHA [7-13]. Studies in human infants and children are challenging due to the inability to access neural tissues, the many factors that impact circulating DHA, incomplete knowledge of the circulating DHA pool or concentration that reflects an insufficient supply of DHA for the CNS, and considerable individual and environmental variables that impact child development. Regardless, some studies have shown that higher DHA intake or status during pregnancy is associated with better cognitive performance and improved attention in infants and children [14-19].

Importantly, CNS development in humans has a long time-course, beginning *in utero* and continuing after birth [20], with an increase in DHA rich synapses until around 5-7 y [21]. Therefore, post-weaning, infants and children require ω-3 fatty acids in their own diet to support continued CNS development. While little is known about ω-3 and ω-6 fatty acid intakes in children, it is clear that fish is a rich source of DHA, and DHA consumption does result in higher blood DHA [22]. However, while DHA can be synthesized endogenously from dietary α-linolenic acid (18:3ω-3, ALA), a high dietary intake of the ω-6 fatty acid, linoleic acid (18:2ω-6, LA) may antagonize metabolism of ALA to DHA or acylation of ω-3 fatty acids, possibly resulting in lower plasma and cell membrane DHA [23-25]. Higher DHA intakes or status of children have also been associated with better indices of neural development [26-28]. Determining a critical time period when the developing brain is most vulnerable to inadequate DHA is further complicated by potential similarities between the maternal and child ω-3 fatty acid intakes. Since it is possible mothers with low DHA intakes would have children with low DHA intakes, it is unclear if positive associations of DHA intake or status on CNS outcomes may be explained by the DHA supply during pregnancy, infancy or childhood.

The overall objective of this research was to address the question of whether or not ω-3 fatty acid deficiency sufficient to influence early infant and child CNS development occurs among pregnant women in our community. This research was also designed to determine if inadequate DHA during prenatal development or children's own DHA status and/or intake affects neurodevelopment in young children.

1.2 Literature Review

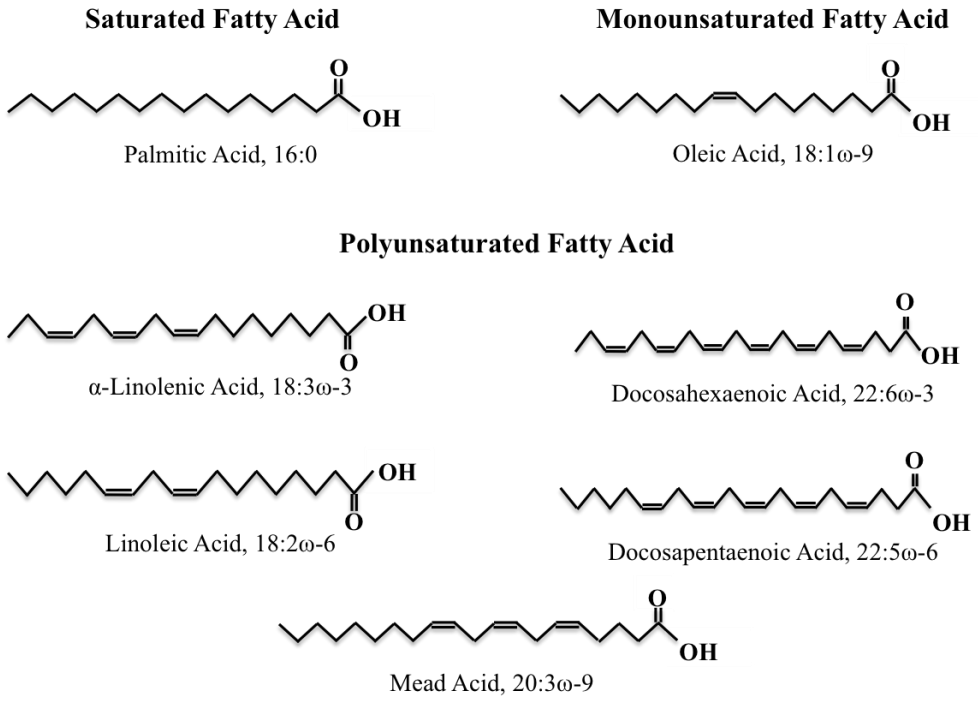
As in other animals, ω-3 and ω-6 fatty acids cannot be synthesized by humans, explained by the lack of desaturases required to insert a double bond at carbon 3 or 6, respectively, from the carboxyl end of an 18 carbon chain fatty acid. Knowledge and acceptance of the dietary ω-3 and ω-6 fatty acid essentiality extends well over half a century [29], although much has yet to be understood on their roles, metabolism, interactions with each other, other dietary components, and genetic variation. The ω-3 fatty acid docosahexaenoic acid (DHA) is a 22-carbon fatty acid with six double bonds which can be in the diet, or synthesized from 18, 20, or 22 carbon ω-3 fatty acids. DHA shows unique enrichment in phospholipids (PL), and is particularly high in certain membranes and PL of the brain and retina. This literature review provides background on fatty acids and their metabolism, focusing particularly on ω-3 and ω-6 fatty acids, which is followed by review of DHA in early CNS development and function, and current understanding of the effects of diet on DHA and its assessment in humans, particularly infants and children.

1.2.1 Fatty acids: Definition and Classification

Fatty acids are hydrocarbon chains with a methyl group on one end and a carboxyl group on the other. Fatty acids are generally classified as short, medium, long and sometimes very long chain based on the number of carbons in the chain. Fatty acids with six or fewer carbons are classified as short chain and those with seven to 12 carbons as medium chain fatty acids. Fatty acids with 14 or more carbons are typically referred to as long-chain fatty acids, sometimes with ω-3 and ω-6 fatty acids with 20 or more carbons referred to as very long chain. Fatty acids are further classified by the position of the first double bond from the methyl end of the carbon chain and the number of double bonds. Saturated fatty acids (SFA) contain no double bonds, monounsaturated fatty acids (MUFA) have one double bond, and polyunsaturated fatty acids

(PUFA) have two or more double bonds. Unsaturated fatty acids are classified into groups using either 'ω' or 'n', for example, ω-3, ω-6, ω-9, with the first double bond at the third, sixth, or ninth carbon, respectively, from the methyl end. A common SFA (palmitic acid, 16:0), MUFA (oleic acid, 18:1ω-9), and PUFA (for ω-3 α-linolenic, ALA, 18:3ω-3; ω-6 linoleic, LA, 18:2ω-6; and ω-9 mead, 20:3ω-9) are shown in Figure 1.1.

Figure 1.1 Schematic of common saturated, unsaturated, and polyunsaturated fatty acids.



In addition to a major source of energy and storage in adipose tissue, fatty acids are present in PL which themselves are complex and have diverse roles in the body. Particularly relevant to this research, PL are a fundamental component of all cell and sub-cellular membranes in which they maintain cellular compartments and also contribute to membrane function, including providing precursors for numerous bioactive metabolites, for example eicosanoids and docosanoids [30,31]. Sphingolipids and glycerophospholipids, differ from triacylglycerols (TAG), as they contain a phosphate group (Figure 1.2). Triacylglycerols have a 3-carbon glycerol backbone bound to three fatty acids, while glycerophospholipids, have a fatty acid esterified at each of the stereo-specifically numbered (*sn*)-1 and *sn*-2 positions, with a phosphate

and specific component such as choline, ethanolamine, serine, or inositol at the *sn*-3 position (Figure 1.3). In sphingomyelin (SPH), the phosphate group is attached to a nitrogen-containing sphingosine backbone linked to a single fatty acid on one side, and choline on the other.

Figure 1.2 Schematic of a triacylglycerol and sphingomyelin.

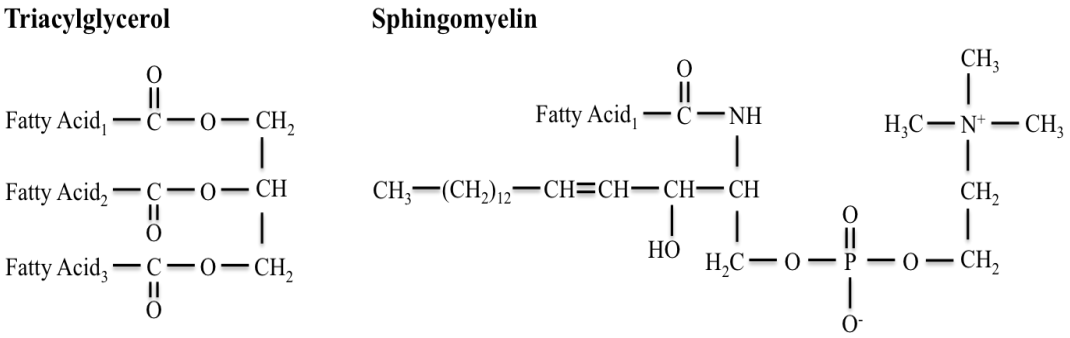
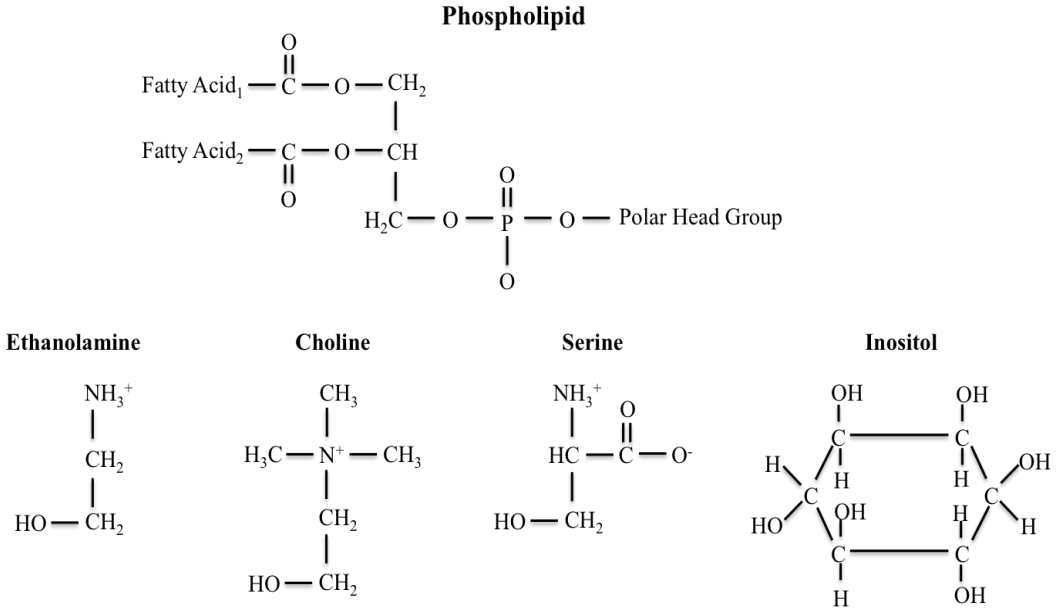


Figure 1.3 Schematic of a phospholipid and polar head groups.



1.2.2 Essential ω-3 and ω-6 Fatty Acids

While there is considerable knowledge on the functional importance of DHA, particularly in the brain and retina, the importance of dietary sources of different ω-3 fatty acids, their interaction with ω-6 fatty acids, and transfer to the CNS to support DHA needs is still incompletely understood. The following section reviews this, as it is a part of understanding the current research that assessed diet and biochemical markers of DHA status.

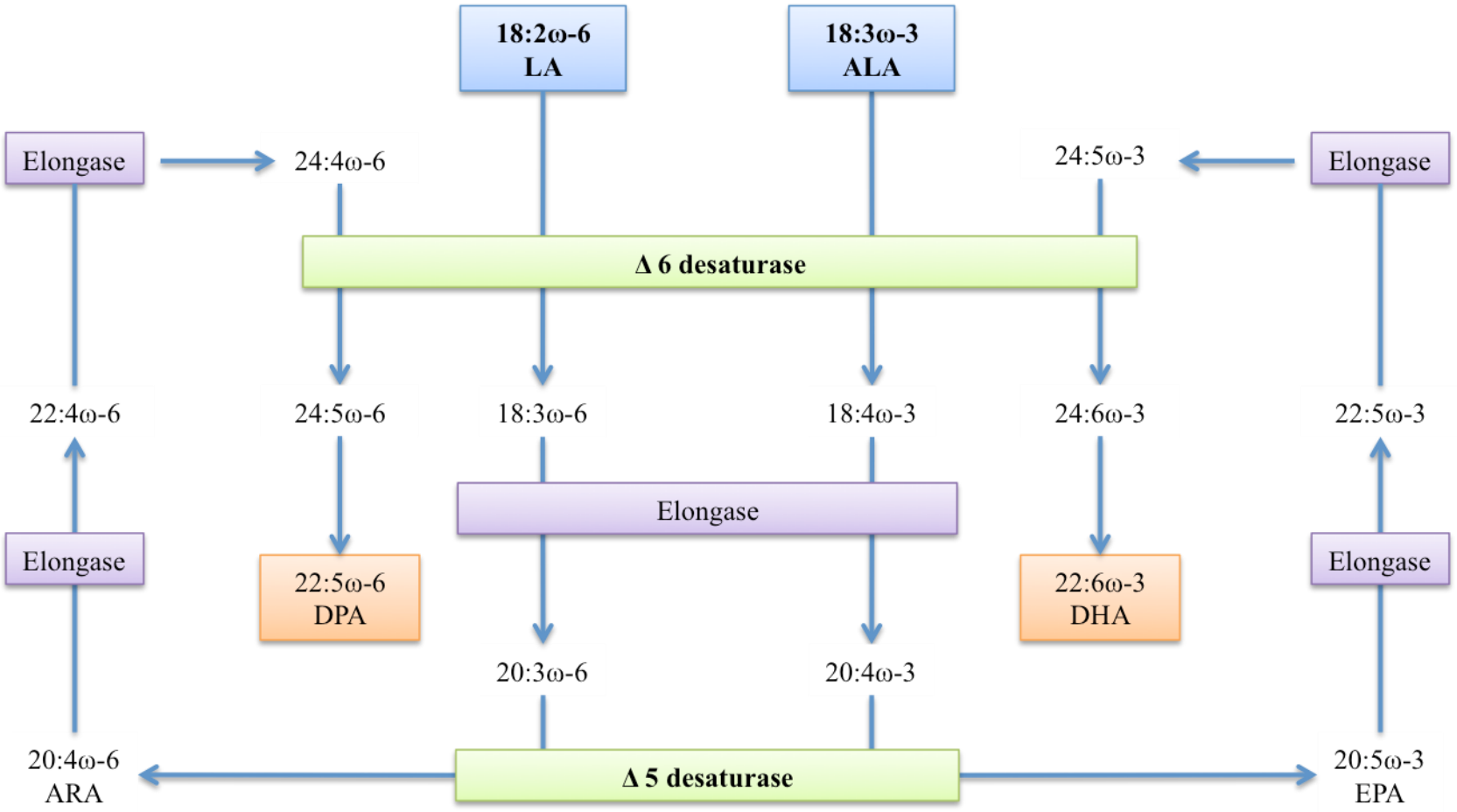
1.2.2.1 ω-3 and ω-6 Fatty Acid Metabolism

Dietary fatty acids are consumed primarily esterified in TAG, with smaller amounts in PL. Much smaller amounts of fatty acids may be eaten as non-esterified fatty acids (NEFA), mono- or di-glycerides, cholesterols esters, and some other esterified nutrients, for example retinyl esters, although the significance of any of these fatty acid sources is not known. Triacylglycerol fatty acid digestion in humans typically begins in the stomach with gastric lipase, which partially hydrolyzes TAG through hydrolysis of the *sn*-3 position fatty acid. In the small intestine, lipids are emulsified by bile salts from the gall bladder to form mixed micelles with hydrolysis by pancreatic lipase to release fatty acids from the TAG *sn*-1 and *sn*-3, also releasing *sn*-2 monoacylglycerol. Pancreatic phospholipase A_2 hydrolyzes dietary and biliary PL at the *sn*-2 position, resulting in a NEFA and an *sn*-1 lysophospholipid. The lipid digestion products are then absorbed into the enterocyte through facilitated transport and passive diffusion with NEFAs and *sn*-2 monoacylglycerols, then re-synthesized into TAG [32]. The TAG are then packaged with PL, cholesterol, apolipoproteins, and fat-soluble vitamins into chylomicrons for release to the lymphatics, which enter the general circulation at the thoracic duct. Once in the blood, chylomicrons obtain apolipoprotein C-II (cofactor for lipoprotein lipase activity) and apolipoprotein E from high-density lipoproteins (HDL), thus transforming into a mature chylomicron [33]. Chylomicron TAG are then hydrolyzed by lipoprotein lipase (LPL) at the endothelial cell surface of extrahepatic tissues, including skeletal muscle, heart, and adipose, with the resulting chylomicron remnants taken up from circulation by the liver [33,34].

Both ALA and LA are considered essential fatty acids and must be obtained from the diet in humans, like all mammals, because of the lack of Δ-12 and Δ-15 desaturase enzymes required to insert a double bond at the third and sixth carbon, respectively, of an 18-carbon chain.

Desaturation and elongation of ALA and LA to longer chain, more unsaturated fatty acids is considered to use the same desaturase and elongase enzymes [35]. The first step of desaturation in this pathway is rate-limiting and involves the introduction of a double bond in ALA to form stearidonic acid (18:4ω-3, SA) and in LA to form ϒ-linolenic acid (18:3ω-6, GLA) by delta (Δ)-6 desaturase (D6D) (Figure 1.4). The 18 carbon chains are then lengthened by addition of two carbons by elongase (ELOVL), followed by desaturation of 20:4ω-3 to eicosapentaenoic acid (20:5ω-3, EPA) and 20:3ω-6 to arachidonic acid (20:4ω-6, ARA) by Δ-5 desaturase (D5D). ARA can be elongated to docosatetraenoic acid (22:4ω-6) then 24:4ω-6. Further metabolism of EPA involves elongation to 22:5ω-3. Synthesis of 22:5ω-6 and DHA from 22:4ω-6 and 22:5ω-3, respectively, is complex and involves additional elongation to 24:4ω-6 and 24:5ω-3, then D6D producing the double bond to form 24:5ω-6 and 24:6ω-3, which is followed by peroxisomal 2 carbon removal [35]. The ω-3 and ω-6 fatty acid desaturation and elongation is considered to use the same enzymes, but also involves competition between LA and ALA, and feedback regulation of the enzymes by their products. For example, EPA and DHA inhibit or reduce EPA, DHA, and ARA synthesis [23,24,36,37].

Figure 1.4 Schematic of ω-3 and ω-6 fatty acid desaturation and elongation[1].



[1] Adapted from Innis, 2003; Novak, 2012 [35,38].

Early work on dietary requirements for ω-6 and ω-3 fatty acids showed that only low intakes of ω-6 fatty acids were needed, likely less than 2% dietary energy, to meet ARA requirements and prevent ω-6 fatty acid deficiency [39-42]. Acknowledgements of ALA requirements came somewhat later, with reports of approximately 0.4 % energy from ALA needed to prevent deficiency in animals and humans [41,43-45]. Although the essentiality of the ω-3 fatty acids was not yet recognized, competition between LA and ALA metabolism was first reported by Mohrhauer and Holman in 1963. The authors reported a reduction of ω-6 fatty acid products in rats when ALA was increased in the diet and LA was constant [46]. Rahm and Holman (1964) then showed the opposite also occurred, when dietary ALA was constant and dietary LA increased, there was a reduction of ω-3 fatty acid products [47]. Numerous studies have since reported competitive interactions in the metabolism of ω-3 and ω-6 fatty acids in animals [2,45,48-52]. However, not all organs show the same response, thus the implications of the competition appears to be tissue specific. For example, Arbuckle et al (1994) showed lower brain DHA in neonatal piglets fed formula with an LA/ALA ratio of 22/1 and 37/1 compared to 4/1 and 8/1 and sow-fed controls, whereas only the 37/1 formula led to lower DHA in the retina [48].

Studies on the competition between LA and ALA in humans are more complex due to the varying amounts of preformed ω-6 and ω-3 fatty acid products in the diet, and ability to assess tissues for ethical reasons. In one small study (n = 7), men were provided diets with an LA/ALA ratio of 8/1 or 30/1 for 12 days, then given deuterated LA + ALA (n = 4) or ALA (n = 3) [23]. The study reported that the high LA/ALA diet led to a 65% reduction in plasma total lipid ω-3 fatty acid metabolites. The analysis of percent deuterated fatty acids showed that only deuterated 20:5ω-3 was significantly reduced with means (SD) of 3.4 (0.5) % and 8.0 (1.5) % total fatty acids ($P = 0.005$) in the high and low ratio groups, respectively [23]. Deuterated DHA was not significantly different between the groups, likely explained by the high variability of plasma lipid DHA concentration in both groups [23]. Two other studies with adult men showed that with a constant intake of ALA between 1-2 % energy, decreasing LA intakes from 10-12 % energy to 4-5 % energy led to an increase in plasma PL 20:5ω-3, but had no effect on plasma PL 20:4ω-6 or 22:6ω-3 [25,53]. It may be possible that 1-2 % energy from ALA was sufficient to meet DHA requirements, or that the combined intake of ALA and LA even at 4-5 % energy from LA may

have saturated D6D, thus inhibiting the production of DHA from EPA. In contrast to studies in adults, higher circulating levels of DHA and lower ω-6 fatty acid products, including ARA, were achieved in infants fed formulas with a reduced ratio of LA/ALA of 3-4.8/1 compared to formulas with an LA/ALA ratio of 19/1 or greater [54,55]. Thus, it appears that competition between ALA and LA can affect circulating levels of the ω-3 and ω-6 fatty acid products, but the impact of this competition on tissues, including the brain, as well as the optimal ratio to promote adequate accretion of both ω-3 and ω-6 fatty acid products in humans remain unknown.

In addition to the metabolic competition between LA and ALA, single nucleotide polymorphisms (SNP) in fatty acid desaturase (FADS) and elongase (ELOVL) genes have been associated with altered measures of ω-3 and ω-6 fatty acids. Several studies have shown higher ALA and LA and lower ω-3 and ω-6 fatty acid products in homozygous minor allele carriers (mm) of several SNPs for FADS2 (encodes D6D) and FADS1 (encodes D5D) compared to homozygous (MM) or heterozygous (Mm) major allele carriers in multiple pools including plasma, red blood cells (RBC), adipose and human milk, but the results are less clear for DHA [56-66]. Although one US study by Scholtz et al (2015) reported that the RBC DHA of pregnant women was approximately 20% lower in mm carriers of a SNP in FADS1, other studies reported more modest negative associations of plasma or RBC DHA with the mm allele of an FADS or ELOVL SNP [57,60,62-64], and others reported no association [56,58,59,61,65]. In contrast, current studies show plasma and RBC ARA are consistently lower in mm carriers of an FADS SNP [57-64,66], with one Italian study reporting a difference of 30% in plasma ARA between mm and MM carriers [58].

As the effect of a SNP altering DHA synthesis may only be observed in individuals not consuming a pre-formed DHA source, the discrepancy between studies on the association of genetic variability and biochemical measures of circulating DHA could be explained by differences in dietary DHA intake among and within populations. However, no study to date has reported the association of a SNP in FADS on biochemical measures of DHA for individuals who consume no DHA. Although it is clear that SNPs in some FADS and ELOVL genes are associated with altered ω-3 and ω-6 fatty acid metabolism, further study is required to understand if genetic variability may place some individuals at risk for low circulating levels of DHA, as well as the implications for specific tissues. Understanding of the roles of DHA in the brain,

retina and certain other tissues in humans is limited by an incomplete knowledge of the dietary and genetic variables that may contribute to low circulating levels of DHA. However, because DHA is present in some animal tissue lipids, particularly fish, a dietary intake of DHA is expected to overcome any insufficiencies of ALA, a high LA/ALA ratio or genetic variables that limit ALA desaturation and elongation. Thus a major question is whether or not the current food supply and diet limits the synthesis and/or circulating levels of DHA.

1.2.2.2 Food Sources and Intake of ω-3 and ω-6 Fatty Acids

Both ALA and LA are synthesized by the activity of Δ-12 desaturase and Δ-15 desaturase, respectively, which confer plants, but not mammals, the ability to form double bonds at the Δ^{12} or Δ^{15} positions, respectively, of an 18 carbon chain fatty acid [67,68]. In plants, ALA and LA are primarily present in cell membranes, but the amount may vary by species, with the quantity of ALA and LA in leaves being low. For example, 30 g (250 mL) of raw spinach contains 26.7 and 4.2 mg of ALA and LA, respectively, with 122 and 26.7 mg in 30 g of raw purslane [69]. In contrast, much higher amounts by weight of ALA and LA are present in plant seeds, with 30 g of peanuts containing 1 mg ALA but 4.68 g of LA and 30 g of walnuts containing 11.4 g and 2.72 g of ALA and LA, respectively [70]. ALA and LA are transferred up the food chain from plants to the tissue lipids of animals that consume them, thus ALA and LA are also present in meats, but in amounts that vary by species and their diet. However, the amount present in meats is low (i.e. < 1 g ALA + LA / 100 g) [70]. Thus, the limited quantity of ALA and LA in natural food sources suggests that historical diets (i.e. >150 years ago) were likely lower in these fatty acids than current diets.

The processes enabling the commercial refinement of vegetable seed oils were introduced in the late 1800's, leading to a dramatic increase in the availability of ALA and LA in the modern food supply of 'Western' countries [71,72]. One estimate suggests that there has been a 2051% increase in estimated annual per capita consumption of all oils from 1909 to 1999, largely explained by a rapid increase in soybean oil consumption beginning around the 1950's in the US [72]. The ALA and LA content of vegetable oils varies, with the fatty acid compositions of common vegetable oils shown in Table 1.1. Briefly, rich sources of ALA include flax, canola and soybean oils, and rich sources of LA include soybean, corn, and safflower oils. By 1999,

soybean oil had become the primary source of both ALA and LA in the US food supply, with the availability of ALA increasing from 0.35 to 0.72 % energy and of LA from 2.23 to 7.21 % energy from 1909 to 1999 [72]. In addition, the LA/ALA ratio increased from 6.46 to 10.0 [72]. More current estimates of LA and ALA availability in the food supply are not available, however, in 2010 estimated per capita consumption of all salad and cooking oils was 24.4 kg/person/y, a 66% increase from 1999 [73]. Data on the availability of ALA and LA in Canada is not currently available, however, the current Canadian food supply likely resembles that of the US with respect to the use of vegetable oils. In contrast to the increased ALA and LA availability, the availability of their products, EPA, DHA, and ARA, has been reported to have decreased by 60, 30, and 20 %, respectively, from 1909 to 1999 [72].

Unlike plants, mammals, including humans, express Δ-6 and Δ-5 desaturase enzymes enabling the further metabolism of ALA and LA into long chain metabolites. However, as described in section 1.2.2.1, increased availability of ALA and LA may interfere with DHA synthesis, suggesting that an exogenous source may be important in some individuals or populations. Dietary sources of ω-3 and ω-6 fatty acids are shown in Table 1.2. Fish and other aquatic animals provide the richest dietary source of both EPA and DHA. Fatty fish, including salmon and herring, provide the highest amount of EPA and DHA, with a 150 g serving of wild salmon containing approximately 2100 mg EPA and DHA, compared to 136 mg and 91 mg in 150 g of tilapia and shrimp, respectively. Although salmon are carnivorous, current aquaculture practices feed foods containing vegetable oils and grains to farmed salmon, resulting in an altered fatty acid composition of the fish [74,75], as shown in Table 1.2. The relative amount of EPA and DHA is higher in wild salmon, but farmed salmon can contain higher absolute amounts, providing almost 3000 mg EPA + DHA per 150 g serving [70]. The higher EPA + DHA content in the farmed salmon is due to a higher amount of EPA in farmed fish, which may be explained by the addition of EPA-rich fish oil to their diets [74,76]. It should also be noted that the total lipid content of farmed salmon is two-fold higher than their wild counterparts [70,74]. In contrast, 150 g of farmed salmon provides only 138 mg of ARA compared to 400 mg in the same portion of wild salmon.

Small amounts of DHA can also be found in eggs and poultry, though these amounts also depend on the hens' diets [77-79]. For example, one large conventional egg contains

approximately 60 mg of DHA compared to more than 100 mg in an egg from a hen fed a diet enriched in ALA [79]. With increased awareness of the biological roles of DHA, there has also been an increase in the availability of foods fortified with DHA, including yogourt, margarine, and orange juice. However, the amounts they contain are relatively low (e.g. < 50 mg/serving), and the extent that these foods provide a source of DHA to the population is unknown. Although these dietary sources provide low sources of DHA compared to fatty fish, they may be important for individuals with low endogenous DHA synthesis who do not consume fish. A dietary source of ARA is also provided by fish, eggs, poultry, and dairy products, and in contrast to EPA and DHA, ARA is found in pork and ruminant meats [70].

In summary, dramatic changes in the food supply over the last 100 years, at least in the US and Canada, have led to an increased availability of the ω-3 and ω-6 fatty acid precursors and a lower availability of pre-formed products. The prevalence of vegetable oils high in LA in the food supply has led to an increased ratio of LA/ALA, but the implications on ω-3 fatty acid metabolism are not yet known. However, the uneven distributions of EPA and DHA in the food supply in addition to their decreased availability may place some individuals or populations at risk for inadequate circulating levels of ω-3 fatty acids.

Table 1.1 Table of common vegetable oil fatty acid compositions with human and cow milk reference.

	Vegetable Oil[1]							Milk	
	Canola	Soybean	Olive	Corn	Coconut	Flax	Safflower	Human[2]	Cow[1,3]
Saturates									
16:0	4.19	10.5	11.3	10.6	8.20	5.11	4.29	19.4	0.91
18:0	2.31	4.44	1.95	1.85	2.80	3.37	1.92	7.20	0.41
Monounsaturates									
16:1	0.28	0.00	1.26	0.11	0.00	0.06	0.00	0.30	0.08
18:1	59.8	22.6	71.3	27.3	5.80	18.3	14.4	33.9	0.70
20:1	1.08	0.23	0.31	0.13	0.00	0.00	0.00	0.40	0.02
Polyunsaturates									
18:2ω-6	18.8	51.0	9.76	53.2	1.80	14.3	74.6	12.1	0.08
18:3ω-3	7.64	6.79	0.76	1.16	0.00	53.4	0.00	0.10	0.02
20:4ω-6								0.40	0.00
20:5ω-3								0.10	0.00
22:6ω-3								0.20	0.00

All values are g/100g portion, with the exception of human milk, which is % fatty acids.
[1] All oils and cow's milk from USDA database.
[2] Human milk data is from Innis, 1999 [80].
[3] Milk Fat is 3.25 % (whole milk).

Table 1.2 Content of ω-3 and ω-6 fatty acids in foods.

	ω-3 Fatty Acids			ω-6 Fatty Acids		Total Fat
Foods[1]	ALA	EPA	DHA	LA	ARA	
Atlantic Salmon, wild	0.30	0.32	1.12	0.17	0.27	6.34
Atlantic Salmon, farmed	0.17	0.86	1.10	0.90	0.09	13.4
Coho Salmon, wild	0.16	0.43	0.66	0.21	0.13	5.93
Tilapia, wild	0.03	0.01	0.09	0.16	0.02	1.70
Halibut, wild	0.01	0.07	0.13	0.02	0.01	1.33
Tuna, canned	0.00	0.03	0.20	0.01	0.02	0.96
Anchovy, wild	0.00	0.54	0.91	0.10	0.01	4.84
Shrimp, mixed species	0.01	0.07	0.07	0.09	0.03	1.01
Fish Sticks	0.24	0.05	0.09	7.29	0.01	16.2
Eggs, conventional	0.03	0.00	0.04	1.15	0.14	9.94
Eggs, omega-3[2]	0.18	0.01	0.22	1.01	0.18	7.70
Milk, 3.25%MF	0.02	0.00	0.00	0.08	0.00	3.25
Cheese, cheddar	0.12	0.01	0.00	0.78	0.05	33.8
Chicken, breast	0.02	0.01	0.03	0.55	0.08	3.08
Beef, flank steak	0.06	0.00	0.00	0.15	0.06	8.29
Pork, leg, loin, shoulder	0.02	0.00	0.00	0.44	0.05	4.86

Values are g/100 g edible portion.
[1] All data is from the USDA nutrient database [70].
[2] Omega-3 egg is from Nain S, 2012 [79].

Dietary sources and intakes of ω-3 and ω-6 fatty acids of different populations and groups are typically assessed using one or more of several approaches. These include 24 hr food intake recalls, weighed food records, duplicate portions, daily food records for typically 3 to 7 days, and food frequency questionnaires (FFQ). The strengths and limitations of estimating food and nutrient intakes in free-living individuals are well-known, with the strength and limitation of each approach influenced by multiple factors, including the food or nutrient of interest, population, time and demands of the subject, and available resources [81-83]. Accurate dietary intake information may be particularly challenging for individual foods and nutrients with a high day-to-day variation. Among these, foods like fish or other seafood, which are sources of DHA, are problematic since fish or other seafood is not typically consumed daily in many populations, including Canada. Fish consumption varies among individuals, groups, and countries. For example, average fish intake among Canadian Inuit, Japanese, and in the US was reported to be 917g/wk, 588 g/wk, and 82.8g/wk, respectively [84-86]. Canadian food disappearance data suggest fish consumption in 2009 was 150 g/wk [87]. However, this is an average of food disappearance from the food supply, which can include individuals with very high and very low fish consumption as well as food wastage.

FFQs and weighed dietary records have been used for assessment of DHA intake by many groups [82,88-94]. FFQs may be done by interview or self administered, assess intake over weeks, months or years, and may quantify intakes by gathering information on portion sizes, or have participants indicate if they consumed small, medium or large portions. Some FFQs may include what is considered all individual foods and beverages, while others include food sources of the nutrient of interest, or groups in which specific food types are combined (e.g. cereals or oils) together. Lack of consensus among studies for assessment of foods and sources of ω-3 fatty acids and DHA add complexity to comparing intakes across and within different populations and groups [82,88].

In addition to limitations in the assessment of DHA intake from an individual or group, the accurate estimation of nutrient intake is further limited by the available food composition databases. Food composition databases, including the Canadian Nutrient File (CNF) and the USDA National Agricultural Library (USDA NAL) contain values for nutrients in the national food supply that are based on the average composition of similar foods (i.e. similar cuts of meat,

milk, muffins, etc.). The use of averages in nutrient databases may have greater implications for some foods than others. For example, the fatty acid composition of animal products may vary by factors including the animals diet, age, and breed or variety [75,77-79,95-98]. More relevant, in vegetable oil containing foods, the ALA and LA content of the food reflects the type of oil used, which may depend on availability and costs; for example, the oils contained in current products available in Canada may be listed in the ingredient list as 'soy/canola.' Thus, the fatty acid composition may be variable even within the same product, requiring laboratory analysis of the specific food eaten by the consumer to determine an accurate fatty acid composition, which is not always practical.

Many studies present intakes of total ω-3 fatty acids, as well as individual ALA, DHA and EPA as means and standard deviations (SD) or standard errors (SE), although several studies including our own, have shown highly skewed intakes of DHA [90,91,94,99-103]. Population data from the 2004 Canadian Community Health Survey (CCHS) showed median (5^{th}-95^{th} %) intakes of ALA and LA of 1.48 (0.77-2.78) g/d and 8.8 (5.1-14.4) g/d, respectively, for females 19-30 y [104]. The contribution of energy from ALA and LA in this group of women was 0.69 (0.46-1.08) % energy and 4.1 (3.1-5.5) % energy, respectively [104]. In the US, the 2009-2010 National Health and Nutrition Examination Survey (NHANES) was reported to show a mean intake of ALA and LA of 1.31-1.39 g/d and 13.4-13.9 g/d, respectively, for women 20-49 y [100]. Information on pregnant and lactating women were not included in the CCHS or NHANES reports, but other studies have reported intakes of ALA from 1.3 to 1.7 g/d and 9.3 to 16.8 g/d for LA for pregnant women in Canada, US, Belgium, and Norway [90-92,101,105]. ALA intakes in Canadian children 4-8 y were a median (5^{th}-95^{th} %) of 1.23 (0.69-2.2) g/d and for LA were 8.4 (5.1-13.5) g/d, with a median (5^{th}-95^{th} %) of 0.58 (0.43-0.81) % energy and 3.9 (2.8-5.4) % energy from ALA and LA, respectively [104]. Results from smaller studies of Canadian children have found similar results, with means of ALA and LA ranging from 0.7-1.7 g/d and 7.4-8.8 g/d, respectively, and LA/ALA ratios of 6.1-10 [94,106,107]. For US children 1-5 y from the NHANES report, mean (IQR) intakes of ALA and LA were 0.86 (0.66-1.02) g/d and 8.47 (6.46-10.1) g/d, respectively, with an LA/ALA ratio of 10.4 (8.28-12.1) [99].

Population DHA intake data is not currently available for Canadians. In the US, the 2009-2010 NHANES survey reported mean DHA intakes of 50-60 mg/d for females 20-39 y

[100]. DHA intakes for pregnant women assessed in studies in several countries have been reported including Belgium at 300 (190) mg/d and Japan at 290 (170) mg/d [mean (SD)], with India at 11.2 (4.9-19.5) mg/d, and Brazil at 26 (0-30) mg/d [median (IQR)] [92,93,108,109]. Two studies have published mean (SD) DHA intakes of pregnant women in Canada, reporting 160 (246) mg/d in Vancouver and 82 (115) mg/d in Guelph, Canada [90,101].

Very few studies have reported DHA intakes of young children, but it is clear that intakes are variable, and many are skewed with a greater proportion of children with DHA intakes on the lower end of the distribution, at least in Canada and the US [94,99,106,107,110]. Not unexpectedly, lower mean DHA intakes in children than those in adults have been reported using one or up to three days of food intakes [99,106,107,110]. A study by our group used an FFQ to collect information to assess DHA intakes in children 18-60 mo, with the mean (SD) DHA intake of 96 (146) mg/d (n = 84) showing the highly positive skewed intake of DHA very clearly [94]. Similarly, a small study of 41 Canadian children 4-8 y in Guelph, Ontario, used a 3-day duplicate portion method and reported a mean (SD) DHA intake of 54.1 (72.7) mg/d, median (range) of 21.9 (1.0-281) mg/d [106]. A mean (SD) intake of DHA of 37 (63) mg/d from a 3-day diet record was also reported for children 4-7 y (n = 91) in Alberta [107]. Again notable, the latter study showed 74% of children had DHA intakes ≤ 30 mg/d, thus showing high positive skewing to low DHA intake. The national 2007 Children's Survey in Australia reported a mean (SD) DHA intake of 35.9 (92.7) mg/d, and median (IQR) intake of 5.1 (0.9-26.5) mg/d for children 4-8 y (n = 1216), also illustrating that at least 75% of the children have intakes well below the group mean [110]. The mean (SD) DHA intake reported from the US NHANES, which used a single 24 h recall, for children 4-5 y was 21 (80) mg/d [99].

In summary, the intake of ω-3 and ω-6 fatty acids is variable within and among different populations. The dietary methodologies used to estimate fatty acid intake within a population also differ, requiring not only knowledge of the dietary habits of the target population but also the rapidly changing food trends, product availability, and the fats and oils used. Although DHA intakes appear to be highly skewed, the majority of studies with estimates of DHA intakes report means and SD or SE, which may not reflect the intakes of the majority of the group, at least in those populations with a current 'Western' dietary pattern. The median intakes of ALA and LA appear to be similar in Canada and the US, as well as among children and adults. However,

based on the limited data available, children have lower DHA intakes than pregnant and non-pregnant adults. Whether or not individuals with the lowest DHA intakes also have high intakes of LA sufficient to compromise DHA synthesis is unknown. The possibility that DHA insufficiency may limit brain development and function is discussed in section 1.2.3.4.

1.2.2.3 Roles of ω-3 and ω-6 Fatty Acids

Early studies on the essentiality of the ω-6 fatty acids showed that dietary deficiency in rodents led to reduced growth, scaly skin and tail necrosis [111], with deficiency signs prevented when either LA or smaller amounts of ARA were fed [29,112,113]. Turpeinen (1938) showed that 33 mg/d ARA could restore weight gain to the same effect as 100 or 200 mg/d LA in ω-6 deficient rats [112]. This was confirmed by Hume *et al* (1940), who additionally reported that 14 mg/d of LA or ARA were equally effective in curing the skin lesions [112,113]. Later work involving infants fed skim milk and patients supported with fat-free parenteral (intravenous) formulas found poor outcomes, particularly a skin rash now well-known as indicative of ω-6 fatty acid deficiency which was prevented by giving LA [114-116]. Burr *et al* (1932) concluded that ALA was also an essential fatty acid with the observation of improved rat growth by addition of methyl linolenate to the fat deficient diets [117]. However, in a subsequent study, Hume *et al* (1938) reported that ALA was not as effective as LA at restoring growth or curing skin lesions in fat deficient rats [118]. It was several decades later before the importance of the ω-3 fatty acids as an essential nutrient was appreciated [119].

In the 1970s, work on ω-3 fatty acids in animals showed that rodents with diets lacking ALA or other ω-3 fatty acids had altered behaviour and learning [9]. Later work in monkeys fed fat as vegetable oil low in ALA extended this to the retina and visual function [10,120,121]. In humans, the potential importance of ω-3 fatty acids for neurologic functioning was first reported in 1982 in case of a 6 year old girl receiving parentral nutrition containing safflower oil for five months [43]. The symptoms, including numbness, weakness, paresthesia, and blurred vision, were alleviated within 12 weeks when the lipid source was changed to soybean oil [43]. Thus, the hypothesis that ALA is an essential nutrient made by Burr *et al* in 1932 was correct, and studies beginning in the 1970s and still proceeding, have made major advances into the complex and diverse roles of ω-3 fatty acids in tissues, including their incorporation in membrane PL.

In 1899, Overton proposed the existence of a lipid barrier between the eukaryotic cell cytoplasm and its environment, and in 1925 it was further extended by Gorter and Grendel that erythrocyte cell membranes were composed of a lipid bilayer [122,123]. It was not until the introduction of the electron microscope in the late 1950s that the complexity of the cellular membrane began to be appreciated, leading to the 1972 fluid mosaic membrane model of the cell proposed by Singer and Nicholson [124]. Around twenty years later, the understanding of cellular membranes beyond simple barriers began to expand, including the roles of membrane proteins and lipids in transport of components into and out of cells, and cell compartments and cellular functions [122]. It is now known that cellular membranes are composed of diverse and different PL and sphingolipids, with the different PL polar head groups and their fatty acids giving potential rise to numerous lipid species, but showing a high degree of fatty acid specificity in different membrane PL [125-128]. A simplified table of the fatty acid composition of brain, heart, skeletal muscle, and RBC phosphatidylethanolamine (PE), as an example, is in Table 1.3.

In addition to their structural role in cell membranes, the functional relevance of the ω-3 and ω-6 fatty acids has extended to include roles in cell signaling [30,31], as precursors for lipid biomediators [129,130], and the regulation of gene expression [131,132]. However, the specific activities of the roles are dependent on the differential functions of each fatty acid as well as the cells in which they are contained. For example, EPA and ARA are well-known precursors for synthesis of eicosanoids, with one of the best-known roles of eicosanoids derived from EPA and ARA involved in inflammatory responses [133,134]. In addition, EPA and DHA can be converted into the E and D series resolvins, respectively, and DHA can be converted into docosanoids [135-137]. The eicosanoids, docosanoids and resolvins are clearly involved in inflammation, but much remains to be learnt on their tissue and immune cell dependent actions. Detailed reviews of the various roles of the ω-3 and ω-6 fatty acids are available [30,138-140]. Current information on the specific roles of DHA in the CNS is reviewed in section 1.2.3.2.

Table 1.3 Phosphatidylethanolamine fatty acids of different tissues.

	Human[1]				Rat[2]		
	Brain	Heart	Skeletal Muscle	RBC	Brain	Heart	Liver
Fatty acid	*g/100 g fatty acid*						
18:3ω-3	0.5	0.8	n/a	0.1	n/a	n/a	n/a
20:5ω-3	n/a	0.5	n/a	0.3	n/a	n/a	n/a
22:5ω-3	n/a	1.7	<0.2	3.3	0.2	3.3	0.4
22:6ω-3	28.6	6.1	4.0	6.7	20.4	17.5	4.3
18:2ω-6	0.5	2.5	13.2	6.2	0.3	5.0	6.7
20:4ω-6	13.2	30.6	24.9	24.5	14.7	18.5	30.3
22:4ω-6	8.3	0.7	n/a	n/a	n/a	n/a	2.9
16:0	5.9	5.4	1.8	16.7	6.6	15.0	13.3
18:0	30.4	30.1	17.6	8.2	23.0	30.2	23.4
18:1	8.7	3.0	5.5	15.2	8.3	7.2	7.1

[1] Brain from Svennerholm, 1968 [125], cerebral gray matter, 26 y female; heart from Rocquelin, 1985 [141], mean, n = 19, 14-75 y; skeletal muscle from Clore, 1998 [142], vastus lateralis, mean, n = 27, 19-43 y; RBC from Jakobik, 2009 [143], mean, n = 36, young adult.
[2] Brain from Letondor, 2014 [144], frontal cortex, adult controls; Heart from Benediktsdottir, 1988 [145], adult controls; Liver from Burger, 2007 [146], plasma membrane, adult controls.

1.2.2.4 Assessing ω-3 Fatty Acid Status and Potential Deficiency

Direct assessment of brain and retinal lipids and fatty acids in living humans to enable studies of the impact of diet is not ethically possible with current tools. Similarly, measures of the developing embryo and fetal brain to address the impact of maternal diet and placental transfer may also be unethical. During pregnancy, direct access to fetal blood is generally limited to collection of cord blood at birth, limiting assessment of fatty acid status throughout gestation. In infants and young children, control of food intake and blood sample collection to specific times, such as fasting, is also generally not ethically acceptable, or practical. Since medium chain fatty acids, and all monounsaturated, ω-3 and ω-6 fatty acids are known to be efficiently absorbed (>80%), even in very young infants [147], measures of plasma lipid total fatty acids are readily altered by the dietary fatty acid composition, and may not reflect what is

taken up and used in specific tissue PL. In human plasma, the major PL are phosphatidylcholine (PC) followed by SPH, with smaller amounts of lyso-phosphatidylcholine (LPC), PE, and phosphatidylserine (PS) [148]. Thus, the PL composition of plasma differs from that of the brain, which contains large amounts of PE and relatively low levels of SPH, limiting its use as surrogate marker for brain DHA [149].

The RBC fatty acids offer several advantages over plasma total or PL fatty acids. Erythrocytes use glucose as a major energy substrate, do not synthesize fatty acids, or oxidize them as an energy source [150,151]. The RBC bilayer has PE and PS, which are enriched with DHA, present mainly on the inner membrane, with PC and SPH, which are low in DHA, primarily on the outer cell membrane [152,153]. Although the lifespan of RBC is approximately 120 days, changes in dietary ω-3 fatty acid intake, including DHA, does result in altered RBC ω-3 fatty acids in as little as three days [154]. Katan MB *et al* (1997) reported that RBC EPA responded more rapidly to supplemental EPA + DHA than RBC DHA, with a doubling of RBC EPA after three days, but the maximum increase of 0.7 g/100g total fatty acids (TFA) in RBC DHA was reached at 180 days in the group taking 9 g/d of fish oil [154]. Another study reported a 300% and 42% increase in RBC EPA and DHA, respectively, after eight weeks of fish oil supplementation (1296 mg/d EPA + 864 mg/d DHA) [155]. In addition, this latter study supplemented a separate group with flaxseed oil (3510 mg/d ALA + 900 mg/d LA) and reported a 33% increase in RBC EPA after eight weeks, but no significant increase in RBC DHA [155]. However, in a recent study by Klingler M *et al* (2013), 510 mg/d DHA for 29 days led to half-maximal glycerophospholipid RBC DHA levels after six days, with the maximal RBC DHA a mean of 1.18 mol % over the baseline values [156]. Thus, recent changes due to diet or DHA supplement on the RBC DHA are not significant, consistent with definitive cell membrane PL and fatty acid regulation of turnover and replacement to meet needs.

In 1959, Horwitt *et al* reported that the lipid composition of the erythrocyte and brain were both altered by dietary fatty acids [157], with subsequent studies showing higher DHA in the RBC and brain of rats and chicks fed cod liver oil compared to other DHA-free oils [158,159]. However, differences in fatty acid composition between RBC and brain were also noted, for example, brain lipids contained very little LA compared to RBC, 1.3 % vs. 13.3 %TFA and 1.1 % vs. 25.8 %TFA in brain and RBC lipids in the rats and chicks, respectively

[158,159]. Notably, in chicks fed 1.2 %TFA LA but no ALA, the addition of 7% cod liver oil led to higher brain DHA and lower 20:4ω-6, 22:4ω-6 and 22:5ω-6. The RBC DHA was also higher in the cod liver oil group, but only RBC 20:4ω-6 was lower with no difference in RBC 22:4ω-6 and 22:5ω-6 [158]. High levels of brain 22:5ω-6 and sometimes 22:4ω-6 or ARA which accompany low brain DHA in response to low dietary ω-3 fatty acids has been reported in several species of animal [7-13,160,161].

Data for the human infant brain, which is limited and based on autopsy data, show that infants fed formula with low ALA, high LA, and no DHA have lower brain DHA, with higher brain ARA, 22:4ω-6, and 22:5ω-6 than infants fed human milk which contains DHA [162,163]. Farquharson *et al* (1995) reported cerebral cortex PE DHA had a mean (SD) of 17.7 (1.3), 13.4 (1.2) and 11.6 (1.6) %TFA for infants fed human milk or formula with 1.5% or 0.4% ALA (P = 0.001), respectively. Cerebral cortex PE 22:4ω-6 and 22:5ω-6 were 12.0 (0.8) % and 3.2 (0.6) %TFA, respectively, for human milk-fed infants compared to 12.6 (1.5) % and 4.8 (0.7) % and 14.3 (1.0) % and 7.0 (1.9) %TFA in infants fed a high or low ALA formula (P = 0.01), respectively [162]. Notably, the sum of 22 C fatty acids among infants fed the different diets were similar, 32.9%, 30.8%, and 32.9 %TFA for infants fed human milk, high ALA or low ALA formula, respectively, suggesting that 22:4ω-6 and 22:5ω-6 replace brain DHA when ω-3 fatty acids are low.

Thus, the data for both humans and animals showing increased CNS 22:4ω-6 and 22:5ω-6 occurs with loss of brain DHA, which is consistent with the ability to desaturate and elongate from the 18 carbon fatty acids, specifically ω-6 fatty acids. Also notable, early studies on the brain in essential fatty acid deficiency showed an increase in 20:3ω-9 and also 22:3ω-9 occurred to replace ARA and DHA [164-166]. These observations are also consistent with desaturation and elongation to maintain 20 and 22 carbon polyunsaturated fatty acids in the brain, with this dependent on the diet ω-3 and ω-6 fatty acids, not inability for desaturation. The site of 22:4ω-6 and of greater relevance, 22:5ω-6 synthesis, and if synthesized for example in the liver and/or intestine, or brain cells, and pathways for transfer to the CNS are as yet unknown. Thus, while both brain and RBC DHA have been shown to be altered by ω-3 fatty acids in the diet, it appears that the brain, but not RBC, readily incorporate 22:5ω-6 and increases 22:4ω-6 when DHA is reduced, with elevations of 22 carbon ω-6 fatty acids potentially indicative of DHA deficiency.

However, the extent to which differences in RBC DHA, 22:5ω-6 and/or 22:4ω-6 reflect differences in neural tissue remains unclear.

The use of RBC DHA as a biochemical marker of neural DHA has been investigated in several animal models, including rats, pigs and non-human primates, with relationships varying from weak to strong (r^2 = 0.18-0.91) [51,144,167-170]. In addition to species, methodological differences among the studies, including neural tissue assessed and fatty acid composition of the diet may explain the variations in correlations. For example, in rats fed diets with and without DHA, the RBC DHA was related to DHA in the hippocampus (r^2 = 0.37, $P < 0.01$) and pre-frontal cortex (r^2 = 0.44, $P < 0.001$), but not the striatum (r^2 = 0.12, $P > 0.05$) [144]. However, scatter plots of the data show clear clustering of the two groups of animals by diet, but a high degree of overlap [144]. Similarly, other researchers have also grouped results for animals fed with a diet with or without DHA together reporting moderate to strong relationships between DHA in RBC and whole brain (r^2 = 0.86) and retina (r^2 = 0.91) of baboons [168], and in the forebrain (r^2 = 0.79) and cerebellum (r^2 = 0.82) of rats [167]. However, in two studies animals were fed with a constant level of LA and varying levels of ALA, but no DHA, and a weak relationship between pig RBC and cerebral hemisphere DHA (r^2 = 0.18) was reported [51], with a non-significant relationship between rat RBC and brain PL DHA (r^2 = 0.42, $P > 0.05$) [170]. Only one study reported the relationship of RBC and brain 22:4ω-6 and 22:5ω-6 in rats fed varying levels of DHA and ARA, reporting a strong positive association of RBC 22:4ω-6 with 22:4ω-6 in the forebrain (r^2 = 80) and cerebellum (r^2 = 0.86), and RBC 22:5ω-6 with 22:5ω-6 in the forebrain (r^2 = 0.84) and cerebellum (r^2 = 0.85) [167]. Thus, it appears that increased RBC DHA may predict increased DHA in some neural tissues when DHA intake is high compared to very low, but the usefulness of RBC DHA as a biomarker of variation in brain DHA may be limited across a range of dietary ω-3 fatty acid intakes.

The relationship of RBC and brain DHA has been assessed in post-mortem tissue of infants, adolescents, and adults, with results suggesting that RBC DHA may not reflect the level of DHA in the brain of humans following their usual diet [163,171]. Makrides *et al* (1994) compared RBC, brain (frontal cortex) and retina DHA for infants who had been fed human milk (contains DHA) (n = 11) or formula (no DHA) (n = 16). Human milk-fed infants had higher mean ± SD DHA in RBC (4.3 ± 0.9 and 3.0 ± 0.7, $P < 0.05$) and prefrontal cortex (8.5 ± 1.2 and

7.5 ± 1.2, $P < 0.05$) than formula-fed infants, with no difference in retina DHA [163]. However, the relationship between RBC and frontal cortex among all infants was weak ($r^2 = 0.11$) [163]. In a study of individuals 2-88 y (n = 58), Carver *et al.* (2001) reported a significant relationship between DHA in RBC and the frontal cerebral cortex of individuals > 18 y, but not those ≤ 18 y [171]. However, correlation coefficients were not reported and interpretation of the regression equation [brain DHA = 3.419 – 0.019(RBC DHA), $P < 0.05$, n = 39] is not suggestive of a strong relationship [171].

In summary, studies have shown that both circulating and brain levels of DHA increase in response to DHA intake, but the response to ALA intake is less clear. Although a positive relationship between RBC DHA and DHA in some neural tissues has been shown, the ability of the RBC to predict DHA in neural tissues may be limited in individuals with variable ω-3 fatty acid intakes, restricting its use to comparisons of individuals with the lowest and highest RBC DHA within a population. Thus, caution should be followed when extrapolating RBC DHA to infer neural DHA in populations with variable ω-3 fatty acid intakes.

1.2.3 The Central Nervous System (CNS) and the ω-3 Docosahexaenoic Acid (DHA)

1.2.3.1 CNS Development

The human CNS begins to develop by the third week of gestation, with the primitive structure of the brain complete by the end of the eighth week of gestation [172]. The development of the brain follows specific timelines that have been summarized into six interrelated processes; these are neurolation, neuronal proliferation, neural migration, apoptosis, synaptogenesis, and myelination [20]. Detailed reviews of brain development are available [20,21,173], with a brief summary here to provide background on the potential implication of dietary ω-3 fatty acid, or other essential nutrient deficiency, and implications of timing and severity of deficiency.

1.2.3.1.1 Prenatal CNS Development

Following differentiation of neural stem cells around 13 days post-conception, the first stages of brain development begin as early as the third week post-conception with formation of neural folds by the neuroectoderm [172]. Fusion of the neural tube occurs in the fourth week,

then neuroblasts and glioblasts differentiate into specific neuronal cell types and glial cells [20]. Neurons proliferate at rapid rates of up to 250,000 neurons/min during the prenatal period, although up to 50% of brain neurons synthesized during early development will die by adolescence, due to naturally occurring neuronal death by apoptosis [20]. Immature neurons begin to migrate along radial glia to their final positions, with neuronal migration peaking between 12 - 20 wk, and largely complete by 26-29 wk gestation [20]. On completion of migration, neurons extend axons and dendrites to facilitate communication with other neurons. Notably, neuronal growth cones, located at the distal ends of the developing neurite, facilitate the elongation of axons to their final destination. Relevant here, the growth cone membrane is rich in ARA and DHA [174], and contains receptors which respond to external signals enabling its navigation through outgrowth, retraction, or a change of direction [175]. Once the neuronal growth tip has made contact with the dendritic spine of its target neuron, a connection, known as the synapse (i.e. synaptogenesis), is established between the neurons [20].

Synaptogenesis is essential to neuronal cell development and function, but timing of synaptogenesis differs across regions of the brain. The earliest temporary junctions are formed around 5 wk gestation, but a more mature period of synaptogenesis from about 18 wk gestation continues until adolescence [20,21]. Notably, the peak rate of synaptogenesis occurs from 34 wk gestation to 24 mo postnatal with formation at rates of up to 40,000 synapses/second [21]. The number of connections formed in early development and present in the young brain far exceed the number of connections in the adult brain [173]. Fewer synaptic connections in the adult brain can be explained by selective pruning of excess and weak synapses occurring with continued brain maturation and functional development past 5-7 y [173]. Rapid transfer of impulses between neurons is enabled by surrounding the axons with myelin, which are plasma membranes enriched in glycosphingolipids and cholesterol [176]. Myelination is a process more gradual in development than synaptogenesis, beginning around 28 wk gestation and continuing into adulthood. For reference, the fatty acid composition of myelin and non-myelin brain lipids are provided in Table 1.4, and show the unique differences in composition of ω-3 and ω-6 fatty acids in the same PL in myelin and non-myelin lipids.

Table 1.4 Phospholipid fatty acid concentration of the human brain gray matter and myelin.

	PE		PC		PS	
	GM	M	GM	M	GM	M
Fatty Acids	*g/100 g fatty acid*					
16:0	10.2	13.8	42.5	32.0	2.8	2.7
18:0	26.7	11.9	11.7	17.3	46.0	44.4
18:1	10.9	57.1	30.6	49.8	7.7	36.9
18:2ω-6	0.2	tr.	tr.	tr.	tr.	tr.
20:4ω-6	18.2	2.8	5.3	tr.	5.4	1.4
22:4ω-6	0.6	tr.	-	-	tr.	tr.
18:3ω-3	-	-	-	-	-	-
20:5ω-3	-	-	-	-	-	-
22:5ω-3	2.3	tr.	-	-	4.6	tr.
22:6ω-3	16.7	1.3	1.0	tr.	23.5	1.1

GM, Gray matter; M, myelin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; tr, trace
Values are g/100g fatty acids of the frontal lobes from one individual, 6 y of age, adapted from O'Brien JS *et al*, 1965 [149].

1.2.3.1.2 Postnatal CNS Development

Although the major scaffolding of the brain is developed in utero, the human infant brain is still immature at birth with approximately only 25% of adult brain weight, i.e. a mean of 370 g vs 1380 g [177]. Brain functional and morphological development in the postnatal period is remarkable, enabling development of higher order functions. As introduced, although synaptogenesis, apoptosis, and myelination begin in the prenatal period, they are highly active after birth. Notably, brain growth is rapid after birth, with brain weight reaching 70% of adult weight (~950 g) at the end of the first year of life [177], and the greatest increase in size is in the cerebellum, followed by the subcortical areas and cerebral cortex, based on magnetic imaging [178]. Synaptogenesis corresponds to an increase in brain grey matter, with brain grey matter maturing faster in some regions than others. For example, maximum synaptic density in the

visual cortex occurs between 4 and 12 mo of age, but in the prefrontal cortex maximum synaptic density occurs between 1 and 2 y of age [179]. The maximum density of brain synapses in the developing infant may be 150% of adult density, with pruning or remodeling of synapses to reduce the number largely activity dependent [20]. Removal of unused or weak synaptic connections is a key process enabling strengthening and efficiency of remaining functional connections [20]. Like synaptogenesis, the timing and extent of myelination is also region specific, beginning with axons in the sensory and motor pathways and ending in the association areas, those areas required for perception and integration activities [20]. Myelination also continues until young adulthood (~20 y) [177].

The cerebrum is the largest, most developed part of the mature human brain and is associated with higher order functioning [180]. The two cerebrum hemispheres each contain four lobes, the occipital lobe, parietal lobe, temporal lobe, and frontal lobe, with each lobe associated with a different cognitive function. Briefly, the occipital lobe is responsible for processing visual information; the parietal lobe is responsible for spatial awareness, perception and the integration of sensations; the temporal lobe is responsible for auditory stimuli, language, and information retrieval; and the frontal lobe is responsible for voluntary movement, memory formation, emotions, and decision-making [180]. The frontal lobe has three further divisions; the motor cortex, pre-motor cortex (both involved in movement), and the pre-frontal cortex, responsible for executive functioning, including planning, working memory, problem solving, inhibitory control and attention [180]. The human prefrontal cortex is the final region of the brain to mature, with a long period of development that continues up to 20-30 y of age [181].

The interactions between the neuroanatomical brain development and acquisition of specialized abilities are complex, and several theories have been proposed for which reviews have been published [182,183]. Although the debate of "nature versus nurture" with respect to child neural and behavioural development remains relevant in some fields, there is growing interest in how intrinsic and extrinsic factors work together to enable the developing brain to acquire higher order functioning [183,184]. The issue of environment, rather than genetics, needs to be considered when examining the impact of dietary variables across or within diverse groups, for example socio-economic status, education, cultural norms, and child-care [184-186]. While genetics may determine an individual's developmental potential, the interaction between

dietary and other environmental factors may influence the effect of a nutrient deficiency on neurodevelopment [184].

Like the brain, the eye begins to develop early during gestation, approximately four weeks post-conception, with the neuronal retina and retinal pigment epithelium developing from an outgrowth of the forebrain [187]. The optic nerve, which transmits information from the retina to the brain, is composed of retinal ganglion cells and is developed by eight wk gestation [187]. Myelination of the optic nerve begins *in utero* and is complete within the first few months after birth [187]. The maturation of the retina is complex, involving the development, reorganization, and migration of photoreceptors; specialized neuronal cells responsible for converting light into neural signals [188-190]. Photoreceptors include both rod and cone cells; rods are located in the peripheral segment of the retina and enable night vision, while the fovea contains a high density of cone cells and is responsible for colour vision and visual acuity [190]. Although present in the fetus by 26 wk gestation [189], photoreceptor maturation is largely postnatal, with maturity of the cones achieved by 5 y and the rods by 13 y of age [188,189].

To summarize, CNS development begins with key steps *in utero*, but an incredible amount of maturity occurs after birth. The regional differences in CNS maturation may give rise to multiple critical periods when the infant is most vulnerable to nutrient deficiencies, but the outcome and potential for reversal may differ depending on when the "deficiency" occurs. A well-known example is the neurological consequences caused by iodine deficiency during pregnancy. In a region with a high prevalence of severe iodine deficiency, supplementation beginning in the first and second trimester improved infant neurologic outcomes, but supplementation provided to the mother in the third trimester, or to infants and children after birth did not improve neurologic status (n = 984) [191]. Similarly, folate supplementation to reduce the prevalence of neural tube defects must be provided before the closure of the embryonic neural tube around 28 days post-conception [192]. Thus, the nature of CNS development, occurring both *in utero* and for years after birth, suggests that the impact of a nutrient deficiency with the potential to effect CNS development and function may depend on timing. This means that careful consideration of the assessments used, and the timing of the inadequacy or adequacy as well as the duration (i.e. constant or transient) must be considered.

1.2.3.2 ω-3 Fatty Acids, DHA, and CNS Development

As introduced (Table 1.3 and 1.4), neural membrane PL in the CNS contain high concentrations of DHA and ARA, but low amounts of other ω-3 fatty acids, including ALA and EPA, and of the ω-6 fatty acid LA. Brain growth requires a net (quantity) increase in DHA, with the brain weight at about 104 g at 25 wk gestation and 370 g at term delivery, 600 g at 6 mo and 950 g at 1 y [177,193]. The most rapid accumulation (g/100 g TFA and g/brain) of DHA coincides with the brain's growth spurt, which begins in the third trimester and continues until the second year of life [1]. From approximately 25 wk gestation until the second year of life, there is approximately a 25-fold increase in the total brain DHA, explained by the increase in brain size and an increase in the relative amount (% fatty acids) of DHA, with the increase in DHA accompanied by a decrease in ARA in the brain fatty acids [1].

Notably, brain grey matter PL contains high proportions of DHA, but the DHA is preferentially esterified to PE and PS, and low in all other lipids [149,194]. The preferential distribution of DHA into PE and PS, not PC, phosphatidylinositol (PI) or SPH, suggests that PE and PS DHA may have important CNS functions [35]. Experimental work in this field has shown that DHA has specific and critical roles in neural development, function and protection, including neurogenesis and neurite outgrowth, neurotransmitter metabolism, and protection against inflammatory insults through the synthesis of neuro-protective molecules [2-6,195-197].

Experimental studies have shown that the fatty acid composition of the rat CNS neuronal growth cone membranes is influenced by the dietary fatty acid intake during gestation [174,195]. Auestad *et al* (2000) showed that pregnant rats fed ω-3 fatty acid deficient diets had offspring with growth cone membranes lower in DHA and higher in 22:5ω-6 when compared to a diet containing ALA, but no difference in the fatty acid composition of the neuron cell body [174]. Innis *et al* (2001) extended these findings to report higher DHA and lower 22:5ω-6 in growth cone membrane PE and PS in newborn offspring of rats fed diets containing ALA or DHA compared to an ω-3 fatty acid deficient diet [195]. As introduced in section 1.2.3.1.1, the neuronal growth cone has an important role in facilitating neuronal connections with the target cells [198], but whether or not lower DHA in growth cone membrane PL may affect the neurons' ability to form the appropriate synaptic connections is unknown. Following contact of the growth cone with the specific target, the processes involved in differentiating the site into a

mature synapse are complex [199], and have been suggested to include a change in the membrane ω-3 and ω-6 fatty acid composition [175]. Neuronal growth cones contain more ARA than synaptosomes, while the synaptic membrane PL, particularly PE, are enriched in DHA [175]. The enrichment of DHA in synaptic membrane PL has been previously reported [200-203], but its specific functional relevance has yet to be elucidated. However, recent *in vitro* studies have suggested that DHA has roles in neurogenesis, neurite outgrowth, synaptogenesis, and synaptic transmission in neurons [3,5,204,205], all of which are important processes in normal brain development [20,173].

In pregnant rats, compared to offspring from dams fed an ω-3 fatty acid adequate diet (2.5 % ALA), ω-3 fatty acid deficient diets (0.03 % ALA) led to offspring with smaller cerebral hemispheres at embryonic day 19 (E19), explained by a reduced size of the areas that develop into the cerebral cortex and hippocampus [5]. Although the overall size of these areas was smaller, the regions that give rise to neurons of the cerebral cortex and hippocampus were thicker, suggesting that the ω-3 fatty acid deficient diet led to the inhibition or delay of neurogenesis [5]. However, whether a delay or inhibition of neurogenesis can be reversed by the addition of ω-3 fatty acids later on is unknown. In another study, rat E18 hippocampal neurons were obtained from pregnant rats fed an ω-3 fatty acid deficient or adequate diet (0.1 mol % or 2.6 mol % ALA), and cultured for six days with or without DHA [204]. Compared to the ω-3 fatty acid adequate neurons, the deficient neurons had shorter total neurite lengths/ neuron, more neurons with shorter neurite lengths, and fewer branches/neuron [204]. Notably, when DHA was added to the neurons from the deficient group, the total number of neurons with longer neurite lengths and branches/neuron increased to the level of the ω-3 fatty acid adequate group, suggesting that altered neurite growth caused by ω-3 fatty acid deficiency during gestation could be reversed [204]. In 2009 these findings were extended in an E18 mouse model showing that the addition of DHA, but not 22:5ω-6, to an ω-3 deficient hippocampal neuron culture led to neurite growth and also to improved synaptogenesis [205]. Together, these studies suggest that ω-3 fatty acid deficiency has the ability to alter brain development, but the functional consequences, as well as the brain's ability to overcome certain insults, remain unclear.

Of functional relevance for the developing CNS, processes including neurite outgrowth, growth cone navigation, and synaptogenesis may be regulated by neurotransmitters, including

dopamine and serotonin [206,207]. Neurotransmitter metabolism has been shown to be altered by the ω-3 fatty acid supply in animals, and these neurotransmitters include dopamine [12,195,208-210], serotonin [195,210], acetylcholine [211,212], and norepinephrine [210]. Several studies have shown that chronic ω-3 fatty acid deficiency for two generations led to a significant reduction in dopamine and dopamine receptor binding in the frontal cortex of young male rats compared to controls receiving an ω-3 fatty acid adequate diet [12,208,209,213]. In addition, the ω-3 fatty acid deficient rats also showed increased levels of dopamine metabolites and dopamine turnover compared to controls [209,213], which may be problematic as increased dopamine metabolism may lead to neurotoxicity through generation of reactive oxygen species and quinones [214]. As hypothesized by Zimmer *et al* (1998), it is possible that ω-3 fatty acid deficiency may interfere with dopamine storage, as dopamine is stored in synaptic vesicles in order to prevent its degradation [209,214]. Similarly, ω-3 fatty acid deficiency has also led to alterations in the serotonergic pathways with reports of increased serotonin receptor density in the frontal cortex [12,208], increased serotonin turnover in the hypothalamus of rats [213], and lower serotonin concentrations in the prefrontal cortex of pigs [210].

Notably, altered dopamine metabolism observed in rats born to dams receiving an ω-3 fatty acid deficient diet was reversed when ω-3 fatty acids were introduced to the diets of dams at different stages of lactation (0, 7 or 14 d), but not when introduced to the offspring at weaning (21 d) [215]. However, levels of prefrontal cortex DHA were restored to levels comparable to offspring in the control diet regardless of when ω-3 fatty acids were introduced into the diet of the offspring. Therefore, although prefrontal cortex DHA was restored to control levels by the addition of ω-3 fatty acids at weaning, by 60 days of age the effect on dopamine release had not been reversed [215]. Whether the neurochemical effect of ω-3 fatty acid deficiency would be recovered later on is not known. Thus, the role of ω-3 fatty acids in neurotransmitter metabolism is complex and not yet understood, but it is clear that ω-3 fatty acid deficiency during early development can alter neurotransmitter concentrations in the frontal cortex of animals.

An important role of DHA has also been suggested in the development of the retina, as the photoreceptor membranes are rich in DHA [216,217]. Notably, the membrane of the retina is unique to other tissues as it contains PL that may have polyunsaturated fatty acids, including DHA, acylated at both the *sn*-1 and *sn*-2 positions [216,218]. The absorption of light by

rhodopsin, the light sensitive protein in photoreceptor membranes, results in a series of conformational changes that initiate the visual signal transmitted along the optic nerve to the brain [219]. Roles of DHA in the retina include photoreceptor differentiation [220], rhodopsin activation [219,221,222], and rhodopsin regeneration [223]. The specific mechanisms of DHA in these processes are complex, and have been described elsewhere [216,219,224]. However, Rotstein *et al* (1998) showed that a supply of DHA promotes photoreceptor differentiation which begins *in utero*, suggesting that DHA deficiency during development has the potential to lead to long-term alterations in retinal function [188,189,220,225]. In addition, it has been suggested that lowered visual signal transduction efficiency in early development may influence neuronal factors including myelination of the optic nerve and synaptic efficiency [225,226]. Thus, DHA deficiency occurring during pre- and/or post-natal development may have the ability to compromise visual function.

1.2.3.3 Transfer of ω-3 and ω-6 Fatty Acids to the CNS

Due to their dietary essentiality, ω-3 and ω-6 fatty acids in human tissues, including the CNS, must originate from the diet and be transported to different organs for incorporation. As of yet, much remains to be understood on the role of plasma lipoproteins and their lipids, plasma NEFA and lysophospholipids, and other factors potentially impacting transfer, such as specific apoproteins and cerebral spinal fluid. Selectivity and mechanism of CNS fatty acid uptake, and the role of the liver and other tissues in providing ω-3 and ω-6 fatty acids to the CNS, as well as the potential role of desaturation of ALA and LA, remain important questions. In the absence of a preformed dietary or supplemental source of DHA, the liver has been suggested as the primary site of desaturation and elongation of DHA from its 18-carbon precursor for export to extra-hepatic tissues, including the CNS [37,227-229]. However, results from several early studies *in vitro* and *in vivo* have shown that some cells in the brain and the retina, including astrocytes and retinal pigment epithelium are capable of synthesizing DHA [230-235]. The presence of D6D activity has also been shown in the rat jejunum and ileum [236], and in the human CaCo-2 cell line in culture [237]. Thus, these studies have shown that the liver is not the only source of endogenously formed DHA, but the extent of extrahepatic contribution of DHA synthesized from ALA and/or EPA to CNS requirements is still not known.

The uptake of fatty acids from the circulation into the CNS is unique compared to other tissues, including the heart and skeletal muscle, due to the highly selective nature of the blood brain barrier (BBB), which separates the blood from the extracellular fluid in the CNS [238]. The BBB is composed of a monolayer of endothelial cells with tight junctions, limiting its permeability of blood components, including fatty acids [238,239]. The BBB also consists of a basement membrane embedded with pericytes and astrocyte end-feet surrounding the vessels [239]. Although the exact mechanism of how DHA crosses the BBB remains a major question, it has been suggested that it may involve passive diffusion or protein-mediated transport mechanisms [240]. Recently, Nguyen *et al* (2014) identified a transporter (Mfsd2a) enriched in the BBB endothelium, which may be responsible for the transfer of DHA to the brain [241]. The brain PL DHA of Mfsd2a knockout mice was approximately 60% lower than wild-type mice, whereas there was no difference in liver or heart DHA [241]. Notably, the reduction of DHA in the Mfsd2a knock-out mice was accompanied by a 34% increase in brain ARA, suggesting that Mfsd2a is specific to DHA and not all long-chain ω-3 and ω-6 fatty acids [241]. However, brain 22:4ω-6 and 22:5ω-6 were not reported, so it remains unknown if these fatty acids were also increased, or if Mfsd2a has a role in the transfer of all 22 C fatty acids.

Understanding of how DHA is taken up into the brain requires knowledge of the preferred source of DHA for the CNS, which remains a question. Studies beginning in the 1970s have suggested that NEFA were the preferred form of fatty acids transported to the brain, based on comparison with their incorporation into TAG or PL [242,243]. Using a rat model, Rapoport *et al* (2001) showed that following the intravenous infusion of radiolabeled albumin-bound DHA, only about 1% of the dose was incorporated into brain lipids, with approximately 90% of the tracer within the brain lipids [244]. However, the use of intravenous infusion of albumin-bound DHA may not be reflective of the various forms of DHA in circulation, particularly in the post-prandial state. In rats gavaged with a mixture of corn oil and labeled [^{14}C] DHA, smaller amounts of the radiolabel were associated with albumin compared with lipoproteins in the first few hours post-ingestion [245]. The subsequent uptake of labeled DHA by retinal outer segments occurred rapidly between two and four hr post-ingestion suggesting that lipoproteins may have been responsible for the delivery of DHA to the retina [245].

Several other studies have suggested that lipoproteins are a major source of DHA for the brain due to the presence of lipoprotein receptors in the BBB [246-249]. However, it has been shown that deletion of LDL and VLDL receptors in rodents does not alter brain levels of DHA [248,249]. Whether or not HDL has a major role in delivering DHA to the BBB is not yet known. A more recent study compared the incorporation of labeled DHA in the *sn*-2 position of PC or TAG in neonatal pigs [250]. This study reported that PC was a more efficacious source of DHA for the brain and retina, with 1.9-, 1.7-, and 2.2-fold greater dose recovered in the cerebral cortex grey matter, synaptosomes and retina, respectively, for PC than for TAG [250].

It has also been shown that DHA esterified to LPC, which is bound to albumin, was preferentially incorporated into the young rat brain compared to unesterified (free fatty acid) DHA, with the recovery of labeled LPC-DHA 10- to 12-fold higher than labeled unesterified DHA [251]. In contrast, the labeled DHA recovered from the liver and heart was lower from LPC-DHA than from unesterified DHA, with no difference in incorporation of DHA from LPC or unesterified DHA in the kidney [251]. These authors also suggest that brain uptake of LPC-DHA occurs intact without prior hydrolysis, before converting them into PC then subsequent hydrolysis [251,252]. Later, these same authors developed an in vitro blood-brain barrier model and reported that the uptake of DHA by astrocytes was higher for LPC-DHA than unesterified DHA, although this was not observed for the endothelial cells [253]. In the study by Nguyen *et al* (2014), analysis by cell culture showed that cells expressing Mfsd2a did not transport unesterified DHA and exhibited an enhanced uptake of LPC-DHA [241].

Thus, it is possible that there are multiple mechanisms for DHA transfer from circulation into the CNS. In addition, it is clear that both unesterified and esterified DHA in circulation may be incorporated into CNS lipids. Further work in this field in understanding how DHA is transferred and in what form for CNS uptake is important and will enhance understanding of detecting dietary and metabolic conditions that may contribute to low brain DHA.

1.2.3.3.1 Placental Transfer of ω-3 and ω-6 Fatty Acids

In utero, nutrients are delivered across the placenta to the developing fetus from the maternal circulation through the umbilical arteries. Detailed reviews of placental transport of fatty acids are available [254-257], and transfer of ω-3 fatty acids from the maternal diet to the

fetus will be briefly described here. Placental transfer of essential fatty acids, including ALA, and DHA may occur through multiple mechanisms, including passive diffusion and facilitated transport [256-258]. Although the mechanisms are not fully understood, the transfer of DHA from maternal to fetal circulation is variable and depends in part on the maternal diet and her plasma DHA [256-258]. Although D6D and D5D activity has been reported in the fetal liver by 17 wk gestation [259,260], studies in animals have shown that fetal DHA accretion is higher when the maternal diet contains DHA than ALA [203,261]. Variability of LA, ALA, and DHA present in the diet limits the comparison of DHA and ALA interventions on human fetal DHA status. In one study, infants born to vegetarian mothers had lower cord blood DHA compared to omnivore mothers, but the infants' DHA status was not measured [262]. Thus, whether fetal desaturation of ALA supplied from the mother is sufficient to meet fetal DHA requirements remains unclear. However, evidence of constrained CNS development related to insufficient DHA in infants or children of mothers following a vegetarian diet has not been reported.

Regardless, studies in humans have shown that supplementation of DHA during gestation increases DHA and lowers 22:5ω-6 in umbilical cord blood of infants compared to a control [105,263-267]. Whether or not umbilical cord DHA is reflective of DHA in the infant CNS remains to be investigated. It has been suggested that cord blood fatty acids may be influenced by factors other than dietary intake, including gestational age at birth, length of labour, maternal diagnosis of gestational diabetes, and labour induction, but these factors have not been fully investigated [268-270]. However, it has been shown that higher cord RBC PE DHA at birth led to higher RBC PE DHA at 6 wk of age, even among infants fed no DHA [268]. Therefore, fetal DHA must originate from ω-3 fatty acids in the maternal diet, and it appears clear that increasing maternal DHA intake leads to an increase in DHA transferred to the fetus. However, the factors affecting both the transfer and fetal accretion of DHA remain major questions. Whether or not the fetal ω-3 fatty acid supplies are insufficient to support early CNS development in some populations, or if the potential effects of inadequate fetal DHA accretion can be reversed with ω-3 fatty acids in diet after birth is also not yet known.

1.2.3.3.2 Early Infant Feeding of ω-3 and ω-6 Fatty Acids

After birth, exclusively human milk-fed infants must obtain ω-3 and ω-6 fatty acids from human milk. Human milk contains a substantial amount of lipid varying from ~2 to 6 g/dL milk, and on average, 98% of milk lipids are in the form of TAG [271-273]. Although human milk contains ALA and DHA, as well as LA and ARA, the ω-3 and ω-6 fatty acids cannot be synthesized by the mammary gland and thus must be obtained from the maternal plasma. The ω-3 and ω-6 fatty acids in milk from different women are variable and may reflect at least in part differences in the maternal diet. The lowest level of DHA in human milk was reported for women following a vegan diet when compared to non-vegan women, with a mean DHA of approximately 0.1 % and 0.4 % total milk fatty acids (TMFA) for each group, respectively [274]. Also notable, human milk levels of DHA had a mean (range) of 1.3 (0.4-3.8) %TMFA in Canadian Inuit women following traditional diets rich in marine foods [275].

Global national mean human milk DHA levels range from 0.17-0.99 %TMFA, with the lowest levels observed in Canada (excluding Inuit) and the US, the highest levels in the Philippines and Japan, and Australia, with the UK, Mexico, China and Chile in between [276]. Levels of human milk ALA and LA were both lowest in the Philippines (0.43% and 7.90 %TMFA, respectively), with the highest ALA in China (2.02 %TMFA) and the highest LA in Chile (17.8 %TMFA), [276]. Mean levels of ARA in human milk appear more stable, with the mean levels ranging from 0.37 % to 0.45 %TMFA among the previous populations [276]. In addition to variability between populations, the fatty acid composition of human milk also varies among women within the same population. A study by our group showed a range of DHA from 0.1 % to 2.6 %TMFA in a group of 103 lactating women in Vancouver, with a more narrow range of ARA (0.2 – 0.8 %TMFA) [80]. Notably, lactating women from a Tanzanian ethnic tribe (n = 20) with a very high mean (SD) fish intake of 7 (0) times per week, also showed high variability in human milk DHA at 3 mo post-partum, with a median (range) of 0.96 (0.38-1.22) %TMFA [277]. However, details of milk collection were not described in this study and methodological factors, including differences in the time of day or hind vs. foremilk, could explain the variability among this homogenous group of lactating women.

Changes in the fatty acid composition of human milk over time have been reported in Canada, Australia and the UK, with a decrease in DHA, ARA and ALA and an increase in LA

[274,278-280]. This is likely explained by changes in dietary patterns including higher intakes of vegetable oils and lower intakes of fish in some populations. Although vegetable oils do contain ALA, levels of dietary LA may be too high to support DHA synthesis if DHA is absent from the diet. In addition, studies have shown that ALA supplementation of lactating women does not increase the DHA content of human milk [278,281]. In a study by our group, supplementation of lactating women with 3 g/d ALA for two weeks led to an increase in the ALA content of human milk, but the DHA content was not different [278]. However, the dose provided was 2-fold higher than the current Canadian recommendation for lactating women, and combined with the variable intakes of LA and ALA among women, may have inhibited maternal DHA synthesis. In contrast, the same study supplemented an additional group of women with 300 mg/d DHA, which resulted in a 2.5-fold increase in mean milk DHA compared to baseline [278]. However, milk DHA levels returned to baseline within two weeks post supplementation, suggesting that milk DHA is dependent on more recent dietary intake [278]. Several other studies have also reported an increase in milk DHA following supplementation of lactating women with 200 to 1300 mg/d DHA for 2 to 12 weeks [282-285]. Human milk DHA has also been shown to be positively related to the circulating DHA of the human milk-fed infant [80,286-289]. In a study by Gibson *et al* (1997), lactating women were randomized to receive a DHA supplement ranging from 0 – 1.3 g/d DHA from day 5 to 12 wk post partum. The RBC DHA of the infants in this study (n = 52) was positively related to human milk DHA ($r^2 = 0.77$, $P < 0.001$), with an apparent plateau reached when milk DHA was 0.8 %TMFA [287].

Commercial formulas for term-born infants reflect the fatty acid composition of the oils used in the formulation, which may vary among brands, but usually include a blend of several oils including palm, soybean, coconut, safflower, and/or sunflower oil. Current major Canadian infant formulas provide ALA in a range of 0.54-1.00 g/L formula (0.66-0.93 % energy), and LA of 5.2-11 g/L formula (6.88-8.33 % energy), with an LA/ALA ratio ranging from 7.86 to 11.5. In addition, infant formula may provide varying levels of DHA and ARA, with a range of 0-115 mg/L and 0-230 mg/L formula, respectively, for infants up to 1 y.

In 1979, Sanders and Naismith observed that infants fed modified cow's milk formula (n = 12) (0.55 % energy LA, 0.24 % energy ALA; 470 kcal/100 g), from birth had significantly lower RBC PE DHA at 14 wk compared to infants who had been human milk-fed (n = 6), with a

mean (calculated SD from SE) of 3.7 (0.72) and 6.2 (1.00), respectively [290]. Notably, evidence of essential fatty acid deficiency was not detected in the formula-fed infants leading to the authors' conclusion that the young infant requirement for LA is < 1 % energy [290], which is considerably less than that provided in current infant formulas. In another early study, infants fed formula with fat derived from vegetable oil and no added DHA had lower RBC PE DHA between 4.5-6 mo compared to infants fed human milk [291]. Since then, numerous studies have reported lower DHA in the circulation and brain of infants fed formulas without DHA compared to infants fed human milk or formulas containing DHA [162,163,292-297]. While it is clear that providing DHA in the infant's diet leads to higher DHA status, the ability to desaturate DHA may be inhibited in some infants fed formulas lacking DHA and containing high levels of LA. For example, for infants fed formula with an LA/ALA ratio of 5/1, RBC DHA was approximately 17% higher than infants fed a formula with a 10/1 LA/ALA ratio [294]. Whether or not the LA and ALA content in infant formulas available in Canada place some infants at risk for inadequate DHA deserves further investigation.

In addition to human milk or infant formula, infants may also obtain ω-3 and ω-6 fatty acids from complementary foods introduced into their diet, currently recommended by Health Canada to be introduced at 6 mo of age [298]. However, recent data from the Canadian Community Health Survey reported that 43% of infants received solid foods by 5 mo, with 11% given solid foods at or before 3 mo of age [299]. The CCHS also reported that among women who initiated breastfeeding, 45% then another 13% introduced other liquids (water, juice, or infant formula) to their infants at or before 3 mo or 4-5 mo of age, respectively [299]. The implications of complimentary food introduction to the infants' ω-3 fatty acid status are not yet known. However, it is possible that partial replacement of human milk or infant formula with other liquids or solid foods such as infant cereal likely low in ω-3 fatty acids would reduce the infants' ω-3 fatty acid intake. Hoffman *et al* (2003) reported that infants receiving a formula with no DHA and an LA/ALA ratio of 9.8 led to a decrease in RBC DHA at 12 mo of age from the pre-weaning RBC DHA ~15-28 wk by almost 50% [296]. In contrast, no differences in RBC DHA between pre- and post-weaning measurements were reported in the infants fed a formula containing 0.21 g/L DHA [296]. Notably, in infants receiving a mean ± SD human milk high in DHA (1.20 ± 0.57 %TMFA), the introduction of a cod liver oil supplement by 3 mo (n = 80) had

no effect on their plasma PL DHA at 3 mo compared to infants who did not receive cod liver oil (n = 53) [289]. However, in the infants fed human milk with lower DHA (0.47 ± 0.40 %TMFA), cod liver oil supplements (n = 50) led to a 10.5% increase in plasma PL DHA than in infants who did not receive cod liver oil (n = 68) [289]. It is unknown if adding additional ω-3 fatty acids to the infant diet will increase the amount of DHA in other tissues.

Although Health Canada recommends iron-rich meat, meat alternatives and iron-fortified cereals as infants' first foods at 6 mo, very little is known about the type and amounts of foods introduced to Canadian infants [298]. Previous infant feeding guidelines recommended iron-fortified cereal between 4-6 mo [300]. The effect of weaning at 4 mo compared to 6 mo on infant DHA status is not known. Available studies have reported that most infants (70-90%) are introduced to iron-fortified cereals, as well as vegetables and fruit between 4-6 mo, with ~ 15% introduced to meat and poultry and <10% introduced to fish [301,302]. In a Vancouver study, 62% of infants had not been introduced to fish by 7-9 mo [301], and in a national Canadian study, 78% of infants had not been fed fish by 12 mo [302]. In contrast, at 6 mo 21% of infants had consumed desserts (including pudding and cookies), and 12% had been given breads/grains (including muffins and regular cereals), with an increase to 82% and 65%, respectively, by 12 mo [302]. This suggests current infant weaning practices may place some infants at risk of inadequate DHA if foods high in LA are fed without a source of DHA.

In summary, although the ω-3 fatty acid requirements after birth remain unknown, it is clear that infants receive variable amounts of ω-3 and ω-6 fatty acids through human milk or infant formula. The introduction of complimentary foods adds further complexity to understanding the ω-3 and ω-6 fatty acid intakes of infants. Although some infants receive DHA from human milk or DHA-containing infant formula, partial replacement with other liquids or other foods may reduce their DHA intake. For infants fed formulas without DHA, introduction of foods low in ω-3 and high in ω-6 fatty acids may alter the infants' endogenous DHA production. However, there is little information on the types and amounts of foods consumed by infants. Thus, it is not yet known if current infant feeding practices provide adequate ω-3 fatty acids to support the rapid brain development that occurs in the first year of life.

1.2.3.4 ω-3 Fatty Acid Deficiency and CNS Development

Much of what we currently understand about the roles of DHA in the CNS has been extended from animal studies, but the trajectory of CNS development differs among species [303]. For example, while the brain growth spurt occurs mainly in the postnatal period of humans, the non-human primate brain is more mature at birth corresponding to the brain growth spurt occurring largely *in utero,* while the rat brain spurt occurs after birth [303]. Thus, the critical periods of when the developing CNS is most vulnerable to a potential deficiency would also differ. As a result, several studies have attempted to equate CNS development among species based on morphological comparisons and timing of specific events [304-306]. Rats are commonly used to study the effect of ω-3 fatty acid deficiency on the CNS, and it has been suggested that the rat brain at postnatal day 1-10 is comparable to human fetal brain in the third trimester, with rat neurodevelopment at 1 wk of age equivalent to human neurodevelopment at birth [306]. However, this is a general estimate as specific CNS regions of rats and humans develop at different rates, and care should be taken when extrapolating from studies in rats or other species to humans [304-306]. Therefore, this section will first review studies of DHA deficiency in developing rodents, followed by non-human primates, then humans.

1.2.3.4.1 DHA Deficiency and CNS Development of Animals

As introduced in section 1.2.2.4, dietary deficiency of ω-3 fatty acids leads to decreased brain and retina DHA, accompanied by replacement with ω-6 fatty acids, particularly 22:5ω-6 and sometimes 22:4ω-6 and ARA [7-13,160,161]. In 1976, Lamptey and Walker showed that low brain DHA in rats due to an ω-3 fatty acid deficient diet beginning *in utero* led to inferior performance on a discrimination-learning task compared to controls [9]. Notably, there were no differences in growth or measures of reflex or neuromotor development between diet groups, but the ω-3 fatty acid deficient diet led to more time standing supported and less time grooming [9]. On a brightness-discrimination learning test, Yamamoto *et al* (1987, 1988) also reported inferior learning ability of ω-3 fatty acid deficient compared to control rats [307,308]. The learning tests used by the latter studies also involved the ability to detect contrast or brightness, leading to suggestion that the observed inferior learning ability was related to rat retinal function, as rats with low brain DHA had abnormal electroretinograms (electrical response to illumination)

compared to controls [45,309]. However, Yamamoto *et al* (1988) provided evidence to support the role of DHA in the brain in learning, showing that the extinction of learning, a process not requiring brightness detection, required more time in the ω-3 fatty acid deficient rats than controls [308]. Following these early reports, numerous studies have shown that ω-3 fatty acid deficient diets have led to inferior performance in tasks of learning [213,310-317], memory [213], locomotor activity [316-318], exploratory behaviour [319], anxiety [313], and olfactory-based discrimination [320], with several reviews available [28,213,321,322].

Notably, studies have shown that some effects of low brain DHA caused by inadequate ω-3 fatty acids *in utero* can be reversed with the introduction of ω-3 fatty acids at lactation or weaning in rodents [313,317,319,323]. In a study by Enslen *et al* (1991), female rats received an ω-3 fatty acid deficient diet during pregnancy and lactation, with the offspring weaned to an ω-3 fatty acid deficient or adequate diet [319]. Although brain levels of DHA, 22:4ω-6, and 22:5ω-6 were restored to control levels by 16 wk of age after the addition of ω-3 fatty acids at weaning (21 d), exploratory behaviour deficits were not reversed [319]. In contrast, rats fed an ω-3 fatty acid deficient diet for two generations with offspring switched to an ω-3 fatty acid adequate diet at weaning showed that brain DHA was only partially recovered by 10 wk, with higher learning performance at 9 and 13 wk than in the ω-3 fatty acid deficient group, and some measures not different from the controls [317]. Similarly, when ω-3 fatty acid deficient rats were switched to an ω-3 fatty acid adequate diet at 7 wk, brain DHA and learning performance was only partially recovered at 9 to 13 wk [317]. However, in ω-3 fatty acid deficient rats cross-fostered at birth to dams with ω-3 fatty acid adequate diets, both brain DHA and learning performance was restored to control levels [317]. Thus, these studies suggest that ω-3 fatty acid deficiency during rapid brain development has the potential to result in lasting deficits to function, which may or may not be recovered through repletion of brain DHA later in development.

Studies on the effect of ω-3 fatty acid deficiency on the non-human primate brain and retina by Neuringer and colleagues began in the 1980s. They showed that maternal ω-3 fatty acid deficiency during pregnancy led to reduced DHA concentrations in the CNS of the monkey's offspring compared to a diet with ALA as a source of ω-3 fatty acids [120]. These important studies provide key information on the effect of dietary ω-3 fatty acid deficiency on the developing CNS of the monkey, particularly involving the retina, including delayed visual

acuity development [10,120], altered electroretinograms [120], deficits in visual attention [121], and stereotyped behaviour [324]. Interestingly, repletion of ω-3 fatty acid deficiency *in utero* by feeding a high ALA diet after birth led to the restoration of DHA similar to control values in the cerebral cortex within 15 weeks, although the retina DHA levels remained 16% lower than the controls after 3 years [325]. Notably, the functional effect of prenatal ω-3 fatty acid deficiency on electroretinogram parameters was not restored to control values by ω-3 fatty acid repletion after birth [309], suggesting lasting effects when deficiency occurs during development.

1.2.3.4.2 DHA Deficiency and CNS Development of Humans

Studies in humans on the role of DHA in the CNS are more challenging due to the inability to access human neural tissues, the need for biomarkers of brain DHA, timing and choice of neurological development tests, and number of participants required in the group which is influenced by confounding variables. The extent to which reduced brain DHA is associated with altered CNS function and implications for reversal in humans is still poorly understood, but several studies have reported that low ω-3 fatty acid intakes in both the pre- and post-natal period are associated with CNS function [14-19,26,27]. This section will briefly review the observational data of ω-3 fatty acid intake or status with infant or child neurodevelopmental outcome, then ω-3 fatty acid intervention trials in the pre- and post-natal period.

A prospective cohort study (n = 8,916) started in 1991 in the UK, Avon Longitudinal Study of Parents and Children (ALSPAC), reported that children of mothers with low ω-3 fatty acid intakes from seafood during pregnancy were at increased risk for sub-optimal scores on verbal IQ tests at 8 y compared to children of mothers with high ω-3 fatty acid intakes [16]. More specifically, children of women consuming 1-340 g/wk seafood had an increased risk of sub-optimum scores on early development tests, including gross and fine motor skills, social skills and communication skills from 6-42 mo, behavioural outcomes at 7 y, and tests of cognition at 8 y when compared to children of mothers who consumed >340 g/wk seafood (equivalent to ~ 5 servings) [16]. An earlier report from ALSPAC described an association between maternal prenatal fish intake and infant performance on tests of language development at 15 and 18 mo [14]. Several other studies have reported associations between maternal DHA intake or blood DHA during gestation and infant and/or child CNS function, including

stereoacuity, visual recognition memory, novelty preference, cognitive development, verbal IQ, memory, novel object search task, and internalizing problem behaviour, with outcomes in infants and children from 6 mo to 9 y [15,17-19,326-329]. Notably, Daniels *et al* (2004) reported that in addition to the association of maternal prenatal fish consumption and infant cognitive development, fish intake of the infants' at 6 and 12 mo was also associated with neurodevelopment assessment scores up to 18 mo [14]. In a study of human milk-fed infants, the RBC DHA of the infants at 2 mo was related to visual acuity at 2 and 12 mo, and language development at 9 mo [330].

In older children, an association was reported between ω-3 fatty acid intake and hippocampal-dependent relational memory in children 7-9 y [26], and between cheek cell DHA and non-verbal IQ in children 8-10 y [27]. In addition, McNamara *et al* (2010) reported a positive association of baseline RBC DHA and several parameters of prefrontal cortex activation and a negative association between baseline RBC DHA and reaction time on a sustained attention test in boys 8-10 y [28]. Although the results from observational studies are highly suggestive of a role for DHA in CNS development, it is also possible that the results may be confounded by other variables associated with diet and CNS development. For example, while fish is a major source of DHA, it is also a source of selenium and iodine, both of which have been suggested to be important during early CNS development [331,332]. Moreover, a lower fish intake has been associated with lower income [333,334]. Lower than higher socioeconomic status in the US has also been associated with negative effects on CNS development, including lower brain gray matter volumes and slower growth, measured by MRI, and lower child IQ [185,186]. Whether this decreased brain volume is caused by nutritional factors, lack of an enriched environment, some other factor(s), or their interaction, is not yet known.

Numerous intervention studies have examined DHA during early development, with the majority of studies conducted during pregnancy and/or early feeding of the infant. One of the major challenges in evaluating this area of research is the lack of consistency in design among the studies, with variations including differences in the amount of DHA provided, the addition of other nutrients or fatty acids in the supplement, the duration of supplementation, outcome measurements, and ages of evaluation. Studies during pregnancy have provided supplementation of 200-2200 mg/d DHA, EPA, or ARA plus DHA, or cod liver oil, began between 12 and 25 wk

gestation, with infants evaluated for a variety of neurodevelopmental outcomes from 1 mo to 6.5 y [105,266,335-350]. In addition, one of the interventions also provided folate [349,350], while two interventions continued supplementation to 3 mo postpartum [105,346]. Notably, no DHA intervention during pregnancy with infant neurodevelopmental assessments has been reported in Canada. Only 2 interventions have been reported for the US [335,336,341], and the remaining conducted in Australia [266,337-340], Norway [105,342,343], the UK [344,345], the Netherlands [346], Bangladesh [347], and Mexico [348], with a multi-site intervention conducted in Germany, Spain, and Hungary [349,350]. As dietary intake patterns may differ among countries, the ability of a group to respond to a DHA intervention would also differ among the groups, adding further complexity to potential comparisons among studies.

The majority of studies on prenatal DHA supplementation have reported no effect of DHA on infant/child neurodevelopment [105,266,337-339,343,344,346-350]. However, positive effects have been reported for infant outcomes including motor abilities and autonomic responses at 1 wk [341], visual acuity at 4 mo [336], problem solving at 9 mo [335], hand-eye coordination at 2.5 y [340], and the mental performance index of the Kaufman Assessment Battery for Children at 4 y [342]. In studies reporting no effect of DHA supplementation, analysis beyond mean group comparisons suggests positive associations between infant and child development and DHA during gestation [266,337,343-346,349,350].

A study by Makrides *et al* (2010) provided 800 mg/d DHA + 100 mg/d EPA or a placebo to women by 21 wk gestation until infant birth, assessing infant neurodevelopment with the Bayley Scales of Infant Development (BSID) at 18 mo [266]. Although there was no difference in mean scores between the groups, fewer infants in the DHA group had cognitive development scores indicative of delay compared to the placebo group (3.13% vs 6.40%) [266]. In addition to group comparisons of neural development, several studies have examined the relationship between outcome and maternal or infant DHA status. In the study by Malcolm *et al* (2003), cord blood RBC DHA did not differ between infants whose mothers had been given fish oil or a placebo during gestation, but the cord blood RBC DHA was positively associated with visual development scores at 50 and 66 wk postnatal [344]. In another study, mean child scores on the Kaufman Assessment Battery for Children did not differ between the DHA and placebo groups, nor were scores related to cord blood fatty acids [350]. However, children above the 50th

percentile of Mental Performance Index scores had mothers with higher RBC PE and PC DHA during pregnancy than children below the 50th percentile [350].

Several studies have examined the effect of increasing DHA in human milk on infant CNS development [287,288,351]. In a group of lactating women supplemented with 0-1.3 g/d DHA from birth to 12 wk postpartum, infants showed no differences between groups on developmental tests, although infant RBC DHA at 12 wk was positively related to the Mental Development Index (MDI) of the BSID [287]. In contrast, Jensen *et al* (2005) reported higher MDI scores in the BSID at 30 mo, with no effects detected on other neurodevelopment indexes at 4, 9 or 12 mo, in infants of mothers supplemented with 200 mg/d DHA compared to a placebo for 4 mo after birth [288]. Notably, Lauritzen *et al* (2005) randomized women in Denmark to receive 900 mg/d DHA or placebo from 9 d to 4 mo postnatal [351]. Although the infant RBC DHA increased by 40% at 4 mo in the DHA group, there were no differences in problem-solving scores at 9 mo [351]. While this study enrolled women with intakes <400 mg/d ω-3 long-chain fatty acids, in the placebo group mean milk DHA was 0.4 %TMFA [351], which is higher than mean levels in Canada and the US [276]. Thus, it may be possible that the Denmark diet meets DHA needs for infants.

Numerous intervention studies have assessed the effect of DHA in infant formula on infant neurodevelopment, with some studies providing formula containing only DHA at a range 0 – 0.96 %TFA [297,352-358], while many studies have used a formula containing DHA (0 – 46% TFA) with ARA (0 – 72%) [292,295,296,352-357,359-365], and with formula intervention ranging from 2 to 12 mo, beginning within 1 wk after birth. Several studies have reported no effect of formulas containing DHA compared to no DHA on infant neurodevelopment [292,295,352,353,355,356,359-363]. Others have reported positive effects of DHA on sustained visual attention at 4, 6, and 9 mo [365], visual acuity scores at 12 mo and 4 y [296,297,357], BSID MDI at 18 mo [354,358] and processing speed at 6 y [364]. Although most of the studies assessed neurodevelopment after weaning, infant feeding practices were not reported, and may have contributed to variation in study findings. More detailed reviews of the effects of DHA in infant formula have been published for both term and pre-term infants [225,366-368].

Brain development with age in early childhood has also led several investigators to assess the efficacy of DHA supplementation during childhood [27,28,369-378]. Several of these

studies provided DHA with other nutrients, including iron [376], micronutrient mixes [377,378], gamma linolenic acid [375], and vitamins A, C, D and E [27]. For the studies supplementing only DHA [28,369-371,374], or DHA together with EPA [372,373] children were 4-14 y, were given 127 – 1200 mg/d DHA for 8-16 wk usually as a supplement with two studies that used fortified foods [371,372]. Briefly, children who received DHA gained improved verbal learning ability and protection against declines in spelling ability of children 7-9 y [371], improved brain activation in the prefrontal, occipital, and cerebellar cortex of boys 8-10 y [28], and reduced physical aggression and impulsivity in girls and improved school attendance rate of children 9-12 y [373]. Although Ryan *et al* (2008) in the US reported that 400 mg/d DHA for 16 wk had no effect on mean scores of developmental tests, whole blood DHA was weakly, but positively, related with vocabulary scores on the Picture Peabody Vocabulary Test (PPVT) ($r^2 = 0.16$, $P = 0.04$) at 4 y [369]. Notably, a study by Richardson *et al* (2012) in the UK randomized children below the 33rd percentile on an age-standardized word-reading test to 600 mg/d DHA or a placebo for 16 wk. The DHA intervention led to improved reading scores for children with baseline scores <20th percentile, with a 1.9 mo gain in reading age for children with a baseline reading score <10th percentile [370]. However, the intervention had no effect on scores of children with baseline reading scores between the 20th – 30th percentile [370]. Although it appears that increasing DHA intake may be associated with increased brain function of some children, associations between outcome and RBC DHA are complex. Possibly, studies reporting positive effects of DHA on child neurological outcomes may have been conducted in populations with low DHA status, enabling a response to increased DHA. For example, in the US study by McNamara (2010), baseline RBC DHA was a mean (SD) of 3.3 (1.3) %TFA [28] compared to 5.9 (1.0) and 8.3 (1.3) in children from Sweden [379] and Korea [380], respectively.

1.2.4 Dietary Recommendations for ω-3 and ω-6 Fatty Acids

One of the major hurdles to establishing dietary ω-3 fatty acid recommendations is that it is unclear how much is needed and what dietary factors impact DHA status. In 2002, the Food and Nutrition Board of the Institute of Medicine released the Dietary Reference Intakes (DRI) as recommendations for nutrient intakes in Canada and the US [381]. This report stated a lack of evidence for determining ω-3 fatty acid requirements, with ALA deficiency considered non-

existent in the free-living population. The 2002 DRI for ω-3 fatty acids is an adequate intake (AI) of ALA. The AI of ALA for pregnant and lactating women is 1.4 and 1.3 g/d, respectively, 1.1 g/d for non-pregnant, non-lactating women 19-50 y, and 0.9g/d for children 4-8 y [381]. The AI of ALA is based on the median intake by respective life-stage assessed by the US Continuing Survey of Food Intakes by Individuals in 1994-1996 [381]. For infants 0-12 mo, the AI of ALA is 0.5 g/d and was based on the average concentration of ω-3 fatty acids in human milk and ω-3 fatty acids from complimentary foods in post-weaning infants [381]. Due to the ability of EPA and DHA to reverse ω-3 fatty acid deficiency, the DRI states that intakes of EPA and DHA can contribute up to 10% of the total intake of ω-3 fatty acids towards the AI of ALA [381].

An AI was also set for the ω-6 fatty acids. For pregnant and lactating women, the current AI for LA is 13 g/d, with 12 g/d for non-pregnant and non-lactating women 19-50 y, 10 g/day for children 4-8 y, and 4.4 and 4.6 g/d for infants 0-6 mo and 6-12 mo, respectively [381]. In addition to the AI, an Acceptable Macronutrient Distribution Range (AMDR), a range of intakes with reduced risk of chronic disease, was set for both the ω-3 and ω-6 fatty acids. The AMDR for ω-3 and ω-6 fatty acids is constant for all life-stages and is 0.6-1.2 % energy and 5-10 % energy, respectively, with the lower limit an approximation of the AI and the higher intake equivalent to the highest intakes in the US and Canada [381].

In addition to the DRI, several national and international expert groups have endorsed fish intake for health promotion and to reduce the risk of cardiovascular disease (CVD) [382-384]. The first documented recommendation for two servings of fish per week was in the 1994 Report of the Cardiovascular Review Group Committee on Medical Aspects of Food Policy in the UK [385]. The recommendation was to increase the average intake of long chain ω-3 fatty acids to 1.5 g/wk, with an additional recommendation to double the populations' current fish intake to two portions per week aimed at reducing the risk of CVD [385]. The recommendation has since been adopted by expert groups worldwide, including Health Canada [382], the World Health Organization (WHO) [383], the American Heart Association [386], USDA [384], and Dietitians of Canada and the American Dietetic Association [387]. Health Canada recommends a total of 5 oz/wk fish for all individuals > 2 y [382], and the USDA recommends 8 oz/wk seafood for most individuals with additional recommendations of "less for children" and 8-12 oz/wk for pregnant and lactating women [384].

Some groups have provided recommendations for EPA+DHA intake, ranging from 110 to 450 mg/d for pregnant and lactating women (Table 1.5), and 40 to 450 mg/d for children 1 to 10 y (Table 1.6). Despite the lack of evidence for dietary DHA during pregnancy and lactation, several international groups have specified infant neurodevelopment outcomes as justification for these recommendations, including the American Academy of Pediatrics [388], the International Society for the Study of Fatty Acids and Lipids [389], the European Food Safety Authority [390], and governmental bodies including the USDA [384], the French Food Safety Agency [391], and the Superior Health Council of Belgium [392], with several recommendations also including the prevention of CVD or chronic disease in women [384,390-394]. Most EPA and DHA recommendations for children have been extrapolated from adult values based on the reduction of CVD or chronic disease risk [383,386,390,391,393-397]. The European Food Safety Authority recommends 100 mg/d DHA for infants 7-24 mo to support visual function during complementary feeding [390], and the French Food Safety Agency recommends 70 mg/d DHA for 6-36 mo to ensure accumulation of DHA in cerebral membranes [391].

In summary, the current dietary recommendations in Canada and the US for the ω-3 and ω-6 fatty acids are based on observed intakes, and not on physiological requirements. In addition, the recommendations are largely based on reducing CVD risk of adults. Whether or not these recommendations are sufficient to provide DHA required by the developing CNS of the infant and young child is not yet known. It is also unknown if recommendations for intakes of ALA and LA are so high as to compromise endogenous DHA synthesis. Regardless, several expert groups have recommended a dietary intake of EPA and/or DHA for pregnancy, lactation and early childhood. Although it is clear that dietary DHA does lead to an increased circulating DHA, there is also a lack of evidence of compromised infant and child neurodevelopment in populations that do not eat fish. Thus, while dietary DHA may reduce the risk of DHA deficiency in some individuals, dietary recommendations need to consider the unique needs for DHA in the developing brain.

Table 1.5 Recommendations for dietary intake of EPA and DHA and/or fish for pregnant and lactating women.

Expert group		Amounts	Justification
American Academy of Pediatrics, 2012 [388]	Lactation	200-300 mg/d DHA or 1-2 portions fish/wk	Providing adequate DHA in human milk for infant neurobehavioural benefits.
US Dept of Agriculture, 2010 [384]	Pregnancy and Lactation	8-12 oz/wk seafood	Prevention of heart disease and improved infant visual and cognitive development.
European Food Safety Authority, 2010 [390]	Pregnancy and Lactation	100-200 mg/d DHA + 250 mg/d EPA+DHA	Prevention of coronary heart disease (250 mg/d) and to account for oxidative losses of maternal DHA and fetus/infant DHA requirements.
French Food Safety Agency, 2010 [391]	Pregnancy and Lactation	250 mg/d DHA 250 mg/d EPA+DHA	Child visual and cognitive development in addition to lowering chronic disease risk in adults.
International Society for the Study of Fatty Acids and Lipids, 2007 [389]	Pregnancy and Lactation	200 mg/d DHA or 1-2 servings/wk fish	Based on studies of ω-3 LCPUFA during pregnancy and/or lactation and infant outcomes including visual and cognitive development
Australia Department of Health and Ageing, 2006 [394]	Pregnancy Lactation	115 mg/d EPA+DPA+DHA 145 mg/d EPA+DPA+DHA	Prevention of chronic disease in women and to account for increased body weight during pregnancy and infant needs in lactation.
Superior Health Council of Belgium, 2004 [392]	Pregnancy and Lactation	250 mg/d DHA	Supplying DHA to fetus/infant for neurological development.
Health Council of the Netherlands, 2001 [393]	Pregnancy and Lactation	200 mg/d ω-3 fatty acids from fish	Prevention of CVD.

Table 1.6 Recommendations for dietary intake of EPA and DHA and/or fish for children 1-10 yr of age.

Expert group	Age	Amounts	Justification
European Food Safety Authority, 2010 [390]	7-24 m 2-18 y	100 mg/d DHA 250 mg/d EPA+DHA or fatty fish 1-2/wk	Visual function during complementary feeding period. Consistent with adult values for CVD prevention.
French Food Safety Agency, 2010 [391]	6 m-3 y 3-9 y	70 mg/d DHA 250 mg/d EPA+DHA	To ensure accumulation of DHA in cerebral membranes. Extrapolated from adolescent values and based on prevention of different risks including CVD prevention.
WHO, 2008 [383]	2-4 y 4-6 y 6-10 y	100-150 mg /d 150-200 mg/d 200-250 mg/d	Extrapolated from infant and adult values and based on the prevention of chronic disease
FAO, 2008 [395]	2-4 y 4-6 y 6-10 y	100-150 mg/d 150-200 mg/d 200-300 mg/d or fatty fish 1-2/wk	Adjusted for age and based on the prevention of chronic disease
Health Canada, 2007 [382]	>2 y	fish 2/wk (1 fatty fish)	-
American Heart Association, 2006 [386]	>2 y	fish 2/wk (preferably oily)	Extrapolated from adult values based on reduced risk of sudden death and death from coronary artery disease
Health Council of the Netherlands, 2006 [396]	>1 y	450 mg/d of EPA+DHA or fish 2/wk (1 oily)	To reduce the risk of coronary heart disease
Australia Department of Health and Ageing, 2005 [394]	1-3 y 4-8 y	40 mg/d EPA+DPA+DHA 55 mg/d EPA+DPA+DHA	Median population intakes of Australians from National Nutrition Survey of Australia of 1995

1.2.5 Summary

Although DHA accumulates in the CNS during development beginning *in utero,* brain development continues rapidly throughout early childhood, with an increase in DHA rich synapses continuing well after birth. Experimental work has shown that DHA has critical roles in neural development and function, and decreased DHA in the developing brain may impair neurogenesis and alter metabolism of neurotransmitters. Studies in humans have shown positive associations between dietary fish and/or DHA intake of the mother during gestation and visual and neurodevelopment in the infants. Similarly, higher fish and/or DHA intakes or status of children has been associated with better indices of neural development. Results of interventions of DHA during gestation, infancy or childhood have yielded mixed results. However, whether circulating levels of DHA are sufficiently low enough to alter brain DHA, neurodevelopment or function in infants or young children remains unclear. DHA metabolism is not fully understood, and the factors that affect variability in levels of blood and tissue DHA, including potential effects of other dietary fatty acids and genetic variability are unclear. Although ω-3 fatty acids are critical for optimal brain development and function, which fatty acids and the required amount are still uncertain. Regardless, several expert groups recommend that pregnant and lactating women and children >2 y consume fish and/or DHA as part of the daily or weekly diet.

1.3 Purpose of the Research

Human CNS development follows a long developmental timeline beginning as early as eight weeks gestation, with the completion of some processes occurring as late as 20-30 years after birth. However, the majority of studies assessing the role of DHA in the CNS have been conducted in a finite time period, for example, gestation or early infant feeding. Despite evidence from randomized interventions of DHA in early childhood showing positive effects on neurodevelopmental outcomes, the role of the child's diet to support continued CNS DHA accretion beyond gestation or early infancy is poorly understood. In addition, the majority of randomized DHA intervention trials have been designed as efficacy trials, with the underlying assumption that all individuals in the intervention group have the ability to respond to the intervention. Similarly, because variable amounts of DHA are present in the diets of individuals, the placebo group is not a "non-exposure" group, and both the placebo and intervention groups

may contain individuals who are not deficient and would not benefit from intervention. This is problematic for DHA since there are no biomarkers of deficiency or sufficiency, and whether or not DHA deficiency is present within a population is unknown. Observational studies have reported positive associations of DHA intake and/or status during pregnancy with CNS outcomes measured in infants' and young children. However, associations between maternal and child diets have also been reported. Thus, since it is possible mothers with low DHA intakes would have young children also with low DHA intakes it is important to understand if positive associations of DHA intake or status on CNS outcomes in children are due exclusively to the DHA supply during pregnancy or infancy, or a later effect in childhood. Therefore, the purpose of this research was 1. To address the question of whether or not ω-3 fatty acid deficiency sufficient to influence early infant and child CNS development occurs among pregnant women in our community, and 2. To address if inadequate DHA during prenatal development or children's own DHA status and/or intake affects cognitive performance when assessed at 5-6 y.

1.4 Research Rationale and Objectives

1.4.1 Rationale and Objectives for Chapter 2

Rationale: The enrichment of DHA in the brain and retina has led to investigations of its role in neurodevelopment, with evidence suggesting that DHA is important during early development. As noted, observational studies have shown positive associations of infant and child CNS outcomes with fish consumption during pregnancy, while randomized interventions of DHA during gestation have yielded mixed findings. Despite the lack of evidence on which to base dietary recommendations for ω-3 fatty acids, several national and international expert groups have recommended intakes of DHA or fish during pregnancy and lactation. These recommendations may be problematic, as they assume that DHA must be consumed in the diet, and evidence of altered neurodevelopment in populations that do not consume fish, vegetarians for example, has not been reported. In addition, it is unknown if current intakes of ω-3 fatty acids during pregnancy are inadequate to constrain infant development, perhaps because ω-3 fatty acid intakes are too low, or the intake of ω-6 fatty acids is too high relative to ω-3 fatty acid intakes. Examining the role of DHA during development on the CNS is complex, as there is no biomarker of DHA deficiency or sufficiency. This means that the prevalence of DHA deficiency

in a population is unknown and may explain at least in part the discrepancy of findings among randomized intervention trials, since only DHA deficient individuals would have the ability to respond to a DHA intervention. This study uses an intervention to identify the presence of ω-3 fatty acid deficiency, specifically DHA of the CNS, when neither the occurrence of deficiency nor proportion of individuals affected is known. More specifically, the research described in Chapter 2 was designed to determine if DHA inadequacy sufficient to constrain infant neurodevelopment occurs among some pregnant women in Vancouver.

Objectives:

This research involved a randomized, double-blind, controlled trial of 400 mg/d DHA or a placebo from 16 wk gestation until term infant birth to:

1. Determine if fetal DHA inadequacy sufficient to constrain infant neurodevelopment occurs with comparison of maternal placebo and intervention of 400 mg/d DHA

Aims:

This research aims to:

1. Use estimated fatty acid intakes, including DHA, from a food frequency questionnaire administered at 16 and 36 wk gestation
2. Use maternal venous blood RBC fatty acids, including DHA, collected at 16 and 36 wk gestation
3. Assess results for different domains of neurodevelopment, including visual acuity, language, and cognition to determine the distribution of developmental test scores among infants at 2, 6, 9, 12, 14, and 18 mo of age
4. Use a risk reduction model to determine the risk that an infant from the maternal placebo group will fail to achieve a score in the highest quartile of all infants

Null Hypotheses:

1. The level of RBC DHA among pregnant women will not differ between 16 and 36 wk gestation, or between women assigned to 400 mg/d DHA or placebo at 36 wk gestation
2. The distributions of scores on tests of infant neurodevelopment will not differ between infants born to women assigned to 400 mg/d DHA or a placebo

3. Fetal DHA inadequacy sufficient to constrain infant neurodevelopment does not occur in our population

1.4.2 Rationale and Objectives for Chapter 3

Rationale:

Chapter 2 demonstrated that in a group of pregnant women in Vancouver, DHA was low enough in some individuals to constrain the infant neurodevelopment up to 18 mo. However, human brain development continues well past infancy and it is unknown if inadequate DHA during gestation has lasting effects on the child's CNS. In addition, the post-weaning (and formula-fed infant) diet must provide ω-3 fatty acids, but the role of the child's DHA intake / status on neurological function is not known. Although epidemiological studies have shown that fish/DHA intake during pregnancy and/or lactation is associated with child outcomes up to 8 y, it seems logical to assume that the diets of many of the children will resemble that of their mothers. Therefore, separating the effects of the maternal ω-3 fatty acid intake during pregnancy or lactation from the post-weaning ω-3 fatty acid intake of the infant/child on continued brain development is challenging. To address this, the purpose of this study was to determine if effects of fetal DHA inadequacy observed on infant development persist into early childhood, while addressing a potential relationship between maternal DHA and child DHA intake and status.

Objectives:

This research involves a prospective cohort of healthy children 5 y + 9 mo of age (5.75 y) whose mothers were given 400 mg/d DHA or a placebo during pregnancy to:

1. Assess if fetal DHA inadequacy has effects on child neurodevelopment
2. Assess if a potential relationship between maternal DHA intake and RBC DHA during gestation and child DHA intake and RBC DHA occurs

Aims:

This research aims to:

1. Use tests of neurodevelopment, including attention, language, working memory and learning, to assess the distribution of developmental test scores

2. Use a risk reduction model to determine the risk that a child from the maternal placebo group will fail to achieve scores in the highest quartile of all children
3. Estimate dietary fat and fatty acid intakes of the child from food and supplements
4. Correlate maternal DHA intake and RBC DHA during gestation with child DHA intake and RBC DHA

Null Hypotheses:

1. Fetal DHA inadequacy sufficient to constrain neurodevelopment will not be detected in children assessed at 5.75 y
2. Maternal intake and RBC DHA during gestation will show no relationship to child DHA intake and RBC DHA at 5.75 y

1.4.3 Rationale and objectives for Chapter 4

Rationale:

The results of Chapter 3 failed to find a difference in risk of failure to achieve a low developmental test score among children from the placebo or DHA groups at 5.75 y. This may suggest that the effects of DHA inadequacy during gestation on CNS development up to 18 mo in Chapter 2 may have been lost or masked by other variables in the children at 5.75 y. However, children of mothers in the highest quartile of RBC DHA at 36 wk had higher scores on some tests of development compared to the lowest quartile of maternal RBC DHA. It is also possible that the participants of Chapter 3 were highly motivated, participating in 8 study visits from 16 wk gestation to 18 mo after birth, and returning at 5.75 y. Thus, it may also be possible that the DHA status of this group of children was not low enough to compromise development, with the majority of children having an RBC DHA > 4.0% TFA. However, child DHA intake and RBC DHA were related to the DHA intake and status of the mother during gestation. Therefore, the study in Chapter 4 involved enrolment of a cross-sectional group of children to determine the potential relationship between DHA intake and RBC DHA on neurodevelopmental test scores in children at 5.75 y. This study also sought to determine if children who participate in cohort studies differ from a cross-sectional group of children.

Objectives:

This research involves healthy children 5.75 y and is designed with the following objectives to:

1. Determine if there are differences in dietary intake, RBC fatty acids or neurodevelopment test scores among children who participated in a prospective cohort and the cross-sectional group of children
2. Determine the potential relationship of DHA intake with neurodevelopment test scores
3. Determine the potential relationship of RBC DHA with neurodevelopment test scores

Aims:

This research aims to:

1. Use a combination of dietary assessment methods to estimate dietary fat and fatty acid intakes from food and supplements, and the major sources of dietary DHA
2. Use data on child RBC fatty acids, including DHA, collected at 5.75 y to determine the association with dietary DHA
3. Use scores on tests of neurodevelopment, including attention, language, working memory and learning, to assess the distribution of developmental test scores
4. Compare dietary and RBC fatty acids and child neurodevelopment test scores of children who participated in a prospective cohort and a cross-sectional group of children
5. Compare DHA intakes and RBC DHA among children with low and high developmental test scores

Null Hypotheses:

1. Dietary and RBC fatty acids and neurodevelopment test scores will not differ between a prospective cohort of children and a cross-sectional group of children
2. Child DHA intake will show no relationship to neurodevelopment test score
3. Child RBC DHA will show no relationship to neurodevelopment test score

Chapter 2: Omega-3 Fatty Acid Deficiency in Infants Before Birth Identified Using a Randomized Trial of Maternal DHA Supplementation in Pregnancy[1]

2.1 Chapter Synopsis

The overall objective of the research described in this chapter was to determine if fetal DHA inadequacy sufficient to constrain neurodevelopment to 18 mo occurs in our community of Vancouver, BC. Identifying fetal DHA inadequacy is complex due to the inability to access neural tissues, incomplete knowledge of biomarkers of deficiency/sufficiency reflecting brain DHA, and many individual and environmental variables that impact CNS development. Since the presence of DHA deficiency in our population is unknown, the study provided DHA to a group of pregnant women to reduce the risk of inadequate DHA, although the DHA intervention is not expected to have benefit for those who are not deficient. Similarly, only infants in the placebo group with inadequate DHA during gestation would be expected to have neural function deficits. Thus, this study was designed to identify fetal DHA deficiency by determining risk of failure to achieve a developmental test score in the highest quartile of scores of infants whose mothers were given a placebo or DHA supplement. The findings showed that DHA supplementation increased the maternal RBC mean DHA at 36 wk gestation and mean RBC 22:4ω-6 + 22:5ω-6 were decreased. However, levels of RBC DHA, 22:4ω-6 + 22:5ω-6 showed variance within each group, with a large overlap in RBC DHA among women in the DHA and placebo groups. The key outcome of this study was that infants in the placebo group, compared to the DHA intervention group, were less likely to achieve a test score in the top quartile of children, particularly for tests of language development. The results of this study suggest that in Vancouver, low fetal DHA occurs in some children that limit infant development to 18 mo. Whether or not fetal DHA inadequacy has lasting, irreversible effects on the brain is not yet known and is investigated in the subsequent chapters. The following introduction, methods, results and discussion are provided from our publication in PLoS One [398].

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[1] *A version of this chapter has been published:*
Mulder KA, King DJ, Innis SM. Omega-3 fatty acid deficiency in infants before birth identified using a randomized trial of maternal DHA supplementation in pregnancy. PLoS One. 2014, 9:e83764. © 2014 Mulder *et al.*

2.2 Introduction

Nutrient deficiencies during development may have long-lasting consequences for the CNS that range from devastating malformations to subtle effects on neural functioning. Persisting CNS deficits due to early deficiency depend on the nutrient and inadequacy at a time vulnerable to morphological or molecular shifts from which recovery is difficult [399,400]. In this regard, DHA, but not other ω-3 fatty acids, is enriched in brain and retina membranes where it functions in early developmental events such as neurogenesis, neurite outgrowth, synaptic plasticity, axonal elimination, and gene expression [5,401-408]. Loss of CNS DHA is compensated for by increased 22:4ω-6, 22:5ω-6 and sometimes ARA in animals and human infants [3,5,120,162]. Although substitution of ω-6 fatty acids for DHA fulfills needs for membrane fatty acids, ω-6 fatty acids differ in functional properties from DHA. This results in a complex problem whereby the consequences of insufficient DHA may reflect both loss of DHA and the actions of the ω-6 fatty acids used in replacement.

Understanding dietary needs for ω-3 fatty acids for CNS development is complicated by the different ω-3 fatty acids in the diet, interactions with ω-6 fatty acids, and genetic and other variables that impact their metabolism. The ω-3 fatty acids are consumed as ALA, mainly in plant oils, and EPA and DHA from animal lipids, particularly fish. Conversion of 18:3ω-3 to DHA occurs in humans, but in a complex pathway requiring D6D, which has at least six substrates [35,409]. Among these, ω-6 fatty acids are important due to the abundant LA in westernized diets, inhibition of 18:3ω-3 desaturation by high LA, and knowledge that ARA, 22:4ω-6 and 22:5ω-6 accumulate, and hence ω-6 fatty acid desaturation occurs, in infants and animals fed diets high LA [3,25,48,120]. When consumed, DHA effectively provides DHA to the developing CNS thus overcoming concerns over ALA inadequacy, ability to synthesize DHA, or high ω-6 fatty acid intakes [3,195,203,261,410].

Our work focuses on whether ω-3 fatty acid deficiency leading to DHA low enough to compromise fetal CNS development occurs during gestation. Observation studies to show a positive association between child CNS development and maternal DHA intake or blood DHA levels during pregnancy [16,18,327,411] provide evidence that insufficient DHA to support child CNS development does occur in some pregnant women. Randomized clinical trials on the efficacy of supplemental DHA during pregnancy in enhancing child CNS performance have

yielded mixed findings [105,266,335,339,340,342,343,346,348-350], but whether the infants in these studies were compromised by underlying ω-3 fatty acid insufficiency before birth is unknown.

The present study is based on a risk-reduction model, and used a randomized intervention with DHA or a placebo to address whether fetal DHA supplies are sufficiently low in some pregnant women as to increase risk that children will not meet their developmental potential. The design recognizes that neither the prevalence nor extent of DHA insufficiency, if present, is known. The efficacy of DHA in enhancing cognitive development can only be tested in individuals known to have deficits in cognitive development. For example, if it is accepted that infants fed formula have cognitive delays compared to human milk-fed infants, then the efficacy of DHA in preventing the cognitive deficits due to feeding formula can be tested by comparing infants fed formula with and without DHA. Equally important, our design incorporates understanding that the placebo group in this study is not a non-exposure group. Variable amounts of DHA are present in the diet of all participants. Further, individuals in the DHA supplement group who are not deficient are not expected to benefit. Because our underlying goals are to demonstrate deficiency and subsequently identify biomarkers of deficiency, maternal blood samples were a pre-requisite to assessment of child outcomes in the present work.

2.3 Subjects and Methods

2.3.1 Ethics Statement

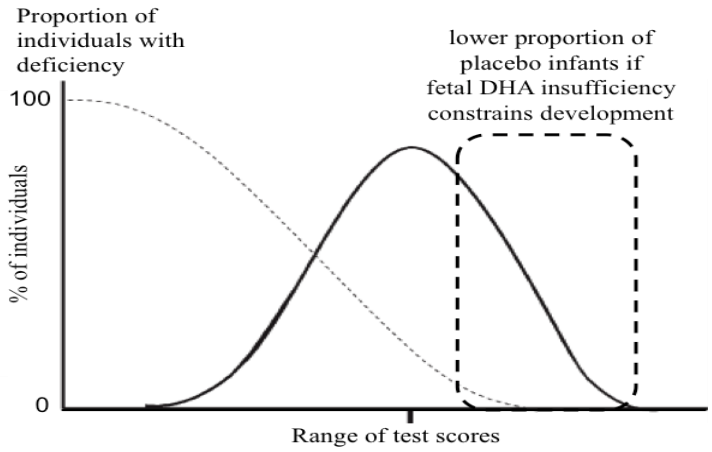
This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were reviewed and approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia (B.C.) and the B.C. Children's and Women's Hospital. Written informed consent was obtained from each woman prior to randomization on behalf of herself and for her infant.

2.3.2 Study Design, Intervention and Subjects

This was a double-blind, single center, randomized study designed to assess whether DHA was sufficiently low among some term infants prior to birth as to limit CNS development to 18 mo. All of the participants were residents of Vancouver, Canada and enrolled between

2004 and 2008. The purpose of randomizing a group of women to DHA was to provide a group of infants in which risk of DHA insufficiency during gestation was considered low. The rate of CNS development differs among infants, giving a distribution of scores within which the best potential development of an individual infant is unknown. As a result, the risk reduction model focused analyses on performance of infants in the placebo group within the distribution of development of all infants in the study. We hypothesize that prenatal DHA insufficiency will constrain child CNS development. If this occurs, we expect that fewer infants in the placebo than DHA supplement group will achieve high neurodevelopmental test scores, giving evidence that DHA deficiency occurs during gestation and constrains child development in our population (Figure 2.1).

Figure 2.1 Schematic to illustrate the concept that individuals with high neurodevelopment test scores are unlikely to be nutrient deficient.



We assessed whether DHA insufficiency sufficient to constrain CNS development occurs based on failure to achieve a neurodevelopmental test score in the upper 25% of infant scores, representing the range of achievement where deficiency is less likely.

As described [278,412], our studies were not designed to test the efficacy of DHA supplements in increasing child CNS abilities, which is a different question and not our hypothesis. Efficacy of an intervention can only be addressed with individuals known to be able to respond to the intervention; in our situation the presence of deficiency and thus the ability to respond is unknown. In the event that no effect is found, the interpretation is that DHA

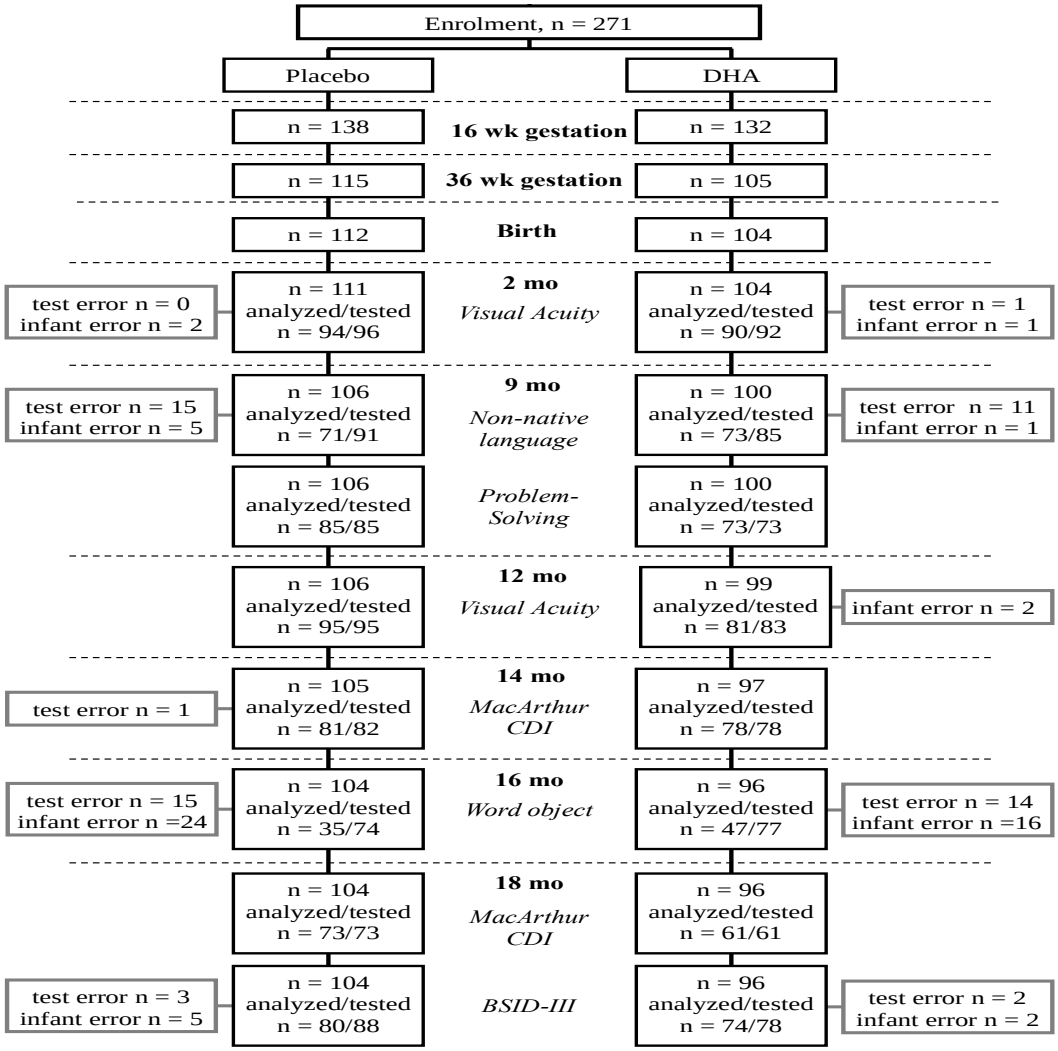
deficiency was not detectable in our study population, not that DHA is not needed by the brain. An assumption of the prevalence of DHA deficiency was used for the purpose of study design. We assumed DHA deficiency in 15% of infants before birth and 35% dropout, with increased risk of not being in the 25% of infants with the highest test scores identified in the placebo group with enrolment of 270 participants.

Eligible participants were ≤16 wk gestation, not taking any lipid or fatty acid supplement, and were expected to deliver one infant at full-term gestation, with no maternal or fetal complications. After enrolment, participants were assigned to 400 mg/d DHA or a placebo using computer-generated, random codes held in sealed, opaque envelopes using a block design. The supplements were provided in identical capsules in bottles with more than sufficient supplements to cover the study interval. Compliance was monitored from the number of returned unused capsules. DHA was given as algal oil triglycerides and the placebo was an equivalent amount of corn and soybean oil blended to reflect the dietary LA/ALA ratio, but in amounts quantitatively insignificant compared to usual intakes [412]. The supplements were identical in appearance, contained an orange flavour mask and were provided by DSM Nutritional Products (formerly Martek Biosciences, MD). We chose a supplement of 400 mg/d DHA since this is equivalent to DHA from about two meals of fatty fish/wk, and four to ten-fold higher than the amount of DHA accumulated/d in fetal tissues [413,414]. Higher amounts of DHA would give greater separation between the intake and blood levels of DHA [22] among women in the DHA and placebo supplement groups. However, this is not a useful strategy for this study because of the underlying understanding that providing more DHA than required by the CNS will not result in enhanced CNS function. Fundamentally, the relationship between the intake of an essential nutrient and functions dependent on that nutrient are non-linear, with a linear dose-response between DHA and child cognitive function unlikely. We do not hypothesize that DHA supplements increase cognitive abilities in individuals who fulfill their DHA requirements. The duration of supplementation was from enrolment until infant delivery. Maternal venous blood and information on dietary intake was obtained at 16 wk gestation prior to commencing supplements, then again at 36 wk gestation.

A flow diagram of subject enrolment, retention in the study, infant developmental tests and reasons tests were not analyzed are in Figure 2.2. Two hundred and seventy one pregnant

women completed the informed consent. One woman withdrew before commencing the study, leaving 138 and 132 women in the placebo and DHA groups, respectively. Blood was obtained and analyzed for 114 and 103 women in the DHA and placebo groups, respectively, at both 16 and 36 wk gestation. Infants of mothers who did not complete the 36 wk prenatal assessment or had complications, including prematurity, likely to interfere with normal development were not included in the follow-up. Maternal socio-demographic characteristics, including age, ethnicity, family income, education, and parity were recorded. Each mother's IQ was assessed using the Test of Nonverbal Intelligence-3 (TONI-3), which assesses aptitude, abstract reasoning and problem solving [415]. Data on birth weight, pregnancy weight gain, and gestation length were collected from hospital charts. Infant weight and length was measured at 2, 6, 9, 12 and 18 mo.

Figure 2.2 Flow diagram of study participants and their infants from enrolment to completion.



Subject withdrawal from 16 to 36 wk was explained for the placebo and DHA groups, respectively, by subject self withdrawal, n = 9, 16; protocol non-compliance, n = 7, 6; preterm delivery, miscarriage, elective pregnancy termination or other pregnancy complications, n = 5, 5; and one woman in the placebo group delivered twins, lost 36 wk blood sample n=1, 0. One subject in the placebo group withdrew between 36 wk gestation and infant delivery, with self-withdrawal and loss to follow-up of infants between 2 and 9 mo of age of n = 5,4; between 9 and 12 mo of age of n = 0,1; between 12 and 14 mo of age n = 1,2; between 14 and 16 mo of age n = 1,1; and between 16 and 18 mo of age n = 0, 0 for the placebo and DHA groups, respectively. Three infants born at term gestation did not meet the protocol requirements for follow up, explained by congenital disorders, n = 1,1 and intrauterine growth retardation, n = 1,0 in the two groups, respectively, and one infant in the placebo group died from sudden infant death syndrome before 2 mo of age. After birth, the number of infants at each milestone, with the number of infants attending assessments, and number of tests incomplete or not analyzed is given. For the placebo and DHA groups, respectively, infant tests done but not analyzed were: at 2 mo, n = 0,1 technical error, n = 2,1 uncooperative infant; at 9 mo non-native contrast test, n = 15,11 technical error, n = 4,0 uncooperative infant, n = 1,0 did not habituate, n = 0,1 exposed to Hindi at home; at 12 mo visual acuity, n = 0,2 uncooperative infant; at 14 mo, n = 1,0 unresolved parent reporting error; at 16 mo, n = 15,14 technical error, n = 18,16 uncooperative infant, n = 6,0 did not habituate; and at 18 mo, BSID-III, n = 3,2 technical error and n = 5,2 uncooperative infant. Technical errors included video-recording and test protocol errors, or external disruption, such as noise causing infant distraction; infant errors include fussy or uncooperative behaviour, and parent interference.

2.3.3 Dietary and Biochemical Analyses

Venous blood samples were centrifuged, the plasma and buffy coat recovered, and the RBC washed in saline and centrifuged three times. All samples were stored frozen at -80°C until analyses. PE is predominately located on RBC inner membrane. As in the brain, the RBC PE contains higher DHA, ARA, 22:4ω-6 and 22:5ω-6 than PC, and shows considerably less variability in response to recent change in fatty acid intake than plasma lipids or RBC PC [155,416,417]. Therefore, RBC PE and PC were separated by HPLC, and their fatty acids analyzed by GLC [3,195,203,330,418]. Maternal dietary intake was assessed at 16 and 36 wk gestation using an FFQ, then analyzed for macronutrient and fatty acid intakes [418]. Infant feeding, including breast-feeding and breast-feeding duration, was collected monthly at a study visit or by telephone if no visit occurred. For this study, breast-feeding was defined as exclusive as long as the intake of formula did not exceed 250 mL/wk.

2.3.4 Assessments of Infant Development

Visual acuity was assessed using the Teller Acuity card procedure at 2 mo ± 3 d and 12 mo ± 3 d using test distances of 38 cm and 55 cm, respectively [330]. Language development was assessed using four approaches: a non-native consonant recognition task at 9 mo ± 3 d [330]; the MacArthur Communicative Development Inventory (CDI) infant scale at 14 mo ± 5 d and infant and toddler scales at 18 mo ± 5 d; a word-object pairing task at 16 mo ± 5 d [419], and Bayley Scales of Infant and Toddler Development, Third Edition (BSID- III) language composite scales at 18 mo ± 5 d [420]. Problem solving ability was also assessed at 9 mo ± 3 d of age [421], and the BSID-III cognitive, fine and gross motor scales were given at 18 mo ± 5 d [420]. The non-native consonant recognition and problem solving tests were videotaped, then the tapes coded and later scored by observers unaware of the infant's study group. The acceptable age range during which each developmental assessment was made was purposefully narrow, based on the premise that DHA insufficiency constrains the rate of child CNS development.

2.3.5 Data Analyses

All data were analyzed on a per protocol basis. Subject characteristics were summarized using descriptive statistics, then compared using independent two-sample t-tests for continuous variables, Mann-Whitney *U* tests for nonparametric continuous variables, and chi-square for categorical data. Data were checked for skewness using the Shapiro-Wilk test of normality prior to analysis. Z-scores for infant weight-for-age, length-for-age and weight-for-length were calculated using the World Health Organization (WHO) Anthroplus anthropometric calculator software (version 1.0.4). Maternal RBC fatty acids and dietary intakes at 16 and 36 wk gestation were analyzed using two-factor ANOVA and Wilcoxen signed-rank test, respectively. Spearman's rank correlation coefficient was used to assess the association between maternal dietary intake of DHA and the RBC PE and PC DHA. Logistic regression was used to assess maternal and infant variables impacting infant test results, and these included maternal age, maternal IQ, maternal RBC DHA at enrolment, infant birth weight, gestation length, infant sex, birth order, and breast-feeding duration. Smoking was not included because only six women reported that they smoked at any time during pregnancy. Of the variables assessed, only infant sex differed between the two randomized groups and showed a potential relationship to infant outcome ($P \leq 0.05$); therefore, sex was included in all subsequent analyses.

The odds ratio and 5-95th confidence interval (CI) that an infant in the placebo or DHA group would fail to perform among the top 25% of all infants was determined with contingency tables and Fisher exact test. For some tests, separation of the cohort into an exact upper 25% and lower 75% of infants was not possible when more than one infant achieved the same test score at the group cut point. In those cases, we defined the upper 25% of infants as the n that gave the lowest possible deviation from n = 25% of all infants tested for that test. Pass/fail results for the problem-solving and non-native language consonant tests were compared between the DHA and placebo supplement groups using chi-square. Data analysis was conducted with IBM SPSS Statistics (Version 20.0.0, 2011. Chicago, IL); differences considered significant at a $P < 0.05$.

2.4 Results

Of the 138 and 132 women randomized to the placebo and DHA groups, 22 in the placebo group and 27 in the DHA group withdrew before 36 wk gestation (Figure 2.2). Subject

withdrawal was explained for the two groups respectively by subject self withdrawal, n = 9 and 16; protocol non-compliance, n = 7 and 6; preterm delivery, miscarriage, elective pregnancy termination or other pregnancy complications, n = 5 and 5; and one woman in the placebo group delivered twins. One blood sample was lost for one woman in the placebo group at 36 wk gestation due to technical error, giving 115 and 105 women in the placebo and DHA groups, respectively, for whom RBC fatty acids were analyzed at 36 wk gestation (Figure 2.2). Diet records were analyzed for all participants with the exception of one woman in the DHA group at each time point. Two blood samples collected at 16 wk gestation were not analyzed due to technical error. One subject in the placebo group withdrew between 36 wk gestation and infant delivery. Three infants born at term gestation did not meet the protocol requirements for follow up, explained by congenital disorders, n = 2, and intrauterine growth retardation, n = 1, and one infant in the placebo group died from sudden infant death syndrome before 2 mo. Retention in the study after birth exceeded 90%, with 111 and 104 infants in the placebo and DHA groups, respectively, at 2 mo and 104 and 96 infants in the two groups, respectively, at 18 mo. The number of infants in the study at 2, 9, 12, 14 and 18 mo, with the number of infants attending assessment visits, tests analyzed, and reasons tests were not analyzed are in Figure 2.2.

The characteristics of the 115 and 105 women in the placebo and DHA group, respectively, who completed the study protocol to 36 wk gestation did not differ for maternal age, IQ, or pregnancy weight gain (Table 2.1). The women were primarily of Caucasian background (73.6%), most with post-secondary education (94.4%). No differences in maternal and family socio-demographic characteristic were found between the randomized groups. For the 216 infants at delivery (Figure 2.2), 113 were girls and 103 were boys. For reference, on average 105 boys are born for every 100 girls in Canada. Since DHA cannot alter sex, it was by chance that there were more infant boys ($P < 0.02$) in the placebo than DHA group. Infant weight, length and weight-for-length z-scores did not differ between the two randomized groups at any age (Table 2.2). Breast-feeding rates were high, with 77% and 70% of the placebo group, and 72% and 63% of the DHA group infants fed <250 mL infant formula at 4 and 6 mo, respectively, ($P < 0.05$).

Table 2.1 Maternal and infant characteristics classified by intervention group.

	Maternal group	
	Placebo n = 111	DHA n = 104
Maternal age, (y)	33.4 ± 3.61	32.6 ± 4.04
Maternal Ethnicity, % Caucasian	73.9	73.1
Parity[1], % 1, 2, >2	47.7, 36.7, 15.6	60.6, 30.8, 8.6
Pre-pregnancy weight[2], (kg)	64.7 ± 12.6	64.8 ± 12.6
Pregnancy weight gain, (kg)	14.7 ± 4.48	14.1 ± 4.82
Maternal IQ[3]	34.0 ± 7.27	34.4 ± 7.58
Infant sex[4], % boys/girls	55.0/45.0	40.4/59.6
Infant birth weight[5], (g)	3497 ± 479	3494 ± 400
Infant birth weight, (z score)	0.36 ± 1.02	0.42 ± 0.78

Values are mean ± SD or % as shown for infants eligible for follow-up and their mothers.
[1] Trend to less first-born infants in the placebo than DHA group, $P = 0.06$.
[2] Pre-pregnancy weight was by self-report.
[3] Maternal IQ was assessed using the Test of Non-Verbal Intelligence-3 (TONI-3) [415].
[4] There were more boys in the placebo than the DHA group, $P = 0.03$.
[5] Birth weight was not available from hospital charts for 2 infants in the DHA group (n = 102).

Table 2.2 Anthropometric measures (z scores) of infants from 2 to 18 mo-of-age.

Age	Measure	Maternal supplement group		P^1
		Placebo	DHA	
2 mo	Weight-for-length	-0.16 ± 1.08 (101)	-0.42 ± 1.20 (90)	0.11
	Length-for-age	0.29 ± 1.08 (102)	0.17 ± 1.04 (92)	0.42
	Weight-for-age	0.06 ± 1.08 (101)	-0.19 ± 1.08 (90)	0.11
6 mo	Weight-for-height	0.04 ± 1.04 (101)	-0.11 ± 1.02 (95)	0.32
	Height-for-age	0.25 ± 1.06 (101)	0.17 ± 1.04 (95)	0.58
	Weight-for-age	0.10 ± 1.01 (101)	-0.06 ± 1.11 (95)	0.30
9 mo	Weight-for-length	-0.04 ± 0.99 (94)	0.17 ± 1.05 (87)	0.16
	Length-for-age	0.22 ± 1.08 (95)	-0.06 ± 1.05 (88)	0.08
	Weight-for-age	0.03 ± 0.99 (94)	0.04 ± 1.11 (87)	0.92
12 mo	Weight-for-height	-0.04 ± 0.99 (93)	0.14 ± 1.09 (81)	0.24
	Height-for-age	0.44 ± 1.11 (94)	0.11 ± 1.06 (84)	0.05
	Weight-for-age	0.15 ± 1.02 (94)	0.12 ± 1.05 (81)	0.86
18 mo	Weight-for-length	0.14 ± 1.05 (70)	0.14 ± 1.05 (74)	0.90
	Height-for-age	0.41 ± 1.14 (82)	0.16 ± 1.11 (76)	0.17
	Weight-for-age	0.27 ± 0.99 (70)	0.21 ± 1.04 (74)	0.71

Data are means ± SD (n) calculated using the WHO Anthroplus anthropometric calculator (version 1.0.4).
[1] There were no significant differences between the groups, by ANOVA, $P \geq 0.05$.

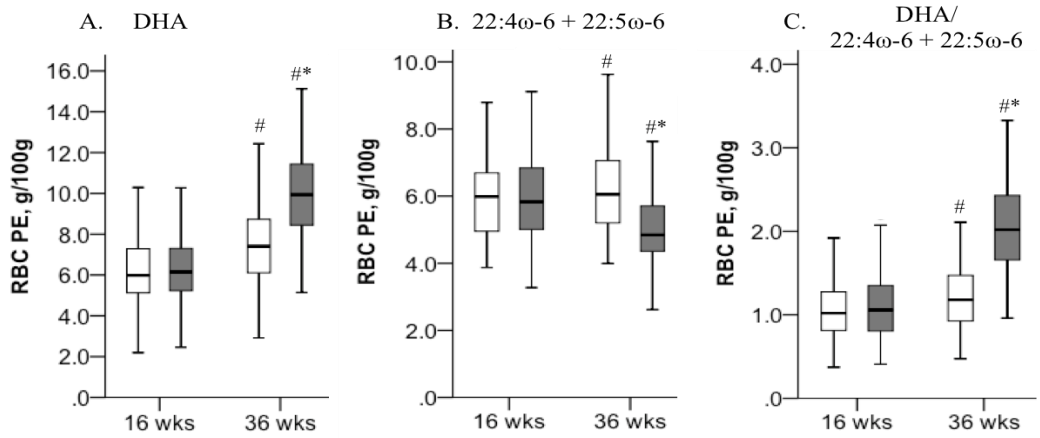
Table 2.3 Dietary ω-6 and ω-3 fatty acids intakes among Canadian pregnant women at 16 and 36 wk gestation randomized to placebo or DHA supplement[1].

Fatty acid	Placebo		DHA	
	16 wk (n = 111)	36 wk (n = 111)	16 wk (n = 103)	36 wk (n = 103)
18:2ω-6, % en	4.98 (2.32-10.3)	5.26 (2.82-9.80)	4.85 (2.96-8.71)	5.31 (2.87-8.74)
18:3ω-3, % en	0.59 (0.29-1.94)	0.61 (0.31-1.69)	0.59 (0.35-1.55)	0.57 (0.35-1.61)
20:4ω-6, mg/d	90.0 (20.0-204)	90.0 (26.0-270)	80.0 (26.0-208)	90.0 (20.0-228)
20:5ω-3, mg/d	40.0 (0.00-224)	30.0 (0.00-160)	50.0 (0.00-314)	40.0 (0.00-162)
22:6ω-3, mg/d	80.0 (0.00-334)	90.0 (10.0-302)	90.0 (6.00-472)	100 (10.0-346)
18:2ω-6/18:3ω-3	7.64 (3.20-14.8)	7.43 (3.58-14.4)	6.84 (3.50-13.2)	7.63 (3.97-13.4)

% en: % total dietary energy
Results are medians (2.5-97.5th percentile range) of fatty acid intakes.
[1] There were no significant differences in intakes between 16 and 36 wk gestation by wilcoxen signed-rank test, or between the two randomized groups by Mann-Whitney *U* test.

The maternal dietary ω-6 and ω-3 fatty acid intakes did not differ between the groups at 16 or 36 wk gestation (Table 2.3). Briefly, fat provided 34.2 ± 6.60 and 33.5 ± 5.74 % energy at 16 wk gestation and 34.7 ± 6.46 and 34.7 ± 5.55 % energy at 36 wk gestation, for women in the placebo and DHA groups, respectively ($P > 0.05$). DHA intakes were skewed, with a median (2.5 - 97.5th percentile range) intake in the placebo (n = 115) and DHA (n = 104) group, respectively, of 80.0 (0 – 334) and 90.0 (6.00 – 472) mg/d at 16 wk and 90.0 (10.0 – 302) and 100 (10.0 – 346) mg/d at 36 wk gestation ($P > 0.05$). Fish (including shellfish) intakes were 141 (0 – 546) and 182 (0 – 708) g/wk at 16 wk gestation and 170 (0 – 570) and 168 (0 – 885) g/wk at 36 wk gestation for the placebo and DHA groups, respectively ($P > 0.05$).

Figure 2.3 Boxplots to show maternal RBC PE g/100g fatty acids for women assigned to placebo (open bars) or supplement of 400 mg/d DHA (closed bars) from 16 wk gestation until infant delivery.



Panels A, B and C are the RBC PE DHA, RBC PE 22:4ω-6+ 22:5ω-6, and RBC PE ratio of DHA/22:4ω-6+ 22:5ω-6, respectively; n = 111 and 111for the placebo group, and n = 102 and 104 for the DHA group at 16 and 36 wk gestation. # Value at 36 wk gestation different from 16 wk gestation within a group, *value for placebo group different from DHA group at the same stage of gestation, P < 0.01, by two way ANOVA.

The maternal RBC PE DHA was 6.25 ± 1.60 and 6.36 ± 1.62 g/100 g fatty acids in the placebo and DHA groups, respectively, at 16 wk gestation ($P > 0.05$), with a significant increase to 10.0 ± 2.06 g/100 g at 36 wk gestation in the DHA group (n = 105, $P < 0.01$) compared to 7.40 ± 2.04 g/100 g fatty acids in the placebo group (n = 115, $P < 0.01$) (Figure 2.3). In contrast, the RBC PE 22:5ω-6 increased from 16 to 36 wk gestation in the placebo group ($P < 0.01$), while 22:5ω-6 and 22:4ω-6 decreased from 16 to 36 wk gestation in the DHA group ($P < 0.01$). Regardless of the difference in the mean levels of DHA in the RBC PE between the groups at 36 wk gestation ($P < 0.01$), the range of RBC PE DHA showed overlap between the groups. Specifically, the 2.5 – 97.5th percentile range of RBC PE DHA was 3.52 – 11.2 and 6.09 – 14.0 g/100 g fatty acids for women in the placebo and DHA groups, respectively, at 36 wk gestation (Figure 2.3). Levels of ARA were not different between the groups at 16 wk gestation, but ARA decreased in the RBC PE in the DHA group to levels below the placebo group at 36 wk gestation ($P < 0.05$, Table 2.4). Results for the analyses of the maternal RBC PC fatty acids are provided in Table 2.5. Dietary DHA intake was positively associated with DHA levels in the maternal RBC PE and PC among all women at 16 wk gestation (rho = 0.341, rho = 0.346, $P < 0.001$, respectively, n = 214) and in the placebo group at 36 wk gestation (rho = 0.286, rho = 0.330, $P < 0.001$, respectively, n = 111) (Figure 2.4).

Table 2.4 Major fatty acids in red blood cell phosphatidylethanolamines (g/100g fatty acids) of Canadian women at randomized to a placebo or 400mg/d DHA from 16 wk gestation.

Fatty acid	Gestation wk 16		Gestation wk 36		
	Placebo n = 111	DHA n = 102	Placebo n = 111	DHA n = 104	*P*[1]
16:0	14.8 ± 1.79	14.8 ± 2.20	15.7 ± 2.23[2]	16.1 ± 2.58[2]	0.22
18:0	7.15 ± 1.43	7.11 ± 1.33	6.67 ± 1.15[2]	6.74 ± 1.16[3]	0.22
18:1	15.3 ± 1.38	15.3 ± 1.20	16.7 ± 1.73[2]	16.5 ± 1.34[2]	0.31
18:2ω-6	4.55 ± 0.80	4.59 ± 0.78	4.71 ± 1.00	4.47 ± 0.77[3]	**<0.01**
20:3ω-6	1.13 ± 0.34	1.15 ± 0.33	0.96 ± 0.58	0.95 ± 0.53[2]	0.71
20:4ω-6	17.2 ± 1.67	17.6 ± 2.00	16.4 ± 1.73[2]	15.7 ± 2.02[2]	**<0.01**
22:4ω-6	5.40 ± 1.10	5.41 ± 1.32	5.46 ± 1.24	4.46 ± 1.01[2]	**<0.01**
22:5ω-6	0.63 ± 0.20	0.65 ± 0.27	0.82 ± 0.24[2]	0.55 ± 0.17[2]	**<0.01**
18:3ω-3	0.25 ± 0.10	0.23 ± 0.07	0.26 ± 0.07[2]	0.24 ± 0.06[3]	0.05
20:5ω-3	0.80 ± 0.37	0.76 ± 0.27	0.69 ± 0.30[2]	0.74 ± 0.26	**0.04**
22:5ω-3	3.12 ± 0.61	3.13 ± 0.55	3.16 ± 0.68	2.63 ± 054[2]	**<0.01**
22:6ω-3	6.24 ± 1.60	6.36 ± 1.63	7.44 ± 1.94[2]	9.98 ± 2.01[2]	**<0.01**

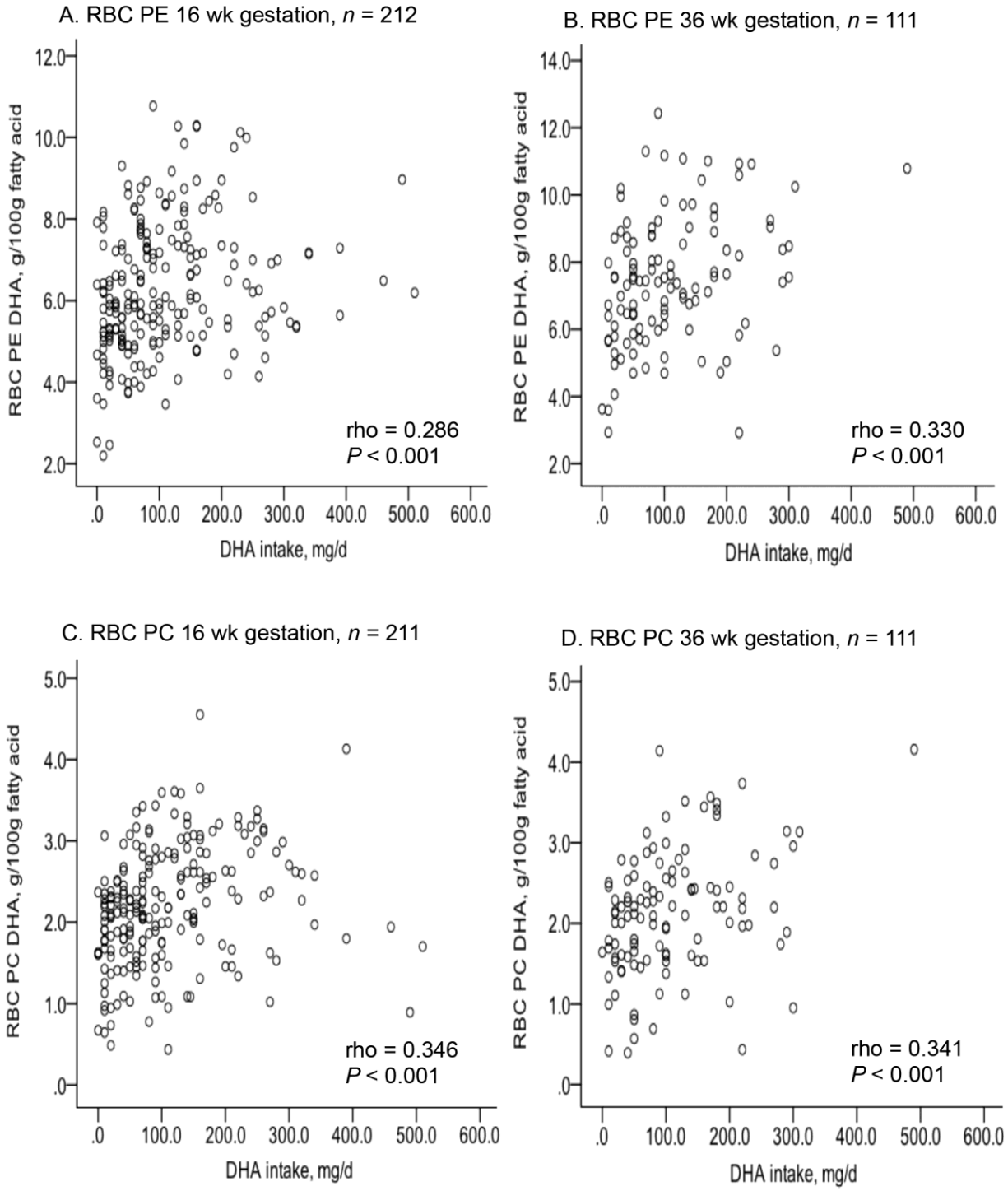
Data are means ± SD, g/100g fatty acids.
[1] *P* value for differences between groups at the by stage of gestation, by ANCOVA.
[2] Value at 36 wk gestation different from 16 wk gestation within a group, $P < 0.01$, by paired t-test.
[3] Value at 36 wk gestation different from 16 wk gestation within a group, $P < 0.05$, by paired t-test.

Table 2.5 Major fatty acids in red blood cell phosphatidylcholine at 16 and 36 wk gestation of women randomized to a placebo or 400 mg/d DHA from 16 wk gestation.

Fatty acid	Gestation wk 16		Gestation wk 36 wk		
	Placebo n = 111	DHA n = 102	Placebo n = 111	DHA n = 104	*P*[1]
16:0	36.2 ± 2.22	36.2 ± 1.60	37.6 ± 1.63[2]	37.7 ± 1.40[2]	0.77
18:0	10.1 ± 0.95	9.96 ± 0.89	8.69 ± 0.75[2]	8.73 ± 0.71[2]	0.36
18:1	19.1 ± 1.95	18.6 ± 1.16	18.7 ± 1.37	18.6 ± 1.54	0.59
18:2ω-6	19.9 ± 2.15	20.5 ± 1.96	20.7 ± 2.43[2]	20.6 ± 2.16	0.18
20:3ω-6	2.63 ± 0.55	2.67 ± 0.66	2.67 ± 0.47	2.56 ± 0.50[3]	**0.01**
20:4ω-6	5.81 ± 1.27	5.80 ± 1.26	5.36 ± 1.13[2]	5.04 ± 1.09[2]	**0.01**
22:4ω-6	0.34 ± 0.32	0.32 ± 0.12	0.28 ± 0.10[3]	0.23 ± 0.08[2]	**<0.01**
22:5ω-6	0.17 ± 0.09	0.17 ± 0.09	0.21 ± 0.11[2]	0.14 ± 0.08[2]	**<0.01**
18:3ω-3	0.34 ± 0.09	0.34 ± 0.18	0.38 ± 0.11[2]	0.34 ± 0.10[2]	**<0.01**
20:5ω-3	0.40 ± 0.26	0.38 ± 0.16	0.32 ± 0.16[2]	0.37 ± 0.20	0.14
22:5ω-3	0.43 ± 0.15	0.41 ± 0.11	0.36 ± 0.12[2]	0.32 ± 0.24[2]	0.19
22:6ω-3	2.19 ± 0.76	2.21 ± 0.68	2.13 ± 0.78	2.82 ± 0.87[2]	**<0.01**

Data are means ± SD, g/100 g fatty acids.
[1] *P* value for differences between groups at the by stage of gestation, by ANCOVA.
[2] Value at 36 wk gestation different from 16 wk gestation within a group, $P < 0.01$, by paired t-test.
[3] Value at 36 wk gestation different from 16 wk gestation within a group, $P < 0.05$, by paired t-test.

Figure 2.4 Scatter plots to show the relationship between dietary DHA intake and the RBC PE DHA as g/100 g fatty acids in panels A and B, and RBC PC in panels C and D, at 16 wk gestation in panels A and C, and 36 wk gestation in panels B and D, women not taking any supplemental DHA.



Correlation coefficients were calculated using Spearman's rank correlation coefficient.

Analyses for categorical results for visual acuity at 2 and 12 mo, the pass/fail results for the problem-solving, non-native language consonant contrast test at 9 mo, and the word-object learning task at 16 mo are described first, followed by the results for the continuous variables (scores) for the CDI and BSID-III. Less than 50% of infants completed the word-object learning task at 16 mo, likely because of the test duration, and results for this test were not further analyzed. Using the results, we defined high visual acuity as an acuity ≥3.3 cycles/degree at 2 mo and ≥13 cycles/degree at 12 mo (Table 2.6). Infant girls showed a non-significant trend to higher visual acuity than boys at 2 mo ($P = 0.07$), but not 12 mo ($P = 0.79$). Infants in the placebo group were at increased risk of not achieving a visual acuity ≥3.3 cycles/degree at 2 mo (OR 2.50, CI 1.02–6.14, n = 184, $P = 0.03$), with no evidence of increased risk of failure to achieve high visual acuity at 12 mo (OR 1.23, CI 0.61–2.49, n = 176, $P = 0.35$, Table 2.6). Analysis of the pass/fail results for the problem-solving at 9 mo found more girls (65%) than boys (49%) were successful ($P = 0.03$), with no difference in success between the placebo and DHA groups when stratified by sex for infant girls ($P = 0.21$) or boys ($P = 0.27$). Similarly, we found no difference in ability to discriminate the non-native language consonant between boys and girls, or between the placebo and DHA groups at 9 mo ($P > 0.05$).

Table 2.6 Risk that an infant in the placebo group would fail to be among infants achieving high visual acuity.

Age mo	Acuity threshold cycles/degree[1]	%Placebo/DHA above acuity threshold	Odds ratio (CI)[2]	*P*
2	≥ 3.3	8.51/18.9	2.50 (1.02-6.14)	**0.03**
12	≥ 13	21.1/24.7	1.23 (0.61-2.49)	0.35

[1] High visual acuity was defined as a visual acuity ≥ 3.3 and ≥ 13 cycles/degree at 2 mo and 12 mo, respectively.
[2] Odds ratios and 5th-95th confidence interval (CI) determined using contingency tables with Fisher exact analysis.

Infant sex was significantly related to the performance on the CDI and BSID-III. Girls scored higher than boys on the CDI infant scale for words produced at both 14 and 18 mo ($P = 0.02$, $P = 0.01$, respectively), on the CDI infant scale for words understood at 14 mo ($P = 0.02$), and the CDI toddler scale of words produced ($P = 0.01$) and the BSID-III receptive ($P = 0.03$) and expressive ($P = 0.02$) language scales at 18 mo. There was no significant difference between

boys and girls on the BSID-III cognitive, fine motor or gross motor scales at 18 mo ($P > 0.05$). Representative frequency plots illustrating the distributions of test scores in all infants, boys and girls in the DHA and placebo groups on languages tests at 14 and 18 mo, and the BSID-III cognitive subscales for which effects of DHA were or were not found, respectively, are in Figure 2.5. Infants in the placebo group were at increased risk of not performing in the highest 25% of infants for words understood (OR 3.22, CI 1.49–6.94, $P = 0.002$) and produced (OR 2.61, CI 1.22–5.58, $P = 0.01$) at 14 mo, and for words understood (OR 2.77, CI 1.23–6.28, $P = 0.03$) and sentences produced (OR 2.60, CI 1.15–5.89, $P = 0.02$), with a similar trend for words produced ($P = 0.07$) at 18 mo (Table 2.7). Infants in the placebo group were also at increased risk of not performing in the highest 25% of infants on the BSID-III receptive language (OR 2.23, CI 1.08–4.60, $P = 0.03$) and expressive language scales (OR 1.89, CI 0.94–3.83, $P = 0.05$) at 18 mo. We found no evidence of increased risk that girls or boys in the placebo group would not be among the 25% of infants with the highest scores on the BSID-III cognitive ($P = 0.70$), fine motor ($P = 0.33$) or gross motor skill subscales ($P = 0.40$) at 18 mo.

Figure 2.5 Representative weighted Kernal density plots to show examples of the distributions of test scores for the DHA and placebo groups for all infants, boys and girls for outcomes in which maternal DHA decreased risk of not achieving high development: CDI, communicative developmental inventories and BSID, Bayley Scales of Infant development receptive and expressive language in panels A-L, or had no effect, BSID-III cognitive in panels M-O.

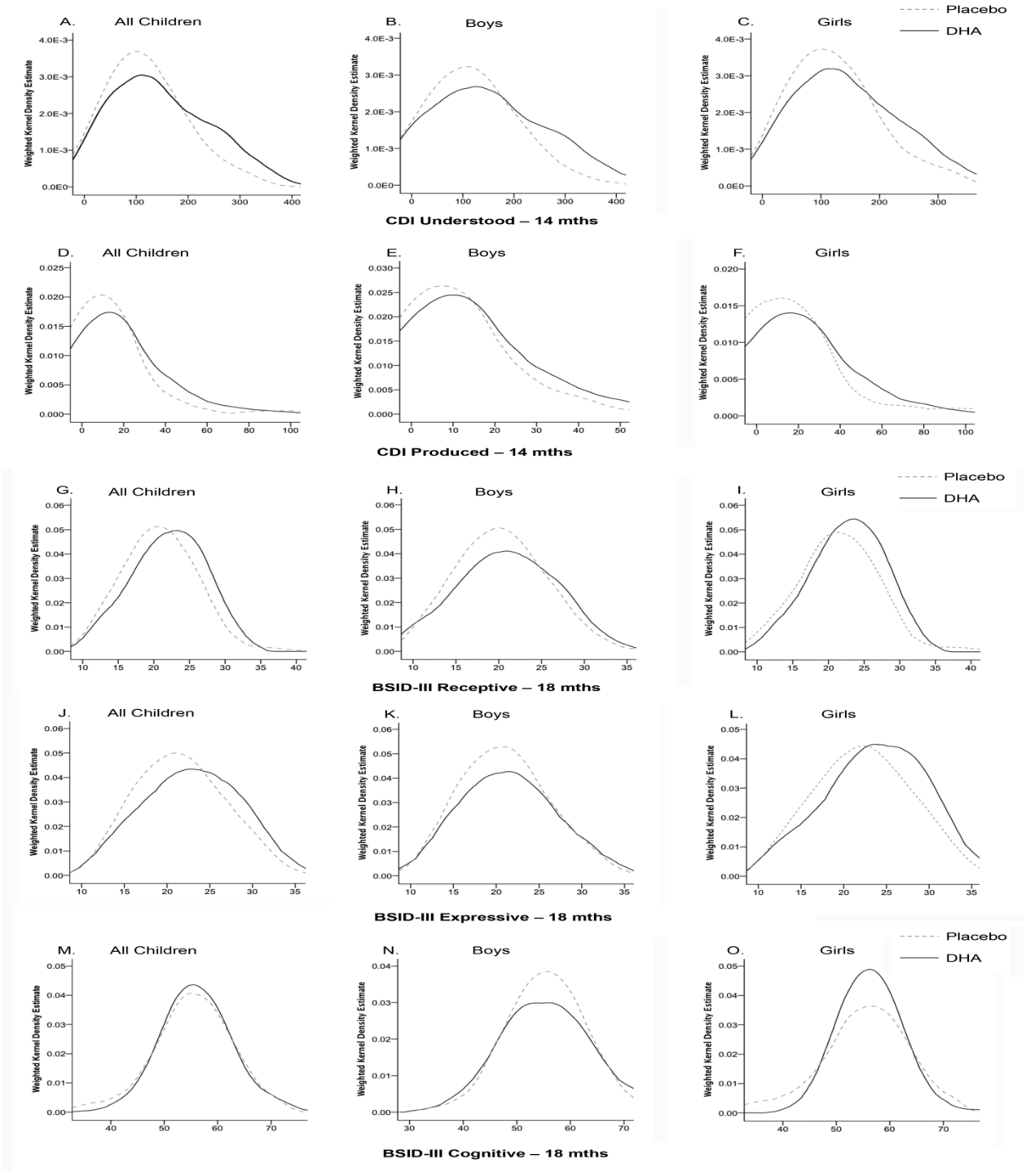


Table 2.7 Risk that an infant in the placebo group would fail to achieve high scores on tests of language, cognitive and motor skill development.

Test	Placebo/DHA in highest quartile[1]	Age	OR (CI)[2]	
	%	mo		*P*
Infant CDI				
Words understood	14.8/35.9	14	3.22 (1.49-6.94)	**0.002**
Words produced	16.0/33.3	14	2.61 (1.22-5.58)	**0.009**
Words understood	18.8/37.3	18	2.77 (1.23-6.28)	**0.01**
Words produced	19.1/37.3	18	2.01 (0.89-4.54)	0.07
Toddler CDI				
Words produced	17.1/35.0	18	2.60 (1.15-5.89)	**0.02**
BSID-III				
Receptive language	20.5/36.5	18	2.23 (1.08-4.60)	**0.03**
Expressive language	24.1/37.5	18	1.89 (0.94-3.83)	0.08
Cognitive	23.1/20.0	18	1.20 (0.55-2.60)	0.70
Fine Motor	25.6/30.1	18	1.25 (0.61-2.55)	0.33
Gross Motor	26.6/29.7	18	1.17 (0.58-2.37)	0.40

CDI, MacArthur Communicative Development Inventory; BSID-III, Bayley Scales of Infant Development III.
[1] High scores were defined as those scores achieved by the highest performing infants whereby n had the lowest possible deviation from 25% of all infants, by sex.
[2] Odds ratios and 5^{th}-95^{th} confidence interval (CI) determined using contingency tables with *P* values determined using Fisher exact analysis.

2.5 Discussion

The present study uses a risk-reduction model to assess whether some infants fail to meet their developmental potential due to insufficient DHA during gestation to meet the needs of the developing CNS. Our study does not address the reasons for inadequate DHA, does not identify dietary requirements, and does not aim to show that supplemental DHA enhances cognitive abilities. However, using a model designed to identify DHA insufficiency in the infant before birth, we provide novel data to show that DHA low enough to limit infant development to 18 mo of age does occur. In the discussion, we highlight the complexity of identifying ω-3 fatty acid deficiency before birth, and raise several points for studies designed with this intent.

In our study, women were enrolled at 16 wk gestation, and randomized to DHA or a placebo until delivery of the infant for the purpose of assessing if fetal CNS development is constrained among infants of women following their usual diet (Figure 2.1). Pragmatically, since DHA consumed by the mother is transported across the placenta [258,289] and provides DHA to the fetal CNS [195,203,261,410], it is expected that maternal DHA supplementation should reduce risk of insufficient fetal DHA. This concept is consistent with observation studies to show lower risk of poor child CNS development in infants and children of women consuming diets rich in fish or marine mammals [16,18,327,411]. However, the corollary that women with low intakes of DHA necessarily equate with fetal DHA inadequacy cannot be assumed from current knowledge. Data to show poor neurodevelopment in children of healthy vegetarians, despite their low diet and blood lipid DHA [274] has not been published. Variable maternal-to-fetal fatty acid transfer, and fetal ω-3 fatty acid metabolism and acylation into neural tissue may also limit extrapolation from measures of maternal DHA to DHA accretion in the fetal CNS. Recognition that assessment of fetal DHA insufficiency differs from assessment in breast-fed and formula-fed infants, children or adults in whom blood levels of DHA can be directly measured is important.

We randomized pregnant women to take four to ten fold more DHA than the estimated fetal DHA needs [413,414], then assessed risk that healthy term-infants born to mothers given a placebo would fail to be among the quartile of infants achieving the highest CNS development (Figure 2.1). Using this approach, we show increased risk of lower language development at 14 and 18 mo on the CDI infant and toddler scales ($P < 0.02$) and on the BSID-III ($P < 0.05$) at 18

mo among infants in the placebo group. Constraint of language development was robust, affecting both boys and girls, productive and receptive language, and evident in well-controlled testing using the BSID-III with testers blinded to the infant group. Visual acuity also showed evidence of delayed maturation in the placebo group when assessed at 2 mo (OR 2.69, CL 1.10–6.54, $P = 0.03$), but not at 12 mo. Previous prospective observation studies [16,330] and clinical trials with preterm infants given DHA [422] have also found language development appears to be sensitive to the early DHA supply. Possibly, these results reflect a role of DHA in the anatomical and molecular factors involved in early language learning. It is of interest that whereas auditory stimuli elicit large event related potentials over temporal brain regions with absent or small responses over occipital regions in the adult brain, 6 mo old infants show equally large response amplitudes over the visual and auditory cortex on auditory stimulation [423]. Maturation and increasing specificity of auditory sensory systems, fundamental to developmental sensitivities of grammatical and semantic/lexical (object-word association) language learning, involve a gradual decrease in responses of occipital regions to auditory stimuli from 6 to 36 mo. Although apparently not studied in humans, experimental work has reported evidence of altered auditory evoked potentials in offspring of animals given DHA during pregnancy [356]. We found no evidence of fetal DHA insufficiency sufficient to constrain cognitive, gross motor or fine motor development when assessed using the BSID-III at 18 mo. The categorical pass/fail results for problem solving and non-native language tests found no group differences, however, tests which do not enable ranking of infant skill development may lack the sensitivity to detect slower rates of CNS development in term infants.

Several studies have addressed the efficacy of maternal prenatal DHA supplementation as a means to increase infant and child neurodevelopment, with findings of no benefit on novelty preference at 6 or 9 mo [105], a small increase ($P = 0.049$) in mean scores on the mental composite scale of the Kauffman Assessment Battery for Children (KABC)-II at 4 y but not at 7 y [342,343], significant positive effects on group mean problem solving, but not novelty preference at 9 mo [335], and no significant difference in group mean test scores on the BSID-III at 18 mo [266,346], Griffiths Mental Developmental Scale or Picture Peabody Test at 2.5 y [340], the Hemple, Touwen, or KABC tests at 4, 5.5 or 6.5 y, respectively [349,350]. In our study, we also found no significant difference in group mean test scores between infants in the

placebo and DHA groups for any test, at any age. Our results thus concur with a conclusion that at a group level, supplemental DHA does not enhance cognitive development of infants born at term gestation [266]. Assuming no random group bias, our results for language development using the BSID-III estimate constraint of development in about 10% of infants in the placebo group, a number too small to detect by group mean comparisons with our sample size enrolled without a priori knowledge of deficiency.

Studies in other species have shown that ω-3 fatty acid deficiency results in increased tissue lipid 22:4ω-6 and 22:5ω-6 [3,5,120,195]. Experimental evidence for a pregnancy-associated increase in hepatic D6D gene expression has been reported [424]. Our results show increased 22:4ω-6 + 22:5ω-6 in RBC PE with advancing gestation in women in the placebo group (Figure 2.3). We interpret this as biochemical evidence of insufficient ω-3 fatty acids to support the gestational increase in fatty acid desaturation, which thus proceeded with ω-6 fatty acids. Whether this was due to insufficient ω-3 fatty acids, excess ω-6 fatty acids or some other factor is unclear. We found no significant relationship between the maternal RBC DHA at 16 or 36 wk gestation and infant CNS development. Regardless of the positive correlation between the maternal DHA intake and the RBC PE and PC DHA ($P < 0.001$), the RBC DHA showed considerable variability among women with similar DHA intakes (Figure 2.4). The RBC DHA levels also overlapped between women in the placebo and DHA group at 36 wk gestation after about 20 wk supplementation (Figure 2.3). Other studies also show high variability in blood lipid DHA and in the response to DHA supplementation. For example, the mean ± 2SD range of plasma phospholipid DHA was 50–153.6% and 79–215.4% fatty acids in 35 wk gestation women assignment to placebo or 1183 mg DHA + 803 mg 20:5ω-3/d from 17–19 wk gestation, respectively [289], and the RBC PE DHA was 2.92–10.2% and 3.46–14.1% fatty acids in term gestation women given a placebo or 500 mg DHA + 150 mg 20:5ω-3/d from 20 wk gestation [267]. Although the reasons for the overlap in blood lipid DHA among women with widely different DHA intakes is not known, it is evident that DHA acylation into blood lipids is more complex than DHA intake. Similar complexity in extrapolating DHA accretion in the fetal CNS from measures of the exogenous, i.e. maternal DHA supply, would seem likely.

This study has several important limitations. High breast-feeding rates, with 63–72% infants still breast-fed at 6 mo may have masked greater constraint of CNS development in

infants with shorter exposure to or no breastfeeding. Brain DHA accretion and neural synapse development continues throughout early childhood. Postnatal compensation or other nutrient deficits would dampen any effect of fetal DHA insufficiency on CNS development assessed after birth. Our definition of failure to be among the quartile of infants with the highest development test scores was arbitrary, and different definitions would yield different results. Although this study was randomized, it is possible that unmeasured variables in the home environment were present between the groups and contributed to the differences found. Regardless, this study was designed with a randomized intervention in a quasi-risk reduction model to provide evidence of fetal DHA insufficiency in our population. Using this approach, we have shown that language development is robustly constrained risk at different ages and with different tests in infants born to women consuming about 5 % energy from LA, 0.59 % energy as ALA and 85 mg/d DHA. We emphasize that we do not hypothesize that DHA enhances child neurodevelopment, but our data support a hypothesis of DHA insufficiency among infants of women following typical western diets for which risk may be reduced by increasing the maternal DHA intake.

Chapter 3: Fetal DHA Inadequacy and Child Neurodevelopment

3.1 Chapter synopsis

The research described in this chapter was designed with several aims to understand if the effects of fetal DHA inadequacy shown in Chapter 2 persist when CNS is assessed at 5.75 y. However, previous studies have shown positive associations between the maternal and child intake of some nutrients. Therefore, this study also addressed the potential similarities in DHA intake and status between mothers and their children. Participants from Chapter 2 were invited to participate in this follow-up study when the children were 5.75 y. Dietary intake information and a blood sample was obtained for the children, and CNS development for the children was assessed using several neurodevelopment tests. The findings in this chapter showed no significant differences in test score performance between children from the maternal DHA or placebo groups, and no differences between the groups of children in the achievement of a test score in the highest quartile, compared to the lowest quartile, of children. However, maternal RBC fatty acids at 16 and 36 wk gestation were related to child performance on some tests. Notably, there was a negative association between maternal RBC 22:4ω-6 and 22:5ω-6 and child performance on a test of visual-motor integration, and children of mothers in the highest quartile of RBC DHA had higher scores on multiple tests, including a language development assessment. In this chapter, significant positive associations of child DHA intake with maternal intakes at 16 and 36 wk gestation are reported. In addition, both DHA intake and RBC fatty acids in the child were related to maternal diet at 16 and 36 wk gestation and RBC PE and PC fatty acids at 16 wk gestation. Thus the results from this study indicate neurodevelopment effects of DHA insufficiency during gestation reported in Chapter 2 may have been lost or masked by other variables that occur in the children at 5.75 y. Associations between child test scores and maternal RBC 22:4ω-6, 22:5ω-6, and DHA/22:4ω-6 + 22:5ω-6 ratio suggest that 22:4ω-6 and 22:5ω-6 may be more sensitive markers of insufficient DHA than RBC DHA alone. Similarly, associations between the mothers during gestation and later child RBC 22:4ω-6, 22:5ω-6, and DHA/22:4ω-6 + 22:5ω-6 also tended to be stronger than the relationships to child RBC DHA. Therefore, in the subsequent chapter (Chapter 4), the relationship between child test scores, DHA intake and status was assessed in a larger cross-sectional group of children together with the prospective group.

3.2 Introduction

The ω-3 fatty acid DHA increases in the brain grey matter and retina PL during early development, with a well-known higher proportion of DHA in the brain and retina PL than other organs. Loss of brain DHA results in replacement with the ω-6 fatty acids 22:4ω-6 and 22:5ω-6 in both animals and humans [9,162], with the loss of DHA and increased 22:4ω-6 and 22:5ω-6 associated with impaired or reduced visual acuity and several aspects of neurological function in animals [9,10,121]. Several randomized intervention trials have reported higher visual acuity and neurological outcomes for infants and children born to mothers given DHA during gestation compared to a placebo [336,340,342], while others have found no benefit [105,266,339]. Similarly, several studies have provided evidence that addition of DHA to infant formula fed in the weeks or months after birth leads to greater visual and neurological development in term and preterm infants compared to formula with no DHA [292,296,357,358,365,425].

It is well known that nutritional deficiency of certain essential nutrients during development can have lasting, irreversible effects on the brain, which do not occur when the same deficiency occurs later on. For example folate deficiency occurring within 28 days post-conception may interfere with closure of the neural tube [192], while iodine deficiency prior to birth is associated with irreversible neurological consequences [191]. Deficiency of DHA in the brain may be caused by an inadequate source of DHA or its precursor, ALA which synthesizes DHA via EPA through desaturation and elongation [35]. A high intake of the ω-6 fatty acid LA has also been shown to be associated with DHA deficiency, with increased ARA in the brain during early development [1]. Loss of neural DHA when an ω-3 fatty acid deficient or high ω-6 fatty acid diet is fed to the mother during gestation has also been shown to alter neurotransmitter metabolism, neural function and behaviour in animals, including rodents and non-human primates [5,9,10,121,426,427]. In humans, several observation and intervention studies of DHA during pregnancy or pregnancy plus the first weeks after birth have reported no effect or a positive effect on neurodevelopment and visual acuity [16,18,19,328,335-340,342-344,365,398]. However, the effects are inconsistent among studies and also in outcome of the same children assessed at different ages.

The infant brain, however, is only about 25% of the adult brain weight at term gestation, being about 370 g, and increasing to about 70% of adult brain weight by 12 mo [177]. Synapses,

which are rich in DHA, increase with remarkable rates of formation of 40,000 synapses/second during the first two years, with structural reorganization and pruning of synapses leading to maximum synaptic density from around 6-7 y [21]. Both observational and randomized intervention studies have provided evidence that DHA intake and status of children up to about 10 y are also related to child neural function, including verbal learning ability [371], language [369], reading [370,375], spelling [375], non-verbal intelligence [27,375] and memory [26]. Reaction time and prefrontal cortex activation during a sustained attention test has also been associated with baseline RBC DHA in boys 8-10 y, and 400 or 1200 mg/d DHA for 8 weeks led to increased prefrontal cortex activation from baseline compared to controls [28]. This suggests that although DHA deficiency during gestation may have lasting effects, continued brain development after birth with child diet potentially impacting brain PL DHA may impact child brain function.

We previously reported that DHA insufficiency in some pregnant women in our population was low enough to limit infant neurodevelopment to 18 mo of age [398]. The primary purpose of the current study was to determine if fetal DHA insufficiency extends to neurological effects in children assessed at 5.75 y. This study also sought to determine whether the child's DHA intake and RBC status was related to the mother's DHA intake and RBC status during gestation.

3.3 Subjects and Methods

3.3.1 Study Design

This was a follow-up of children born to mothers who participated in a randomized, double-blind, placebo-controlled study to assess whether DHA deficiency during pregnancy occurs and is sufficient to limit infant CNS development up to 18 mo [398]. Briefly, pregnant women were randomized to 400 mg/d DHA or a placebo from 16 wk gestation until delivery, with follow-up only of singleton, term-gestation infants, with no congenital or metabolic diseases likely to impact infant neurological development. A total of 200 infants, n = 96 and n = 104 for maternal DHA or placebo supplementation groups, respectively, were invited for follow-up. Of these, 98 parents agreed to participate in this follow-up study that assessed dietary intake, DHA status, and CNS development in children at 5.75 y. New computer-generated codes were made

for all participants to avoid knowledge of the gestational intervention group and infant outcome data to 18 mo.

The present study was conducted according to guidelines laid down in the Declaration of Helsinki, with written informed consent obtained from a parent or guardian for each child before any assessment or data collection. All procedures involving human subjects in this study were approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia and the British Columbia Children's and Women's Hospital.

3.3.2 Subject Characteristics, Dietary Assessment and Biochemical Analysis

Family and sociodemographic information, including income, education, and number of adults and children in the home were updated by questionnaire. Parental IQ was assessed with the TONI-III, which assesses aptitude, abstract reasoning, and problem solving, without dependence on language or formal education [415]. Each child weight and height was measured, and then weight, height, and body mass index (BMI) z-scores calculated using WHO Anthroplus anthropometric calculator (version 1.0.4).

Dietary intake was assessed using a FFQ by face-to-face interview with the parent, using food models and measuring utensils, including all foods and beverages consumed by the child over the previous 4 wk. Dietary intake of macronutrients and fatty acids were quantified using nutrient analysis software (ESHA Food Processor SQL, version 10.10.0.0; ESHA Research), ensuring the database had complete and accurate fatty acid compositions based on product labels for our location, and where necessary our laboratory analyzed the food fatty acids.

Child venous blood (6 ml) was collected with EDTA, centrifuged (2500g, 10 min, 4^{o}C), the plasma and buffy coat separated, aliquoted and frozen at -80^{o}C within 30 min of blood collection. The RBC were washed by re-suspension with saline and EDTA, centrifuged twice, and then the RBC were frozen at -80^{o}C until analysis. RBC total lipids were extracted, then fatty acids quantified by routine gas liquid chromatography [25,38]. Preparation of blood and analysis of RBC was led by technician D.J. King, an expert with clinical samples for fatty acids.

3.3.3 Child Neural Development

Child neural development was assessed using age-appropriate standardized tests, in a dedicated room free of distractions, with a one-way mirror to enable the parent to view their child. The tests included the Kaufman Assessment Battery for Children, 2nd edition (KABC), which enables scaled subset scores to assess cognitive ability. The sequential processing scale measures short-term memory; the learning ability scale measures long-term storage and retrieval; and the simultaneous processing scale measures visual processing. General mental processing ability is also measured by combining all three scales to give a composite Mental Performance Index (MPI) score [428]. The KABC delayed recall was also done, which measures long-term memory. Language development using the Peabody Picture Vocabulary Test-4 (PPVT) [429] and visual-motor integration using the Beery-Buktenica Developmental Test of Visual-Motor Integration (Beery) [430] were also assessed. Finally, the Test of Variables of Attention (TOVA) [431] was used to assess attention and impulsivity, measured by the scores of Errors of Commission and Omission, respectively, and also Response Time, a measure of processing speed, and Response Time Variability, a measure of response time consistency.

3.3.4 Data Analysis

We used descriptive statistics to summarize subject characteristics, and compared variables between the DHA supplement and placebo groups using independent t-tests, Mann-Whitney *U* tests, or chi-square tests, as appropriate. Family and child characteristics including infant birth weight, sex and breast-feeding duration, maternal IQ, number of children and adults in the home were screened for potential associations with child neurodevelopment test scores.

The potential impact of gestation was assessed using contingency tables and Fisher exact tests to determine the OR and 95th percent confidence interval that a child from the placebo group would fail to score in the top quartile of children for each test. Where children could not be separated into an exact upper quartile due to several children with the same score, a cut point "quartile" giving the lowest deviation from 25% of the group was used. Our previous report showed a considerable overlap in maternal DHA status, measured as RBC PE and PC DHA, between the DHA and placebo groups after approximately 20 weeks of supplementation during pregnancy [398]. Thus, Pearson's correlation or Spearman's rank correlation coefficient, as

appropriate, were used to determine the potential relationship between maternal DHA status at 16 and 36 wk gestation and child performance on cognitive tests. In addition, children were grouped in quartiles of maternal RBC fatty acids at 16 and 36 wk gestation, and child test scores were compared between the highest and lowest quartile.

The potential association between the mother's prenatal dietary DHA intake and DHA status with the children's DHA intake and DHA status at 5.75 y was assessed using Pearson's correlation or Spearman's rank correlation coefficient, as appropriate. Due to the 400 mg/d DHA supplementation from 16 wk gestation to term infant birth, the relationship between the maternal RBC DHA, 22:4ω-6, 22:5ω-6, and the DHA/22:4ω-6+22:5ω-6 ratio was addressed for all children at 16 wk gestation, but not 36 wk gestation. Data analysis was done using IBM SPSS Statistics (Version 20.0.0, 2011. Chicago, IL), with significance set at $P < 0.05$.

3.4 Results

A total of 98 children, 5.75 y, from the study on DHA deficiency during gestation participated in this study. No significant differences were found in maternal or infant characteristics at birth or through the 18 mo infant follow-up between children who did not participate in this study (n = 102) and those who did (n = 98). For the group, 74.5% of the children were Caucasian, 12.2% were Chinese, and 13.3% were of other ethnicities; of the children 53.1% were girls, 83.5% of the children lived with two adults, 55.1% had one sibling, 89.8% were human milk-fed at least 3 mo, and had a BMI z-score mean (SD) 0.06 (1.01) at 5.75 y. No differences were found in maternal IQ measures, family characteristics, or the child sex, length of human milk-feeding, weight, height, or BMI assessment, $P>0.05$, between children in the placebo (n = 52) and DHA (n = 46) groups. Cognitive tests were completed for >90% of children, with incomplete tests for PPVT, n = 1; Kaufman ABC Sequential, n = 2; Learning, n = 4; Simultaneous, n = 1; MPI, n = 5; Delayed Recall, n = 1; Beery, n = 0; TOVA, n = 9. The FFQ was analyzed for all children, and blood samples sufficient for fatty acid analysis were obtained from 73 children.

Potential variables associated with child neural development were screened for all cognitive tests and showed a lower reaction time ($P = 0.003$) and higher percent errors of commission ($P = 0.004$) in boys than girls on the TOVA, and a higher PPVT scores for

Caucasian than non-Caucasian children (P = 0.001). Infant human milk-feeding duration showed no association with child development test results; however, only 10.2% were human milk-fed <3 mo. There was no effect of any other collected or measured child or family characteristic on child cognitive scores at 5.75 y.

No significant differences were found in mean test performance between children in the maternal gestation DHA and placebo groups (Table 3.1). We also found no differences between children in the two maternal groups in achieving performance in the upper quartile of test performance (Table 3.2); PPVT (P = 1.00), Beery (P = 1.00), the KABC sequential processing (P = 0.808), learning ability (P = 0.628), simultaneous processing (P = 0.818), mental performance index (P = 0.476), and delayed recall (P = 0.825), or on the TOVA response time variability (P = 0.739), response time (P = 0.717), errors of commission (P = 0.645), and errors of omission (P = 0.111).

Table 3.1 Cognitive scores for all children and by maternal DHA supplement or placebo group.

	n Placebo, DHA	All	Placebo Group	DHA Group	*P*
PPVT	52,45	117 ± 18.2	115 ± 20.6	119 ± 15.0	0.283
Beery[1]	52,46	17.0 (12.5-21.0)	16.0 (12.3-21.0)	17.0 (12.2-20.6)	0.117
KABC					
Sequential	52,44	21.9 ± 5.00	21.9 ± 5.36	21.9 ± 4.59	0.981
Learning	52,42	23.1 ± 5.41	23.0 ± 5.79	23.2 ± 4.95	0.836
Simultaneous[1]	52,45	35.0 (18.4-44.0)	34.5 (14.6-43.4)	36.0 (22.3-44.0)	0.086
MPI[1]	51,42	81.0 (50.4-98.6)	81.0 (42.3-102)	83.5 (72.0-88.0)	0.293
Delayed Recall	52,45	22.2 ± 3.99	22.3 ± 4.30	22.0 ± 3.64	0.725
TOVA					
RT Variability, ms	47,42	662 ± 102	658 ± 101	666 ± 103	0.717
Response Time, ms	47,42	245 ± 57.1	243 ± 48.5	246 ± 65.9	0.739
Errors of Commission[1], %	47,42	9.88 (1.25-31.7)	9.26 (1.23-28.5)	10.2 (1.35-36.2)	0.645
Errors of Omission[1], %	47,42	13.7 (0.78-59.7)	17.3 (0.12-67.0)	9.88 (1.24-59.7)	0.111

PPVT, Peabody Picture Vocabulary Test; Beery VMI, Beery-Buktenica Developmental Test of Visual-Motor Integration; KABC, Kaufman Assessment Battery for Children, 2nd edition; ms, milliseconds; MPI, Mental Performance Index; TOVA, Test of Variables of Attention; RT, Response Time
Data are mean ± SD or median (2.5-97.5 percentile) and compared by student's t-test or Mann-Whitney *U* test.
[1]Data is not normally distributed.

Table 3.2 Risk that a child in the placebo group would be in the lowest quartile rather than the highest quartile of cognitive development test scores.

	Quartile of test performance (DHA/Placebo)				
	1	2	3	4	OR (CI)[1]
PPVT	22.2/26.9	31.1/21.2	22.2/28.8	24.4/23.1	1.28 (0.41 – 4.06)
Beery	10.9/19.2	28.3/34.6	43.5/28.8	17.4/17.3	1.78 (0.42 – 7.47)
KABC					
Sequential	22.7/30.8	29.5/21.2	22.7/26.9	25.0/21.1	1.60 (0.51 – 5.05)
Learning	21.4/26.9	23.8/30.8	28.6/21.2	26.2/21.1	1.56 (0.48 – 5.08)
Simultaneous	15.6/28.8	24.4/21.2	31.1/25.0	28.9/25.0	2.14 (0.66 – 6.98)
MPI	16.7/29.4	26.2/19.6	21.4/25.5	35.7/25.5	2.47 (0.77 – 7.92)
Delayed Recall	26.7/23.1	17.8/25.0	28.6/29.4	28.6/21.6	0.80 (0.26 – 2.41)
TOVA[2]					
Response Time	26.2/25.5	28.6/21.3	21.4/27.6	23.8/25.5	0.92 (0.28 – 2.95)
RT Variability	26.2/23.4	40.3/29.8	28.6/21.3	26.2/25.5	0.91 (0.28 – 2.94)
Errors of Commission	23.8/29.8	31.0/19.1	26.2/21.3	19.0/29.8	0.80 (0.24 – 2.63)
Errors of Omission	21.4/29.8	16.7/31.9	33.3/17.0	28.6/21.3	1.87 (0.57 – 6.11)

Beery VMI, Beery-Buktenica Developmental Test of Visual-Motor Integration; CI, 95% confidence interval; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; OR, odds ratio; PPVT, Peabody Picture Vocabulary Test; RT, Response Time; TOVA, Test of Variables of Attention
Data are % of children from maternal DHA group/% of children from maternal placebo group. The number of children for each test as n = DHA/Placebo are: PPVT, n = 45/52; Beery, n = 46/52; KABC Sequential, n = 44/52; Learning, n = 42/52; Simultaneous, n = 45/52; MPI, 42/51; Delayed Recall, n = 45/52; TOVA scores, n = 42/47.
[1] $P > 0.10$ for each test.
[2] For TOVA scores, better performance is the achievement of a lower score; data was analyzed as the lowest scores in quartile 4, and highest scores in quartile 1.

The assessment of maternal DHA status during gestation and child cognitive performance showed an inverse relationship between the child's Beery score and maternal 16 wk RBC PC 22:4ω-6 (rho = -0.332, *P* = 0.001), RBC PC 22:5ω-6 (rho = -0.264, *P* = 0.010), and a positive association with the maternal DHA/22:4ω-6+22:5ω-6 in RBC PE (rho = 0.247, *P* = 0.016) and PC (rho = 0.275, *P* = 0.007) at 16 wk gestation (data not shown). Maternal 16 wk RBC PC 22:5ω-6 was positively associated with KABC delayed recall scores (rho = 0.208, *P* = 0.046). In addition, the child's Beery scores were inversely related to maternal 36 wk RBC PE 22:4ω-6 (rho = -0.247, *P* = 0.016) and positively related to 36 wk RBC PE DHA/22:4ω-6+22:5ω-6 (rho = 0.221, *P* = 0.032). There were no associations with maternal RBC PC or PE fatty acids of interest at 16 or 36 wk gestation and any other test scores (data not shown).

Next, we assessed child scores on neurodevelopmental tests by quartiles of maternal RBC fatty acids. There were no differences in child test scores by quartiles of maternal RBC fatty acids at 16 wk gestation (data not shown), with the exception of the Beery. Children of mothers in the highest quartile of 16 wk RBC PE 22:4ω-6 + 22:5ω-6 had lower mean scores than the lowest quartile (15.6 ± 1.64 and 17.2 ± 1.64, *P* = 0.002), with similar results for 16 wk RBC PC 22:4ω-6 + 22:5ω-6 (15.6 ± 1.75 and 17.4 ± 2.04, *P* = 0.002) (data not shown). In addition, children of women in the highest quartile, compared to the lowest quartile, of maternal 16 wk RBC PE DHA/22:4ω-6 + 22:5ω-6 had higher Beery scores (17.0 ± 2.14 and 15.7 ± 1.73, *P* = 0.032), again with similar results for 16 wk RBC PC DHA/22:4ω-6 + 22:5ω-6 (17.2 ± 2.21 and 15.7 ± 1.88, *P* = 0.014) (data not shown). Using results for 36 wk gestation, compared to the lowest quartile, children in the highest quartile of maternal RBC PE DHA had higher scores on the PPVT (*P* = 0.02) and KABC Sequential (*P* = 0.045) and Simultaneous (*P* = 0.019) scales, with a non-significant trend for the MPI (*P* = 0.057) (Table 3.3). In addition, the maternal 36 wk RBC PE 22:4ω-6+22:5ω-6 showed lower child Beery scores for the highest compared to lowest quartile (15.8 ± 2.28 and 17.0 ± 1.90, *P* = 0.002) (data not shown). For RBC PC DHA at 36 wk gestation, only a close non-significant trend for higher scores on the KABC simultaneous scale for children in the highest quartile compared to the lowest quartile (35.5 ± 5.50 and 32.4 ± 6.01, *P* = 0.054) was observed (data not shown).

Table 3.3 Child test scores by quartiles of maternal RBC PE DHA at 36 wk gestation.

	Quartiles of maternal 36 wk RBC PE DHA				
	≤6.81	6.87 - 8.44	8.53 - 10.3	≥10.51	P^1
PPVT	108 ± 19.5	120 ± 16.2	117 ± 21.1	120 ± 14.4	**0.020**
Beery[2]	15.9 ± 2.21	16.7 ± 1.72	17.0 ±2.29	16.7 ± 1.95	0.168
KABC					
Sequential	20.4 ± 4.84	21.4 ± 3.63	21.7 ± 5.12	23.6 ± 5.70	**0.045**
Learning	22.1 ± 5.62	24.3 ± 4.91	23.3 ± 5.35	22.4 ± 6.00	0.860
Simultaneous[2]	31.4 ± 6.54	34.6 ± 5.05	34.9 ± 4.79	35.7 ± 5.67	**0.019**
MPI[2]	74.0 ± 12.9	80.3 ± 10.2	80.0 ± 11.8	81.2 ± 12.5	0.057
Delayed Recall	21.8 ± 4.09	23.2 ± 3.50	22.0 ± 4.20	21.3 ± 4.14	0.685
TOVA					
RT Variability, ms	237 ± 56.2	254 ± 49.5	241 ± 67.7	244 ± 58.4	0.668
Response Time, ms	662 ± 119	670 ± 97.1	666 ± 97.8	644 ± 95.9	0.575
Errors of Commission[2], %	10.8 ± 7.61	10.0 ± 5.58	11.6 ± 8.77	11.7 ± 7.40	0.709
Errors of Omission[2], %	21.5 ± 20.6	15.4 ± 12.6	18.8 ± 14.2	16.6 ± 12.3	0.733

PE, phosphatidylethanolamine; PPVT, Peabody Picture Vocabulary Test; Beery, Beery-Buktenica Developmental Test of Visual-Motor Integration; KABC, Kaufman Assessment Battery for Children; ms, millisecond; MPI, Mental Performance Index; TOVA, Test of Variables of Attention; RBC, red blood cell; RT, Response Time
Data are mean ± SD. The number of children for each test are: PPVT, n = 97; Beery, n = 98; KABC Sequential, n = 96; Learning, n = 94; Simultaneous, n = 97; MPI, 93; Delayed Recall, n = 97; TOVA scores, n = 89.
[1] Comparison of child test scores between lowest and highest maternal RBC PE DHA quartiles by student t-test.
[2]Data is not normally distributed and analyzed by Mann-Whitney *U* test.

Since previous studies have shown associations between the maternal and child diet [432-435], we addressed the relationship of child DHA intake and RBC DHA to that of the mother's. DHA intake among the children was skewed, with a median (2.5-97.5 %) of 43.2 mg/d (1.73 − 300) mg/d, and no difference between boys and girls (P = 0.524), or between children in the gestation placebo and DHA groups (P = 0.603) (data not shown). The child DHA intake at 5.75 y was significantly related to their mother's DHA intake at both 16 wk and 36 wk gestation, rho = 0.317, P = 0.002 and rho = 0.256, P = 0.013, respectively (Table 3.4). In addition, the children's DHA intake was related to maternal RBC PE and PC DHA/22:4ω-6+22:5ω-6 at 16 wk gestation, rho = 0.297, P = 0.004 and rho = 0.264, P = 0.010, respectively, (n = 94) (Table 3.4).

Child RBC DHA mean (SD) was 5.20 (1.43) %TFA, with 3.31 (0.68) and 0.63 (0.16) 22:4ω-6 and 22:5ω-6, respectively, and RBC DHA/22:4ω-6+22:5ω-6 of median (2.5-97.5 %) 1.31 (0.60-3.34) %TFA. Child RBC DHA was significantly related to child DHA intake (rho = 0.396, $P < 0.001$). The relationship between child RBC DHA at 5.75 y and maternal DHA intake and RBC DHA at 16 wk gestation is shown in Table 3.5. Child RBC DHA was related to maternal DHA intake at 36 wk gestation (rho = 0.277, $P = 0.021$), but not 16 wk gestation (rho = 0.195, $P = 0.109$). The children's RBC DHA was related to maternal 16 wk RBC PE DHA (rho = 0.239, $P = 0.048$), RBC PE 22:5ω-6 (rho = -0.286, $P = 0.017$), and the RBC PE DHA to 22:4ω-6 + 22:5ω-6 ratio (rho = 0.313, $P = 0.009$), but not RBC PE 22:4ω-6 (r = 0.124, $P = 0.312$). Child RBC DHA was also related to the RBC PC DHA/22:4ω-6 + 22:5ω-6 ratio (rho = 0.306, $P = 0.010$), but not RBC PC DHA (r = 0.219, $P = 0.375$), RBC PC 22:4ω-6 (rho = -0.080, $P = 0.512$), or RBC PC 22:5ω-6 (rho = -0.120, $P = 0.323$) at 16 wk gestation.

Table 3.4 Associations of child dietary DHA with maternal dietary and RBC markers of DHA sufficiency during gestation.

	Child DHA Intake	
Mother	rho	*P*
16 wk gestation DHA intake	0.317	**0.002**
36 wk gestation DHA intake	0.256	**0.013**
16 wk gestation RBC PC		
DHA	0.074	0.481
22:4ω-6	-0.191	0.065
22:5ω-6	-0.183	0.077
DHA/ 22:4ω-6 + 22:5ω-6	0.264	**0.010**
16 wk gestation RBC PE		
DHA	0.197	0.057
22:4ω-6	-0.138	0.183
22:5ω-6	-0.199	0.054
DHA/ 22:4ω-6 + 22:5ω-6	0.297	**0.004**

DHA, docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine
Data are Spearman's rank correlation coefficient, for all mother-child pairs with available dietary DHA and RBC fatty acids, n = 69.

Table 3.5 Associations of maternal dietary and RBC DHA during gestation with the children's RBC markers of DHA sufficiency.

		Child RBC fatty acids			
		DHA	22:4ω-6	22:5ω-6	DHA/ 22:4ω-6 + 22:5ω-6
Mother					
Maternal DHA intake					
16 wk gestation	Rho	0.195	-0.292	-0.333	0.301
	P	0.109	**0.015**	**0.005**	**0.012**
36 wk gestation	Rho	0.277	-0.342	-0.233	0.381
	P	**0.021**	**0.004**	0.055	**0.001**
16 wk gestation RBC PC					
DHA	Rho	0.219	-0.197	-0.208	0.393
	P	0.375	0.101	0.084	**0.001**
22:4ω-6	Rho	-0.080	0.091	0.104	-0.099
	P	0.512	0.453	0.390	0.414
22:5ω-6	Rho	-0.120	0.141	0.153	-0.148
	P	0.323	0.244	0.206	0.220
DHA/ 22:4ω-6 + 22:5ω-6	Rho	0.306	-0.206	-0.194	0.333
	P	**0.010**	0.087	0.107	**0.005**
16 wk gestation RBC PE					
DHA	Rho	0.239	-0.065	0.077	0.226
	P	**0.048**	0.594	0.531	0.062
22:4ω-6	Rho	-0.124	0.373	0.221	-0.329
	P	0.312	**0.002**	0.068	**0.006**
22:5ω-6	Rho	-0.286	0.279	0.278	-0.392
	P	**0.017**	**0.020**	**0.021**	**0.001**
DHA/ 22:4ω-6 + 22:5ω-6	Rho	0.313	0.312	-0.117	0.400
	P	**0.009**	**0.009**	0.337	**0.001**

DHA, docosahexaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine
Data are Spearman's rank correlation coefficient, for all mother-child pairs with available dietary DHA and RBC fatty acids, n = 69.

3.5 Discussion

We used a risk-reduction model to address the potential long-term effect of DHA during gestation on cognitive performance when assessed at 5.75 y. Although we found DHA insufficiency during gestation was associated with a greater risk of failure to achieve a high score in infant neurodevelopment tests to 18 mo [398], evidence of lasting effects in children to 5.75 y was not present. The apparent effects of DHA insufficiency during gestation on CNS development up to 18 mo of age may have been lost or masked by postnatal dietary or other variables in children at 5.75 y. The potential importance of the child's postnatal diet to continued brain development, and the association between the children's dietary habits and that of the mother raises the question of the implications of epidemiological pregnancy studies that consider only the maternal diet or status on long-term child outcome.

The primary purpose of the current study was to determine if DHA insufficiency during gestation had long-term effects on neurological development when assessed in a prospective group of children at 5.75 y. In our previous report we found that infants from the placebo group were at increased risk of not achieving a high visual acuity score at 2 mo, although this was not detected at 12 mo [398]. Infants from the placebo group were also at increased risk of not achieving a score in the highest quartile for tests of language development at 14 and 18 mo [398]. At 5.75 y there was no difference in risk of failure to achieve a high score between maternal DHA and placebo groups on the language test (PPVT) ($P = 1.00$), or any other cognitive test assessed, ($P > 0.05$). As discussed, although studies of the effect of DHA supplementation during pregnancy on infant outcome have reported mixed results, the majority of studies on long-term outcome from 2 to 7 years have reported no benefit of maternal DHA supplementation during pregnancy on tests of child attention [338], working memory and inhibitory control [338], general mental processing ability (KABC MPI), short-term memory (KABC Sequential), and visual processing (KABC Simultaneous) [343] or the Hempel and Touwen neurological examinations [349]. In contrast, one study found higher mean scores for hand-eye coordination in children 2.5 y (n = 72) born to mothers taking fish oil during pregnancy compared to a placebo [340]. One other study reported a slightly higher mean score on general mental processing ability (KABC MPI) in a subset of children at 4 y (n = 84) whose mothers had taken cod liver oil compared to a placebo during pregnancy ($P = 0.049$) [342], although this was

not seen when the children were assessed at 7 y (n = 143) [343].

In most DHA interventions, the placebo groups do not have 'non-exposure,' which leads to overlap of nutrient intake and status with the DHA supplementation group. Although a higher amount of supplemental DHA may mitigate the overlap to some extent, nutrient intake exceeding individual requirements should not have additional benefit. Thus, our study differs from efficacy studies in that it was designed to detect if fetal DHA deficiency extended to 5.75 y, and not to show that DHA supplements during gestation enhance neurodevelopment in childhood. Since both the DHA and placebo groups in our study would likely contain individuals who are not deficient and hence unable to respond to intervention, we did not expect to find differences in mean scores between the groups. It is possible that an intervention in a population with a high prevalence of DHA deficiency may enable the detection of an efficacious response to increased DHA intake. However, a biomarker of DHA insufficiency is currently unavailable.

As expected, our study failed to detect a linear relationship between maternal RBC DHA at 16 or 36 wk gestation and child cognitive scores at 5.75 y. However, the highest quartile of maternal RBC PE DHA at 36 wk gestation was associated with child test scores 10-15% higher on measures of language (PPVT), short-term memory (KABC Sequential) and visual processing (KABC Simultaneous), compared to the lowest quartile. Similarly, Escolano-Margarit *et al* (2011) examined the relationship of maternal DHA status at birth and child outcome at 5.5 y, and reported that linear correlations between maternal RBC DHA and child neurodevelopment were not detected [349]. In the latter study, mean maternal RBC PE and PC DHA were 3.6% and 2.1% higher, respectively, for children with an optimal neurological optimality score (NOS) compared to children with a suboptimal NOS score [349].

Notably, in the present study, visual-motor integration (Beery) scores were negatively associated with maternal prenatal RBC 22:4ω-6 and 22:5ω-6. Both 22:4ω-6 and 22:5ω-6 have been shown to accumulate in the brain when brain DHA is low [162,163]. In animals, increased brain 22:4ω-6 and 22:5ω-6 is associated with functional consequences [9], but it is unknown if the negative effects are attributable to low brain DHA or the high long-chain ω-6 fatty acids. A SNP in FADS and ELOVL genes may also limit endogenous synthesis of DHA, however, 20:4ω-6, 22:4ω-6 and 22:5ω-6 may depend on the same enzymes for synthesis [59,62,63]. However, whether or not SNPs in FADS and ELOVL genes may protect the brain from

accumulating 22:4ω-6 and 22:5ω-6 in the absence of DHA is unclear, and thus further work is required to determine if the effects of low brain DHA are observed if levels of 22:4ω-6 and 22:5ω-6 are proportionately low.

The brain of a healthy term-born infant doubles its birth weight (~370 g) in the first six postnatal months [177], and DHA to support continuing brain growth must be provided by ω-3 fatty acids in human milk or infant formula. Accretion of DHA in the infant brain continues rapidly after birth for about 2 y, with brain PL DHA increasing until approximately 8 y [1,436]. During this period, the brain undergoes morphological and functional changes including synaptic proliferation, remodeling, and pruning [20], but whether prenatal DHA insufficiency alone is sufficient to alter these processes is unknown. Notably, the DHA intake of the present group of children was highly skewed, with a median intake of 43.2 mg/d and 2.5 – 97.5% intake of 0 – 300 mg/d. The DHA intake of children in this study is within the range of international estimates for this age group, but the IQR of 17.4 – 97.4 mg/d is higher than the population IQR for DHA intake of US children 1 – 5 y of 10.8 – 26.2 mg/d (n = 2496) [99]. Although it is not known if the level of intake is sufficient for continued brain development of the young child, positive associations have been found between neurodevelopment and both ω-3 intake and DHA status in children up to 10 y [26,27,369].

This chapter highlights the challenge in understanding if long-term effects on outcomes, particularly those with a developmental time-course extending beyond infancy, are explained by dietary intake during pregnancy or by the child's own diet, if children's diets reflect the diet of the mother. The present study found associations between maternal prenatal and child DHA intake and status, suggesting dietary and potentially genetic similarities between mothers and children. Maternal DHA intake at 16 wk and 36 wk gestation was positively associated with child DHA intake, and maternal DHA intake at 36 wk gestation was positively associated with child RBC DHA at 5.75 y. Although the association between the maternal and child DHA intakes have not previously been published, several studies have reported a relationship between the maternal and child diet [432-435], with associations stronger for the maternal-child diet than the paternal-child diet [432,433]. The Framingham Children's study reported that at 3-5 y, the child's polyunsaturated fat intake was positively associated with the mother's ($r = 0.33$, $P \leq 0.01$, $n = 87$), but not the father's intake ($r = 0.10$, $P > 0.05$, $n = 83$), with similar findings for several

other nutrients [433]. Similarly, the ALSPAC study reported a positive association between the child's intake at 10 y and the maternal prenatal (n = 5717) and postnatal (n = 5593) diet for protein, carbohydrate, and fat, but only child protein intake was associated with the paternal diet (n = 3009) [432]. Notably, the relationship between child and maternal prenatal total fat intake was stronger than the postnatal intake [432]. For a subsample of a prospective cohort study in Italy, fish and seafood intake was lower in the prenatal diet than the postnatal diet, but there was no difference in fish intake between mother's postnatal diet and fish intake of the children at 8-11 y (n = 37) [434].

The results of the present study have shown that the effects of maternal DHA during gestation observed in infants to 18 mo were not identified at 5.75 y. The potential long-term effects of DHA insufficiency may be too small to detect with our sample size and design, or it is possible that the DHA intake of our population was not low enough during gestation to have lasting effects. Human brain development begins as early as the third week of gestation, and while considerable DHA accretion has been reported to occur during the brain growth spurt beginning in the third trimester, it is unknown if a low DHA supply early during gestation compromises embryonic brain development. Most intervention studies of prenatal DHA and infant neurodevelopment began supplementation in the second trimester, and thus our intervention at 16 wk gestation may have been too late if DHA is important for early structural brain development. However, the inverse association of child Beery scores with maternal RBC 22:4ω-6 and 22:5ω-6 may suggest that visual-motor integration development is sensitive to low prenatal DHA, consistent with the time-course of brain maturation, with maturation occurring in the visual cortex before the prefrontal cortex [20]. The association between the maternal and child diet may explain the discrepancy between epidemiologic and intervention studies of prenatal DHA on child outcome, as it seems reasonable to assume that mothers with low DHA intakes during pregnancy may have children with low DHA intakes. Thus, with results of several studies also suggesting that the DHA supply in childhood is associated with brain development after birth, separating the pre- and post-natal effects of DHA supplies on brain development is a challenge. Regardless, evidence that even short-term DHA supplementation in children improved multiple areas of neural function [28,370,371,375], raises the possibility that the child's DHA intake and DHA status at 5.75 y may impact neurological performance.

Chapter 4: The Role of Child DHA Intake and RBC DHA on Cognitive Performance.

4.1 Chapter Synopsis

The research presented in Chapter 4 combines children who were described in Chapter 3 with a new cross-sectional group of children assessed at the same age and time, addressing the potential relationship of child DHA intake and RBC DHA with neurodevelopment. A secondary purpose was to determine if children who participate in long-term studies with multiple measurements differ from a cross-sectional group of children. All methods in Chapter 4 were identical to those in Chapter 3, with the exception that maternal dietary intake and RBC fatty acids were not collected. The study in Chapter 4 also addressed the relationship between RBC fatty acids and DHA intake when assessed using an FFQ as well as one and three 24 h recalls, with the major dietary sources of DHA among the children explored. The results of Chapter 4 showed no relevant differences between the children enrolled during gestation (wk 16) then followed to 5.75 y, and a cross-sectional group of children. For all children, the DHA intakes were highly variable when assessed by FFQ and one or three 24 h recalls, with a stronger relationship between RBC DHA and DHA intake assessed by FFQ. Observed ethnic differences in DHA intake, RBC fatty acids and neurodevelopmental test scores led to analysis of the relationship between DHA intake and RBC DHA with neurodevelopmental test scores for Caucasian children only. Dietary DHA was positively associated with a measure of short-term memory (KABC Sequential), and children with scores on this test in the highest quintile had a higher DHA intake than the lowest quintile. Dietary DHA was not associated with scores on any other test. The RBC DHA was positively associated with measures of language (PPVT) and short-term memory (KABC Sequential) scores, with a trend for general mental processing ability (KABC MPI), and a positive association also observed for RBC DHA/DHA + 22:5ω-6 with scores on measures of language (PPVT), visual-motor integration (Beery), and short-term memory (KABC Sequential). Higher RBC DHA was observed for the quintile of children with the highest scores for short-term memory (KABC Sequential) and general mental processing ability (KABC MPI), with a trend for language (PPVT) scores, compared to the lowest quintile of children. In addition, RBC DHA/ 22:4ω-6 + 22:5ω-6 was higher in the quintile of children with the highest scores for tests of language (PPVT) and short-term memory (KABC Sequential)

than the quintile of children with the lowest scores. The RBC DHA was not related to long-term storage and retrieval (KABC Learning), visual processing (KABC Simultaneous), long-term memory (KABC Delayed Recall), or attention and impulsivity (TOVA) scores.

4.2 Introduction

The ω-3 fatty acid DHA is an important component of neural lipids, accumulating in neural tissue during development. Rapid DHA accretion in the brain begins *in utero* and continues until at least 2 y [1], suggesting that a postnatal source of ω-3 fatty acids to supply the developing brain with DHA is important. DHA can be synthesized endogenously from ALA via FADS and ELOVL enzymes [35]. However, a high dietary intake of LA may antagonize metabolism of ALA to DHA, through saturation of the FADS and ELOVL enzymes [23,36,46,47]. DHA, however, can also be consumed in the diet in the form of animal tissue lipids, particularly aquatic organisms, with smaller amounts in eggs, poultry, and very small amounts in ruminants, depending on the tissue [70]. Decreased brain DHA may therefore result from inadequate ω-3 and/or excessive ω-6 fatty acid intakes, but is likely to occur only when the dietary DHA is very limited. Thus, dietary DHA should support brain DHA needs, regardless of dietary ω-6 fatty acid or ALA intakes.

Studies in animals and human infants have shown lower brain DHA is accompanied by an increase in brain 22:4ω-6 and 22:5ω-6 [9,162,163,437], which in animals is associated with deficits in neural function [9,10,121,195,210,437]. In humans, several observational and intervention studies of DHA in maternal gestation alone or including gestation and the first few weeks after birth have reported no effect [337-339], or effects changing with time or positive effects on neural and visual function of infants and children [16,18,19,328,336,340,342-344,398]. However, the DHA intake or blood lipid status of children has also been shown to be associated with CNS function, including learning ability, language, and non-verbal intelligence, with some studies showing a benefit of DHA supplement [26-28,369-373,375]. This raises the possibility that the pre- and/or post-natal DHA supply have the potential to impact CNS development when assessed in childhood.

Although brain growth is about 70% of adult weight by 1 y [177], early childhood is an important period of brain development, with a rapid increase in synapses rich in DHA continuing

until 6-7 y [21]. This suggests brain DHA availability, and hence diet, may be important for the morphological and organizational changes of brain development continuing in early childhood. However, the dietary, genetic and other variables that impact DHA transfer to the brain and the effect on neurodevelopment remain unclear

Associations between maternal and child dietary intakes complicate interpretation of epidemiologic studies on DHA intake or DHA in blood lipids during pregnancy or childhood on CNS related outcomes in children [432-435]. For example, a positive association was reported for the intake of polyunsaturated fat between mothers and children 3-5 y [433]. It has also been reported that fish intake of mothers and children 8-11 y was not different, and that the mothers' prenatal fish intake was lower than their postnatal intake [434]. Thus**,** understanding if maternal intakes during pregnancy impact the child's diet is clearly important. However, it is also clear that increased DHA intake in childhood can improve neural function in some children [27,28,370], possibly overcoming or correcting effects of inadequate DHA in early development.

As previously noted, the needs of children for ω-6 and ω-3 fatty acids, and specifically DHA for CNS development, knowledge of their dietary intakes and implications for ω-3 fatty acid inadequacy is limited. Therefore, the purpose of this study was to assess the DHA intake and status, and determine the association with neurodevelopment test scores in a group of young children in Vancouver, B.C. In addition, this study addressed if family and individual characteristics of children who participated in the prospective cohort (Chapter 3) and a cross-sectional group had any meaningful differences.

4.3 Methods

4.3.1 Study Design

This study enrolled children 5.75 y with a parent from the community in Vancouver, BC: these participants were a cross-sectional group with no previous involvement in our study. A group of 98 children with a parent who had participated in the study of DHA during gestation (Chapter 3) was also studied at 5.75 y (follow-up group). Inclusion criteria in both groups included term gestation, single birth, no known conditions that may interfere with dietary intake, metabolism, growth, or development, and the parental ability to communicate in English. For the follow-up group, the children must have been participants in the pregnancy study in which

the mother was given 400 mg/d DHA or a placebo from 16 wk gestation until delivery. All participants were assigned a unique, computer-generated code for all study documents and blood samples.

The present study was conducted according to guidelines laid down in the Declaration of Helsinki. All procedures involving human subjects in this study were approved by the Committee for Ethical Review of Research Involving Human Subjects at the University of British Columbia and the British Columbia Children's and Women's Hospital. Written informed consent was obtained from a parent for each child before enrolment.

4.3.2 Subject Characteristics

Sociodemographic and family information, including parental age, ethnicity, income, and the number of adults and children in the home, was collected by questionnaire. We assessed parental IQ with the TONI-III, which assesses aptitude, abstract reasoning, and problem solving, and does not depend on formal education achieved [415]. Child weight and height were measured in light clothing, and weight-for-age, height-for-age, and BMI-for-age z-scores were calculated using the WHO Anthroplus anthropometric calculator (version 1.0.4).

4.3.3 Dietary Assessment

The children's dietary intake over the previous four weeks was estimated by interview with a parent using an FFQ. The FFQ included all foods and beverages consumed, with questions specific to fish type, cooking methods, and the use of fortified foods and supplements. Food models and measuring utensils were used to assist with estimation of portion sizes consumed by the children. Three 24 h recalls were also administered, using the five-pass review technique [438]. The first 24 h recall was given in person, and two 24 h recalls were conducted by telephone on random days over the following two weeks. The children's dietary intake of all nutrients relevant to this research was quantified using nutrient analysis software (ESHA Food Processor SQL, version 10.10.0.0; ESHA Research), using Canadian foods, and checking the fatty acid composition of all foods and beverages for accuracy, modifying if necessary based on labels or laboratory analysis of the product.

4.3.4 Child Neurodevelopment

Child neurodevelopment was assessed using several age-appropriate and standardized tests. These assessments were conducted in a clinical testing room at the Child and Family Research Institute (CFRI) that contains a one-way mirror to enable the parent(s) to view the child from an adjacent room. All tests were administered on the same day by one of two individuals trained in child psychometric testing, with assessment and training led by Kelly Richardson, a trained research assistant with a B.Sc. and M.A. in child development. The tests were the KABC which assesses the intelligence quotient (IQ) for children 2-12 y and provides a scaled score for multiple domains of cognitive ability, including sequential (short-term memory) and simultaneous (visual processing) processing, and learning ability (long-term storage and retrieval) [428]. The sum of these three domains provides a composite mental performance index score (general mental processing ability) [428]. Long-term memory was assessed with the KABC delayed recall scale. Language development was assessed using the PPVT [429], visual-motor integration was assessed with the Beery [430], and attention (Errors of Commission) and impulsivity (Errors of Omission) were assessed using the TOVA, which also provides a measure of processing speed (Response Time) and consistency (Response Time Variability) [431].

4.3.5 Biochemical Analysis

Venous blood (6 ml) was collected from the children by a certified phlebotomist, with EDTA as the anti-coagulant. The plasma and buffy coat were separated by centrifugation (2500g, 10 min, 4^{o}C), and the RBC washed with saline and EDTA and centrifuged twice, and the RBC pellet stored at -80^{o}C until analysis. RBC fatty acids were extracted and quantified by routine gas liquid chromatography [25].

4.3.6 Data Analysis

All data were checked for normality using a one-sample Kolmogorov-Smirnov Test. Child and family characteristics were summarized for each group of children using descriptive statistics. Child and family characteristics, dietary and RBC fatty acids, and child neurodevelopment test scores were compared between the follow-up and cross-sectional children

using chi-square, student's T-test, or Mann-Whitney *U* test, as appropriate. The data for all children were combined for the final analyses (n = 285).

Dietary DHA intakes estimated by FFQ, single 24 h recall and the average of three 24 h recalls were compared using ANOVA, with Bonferroni correction for multiple comparisons. The relationship between dietary DHA for intakes calculated using the three approaches and RBC DHA was determined using Pearson's correlation or Spearman's Rho. The frequency (%) distribution of child DHA intake was determined using increments of 25 mg/d. Foods containing DHA were grouped into categories of similar composition, with categories including fatty fish, lean fish, fish roe, shellfish, eggs, poultry, dairy, baked goods, mixed dishes, fortified foods, and sauces/dressings. The intake of DHA was calculated for each category to determine the major dietary sources of DHA. However, because data for the entire group of children does not reflect individual children, we also ranked the major dietary source of DHA for each child, and then determined the percent of children using each food category as their major source of DHA. Descriptive statistics were used to explore DHA intake and its relation to RBC fatty acids for children with each food category as their major source of DHA. The data on dietary supplements was not included in the analysis as many parents were not able to recall the brand, source and/or amount of n-3 fatty acids contained in the supplement. However, only approximately 20% of the children were reported to have taken omega-3 supplements, and of these the majority of children took supplements containing a small amount of DHA (~25 mg).

The potential association of dietary and RBC fatty acids with child neurodevelopment test scores was determined using partial Pearson's correlation coefficients, adjusting for child or family characteristics found to be associated with child test scores. The relationship between an essential nutrient and neurological function is curvilinear; neither the minimum nor optimum DHA intake, or biomarker equivalent of adequate DHA for the brain has been established. Therefore we sought to determine if dietary and RBC DHA and related ω-6 fatty acids differed between children with the highest and lowest scores for each neurodevelopment test. To achieve this, the mean or median dietary and RBC fatty acids of interest were compared for children in the highest and lowest quintiles for each test using student's t-test or Mann-Whitney *U* test, as appropriate. Data analysis was conducted using IBM SPSS Statistics (Version 20.0.0, 2011. Chicago, IL), with significance set at $P < 0.05$.

4.4 Results

A total of 98 and 187 children, all 5.75 y, were enrolled in this follow-up and cross-sectional study, respectively, total n = 285. A single 24 h recall was analyzed for 272 children, three 24 h recalls were analyzed for 259 children, and the FFQ was analyzed for 280 children. Blood samples were obtained from 245 children. Cognitive tests were completed for >90% of the children, with incomplete tests for PPVT, n = 14; KABC Sequential, n = 10; Learning, n = 15; Simultaneous, n = 5; MPI, n = 24; Delayed Recall, n = 21; Beery, n = 1. One exception was the TOVA, which was incomplete for approximately 20% of children (n = 56).

Comparison of characteristics between the follow-up and cross-sectional groups showed that parents of children in the follow-up group had slightly higher IQ scores (36.1 ± 6.86 and 34.2 ± 6.84, $P = 0.014$), and greater percentage had three or more rather than one or two children (23.7% and 15.1%, $P = 0.021$) (Table 4.1). There were no significant differences between the follow-up and cross-sectional groups for ethnicity, number of adults in the home, child sex, exclusive human milk-feeding for >3 mo, or children's BMI-for-age ($P > 0.05$, Table 4.1). For the entire group of children, 50.9% were boys, 81.6% of the children lived with two adults, and 85.6% had been human milk-fed at least 3 mo ($P > 0.05$). The mean (SD) BMI z-score at 5.75 y was 0.15 (0.96). In addition, 66.7% of the children were Caucasian, 15.8% were Chinese, and 17.5% were of another ethnicity, including East Indian/ South Asian (7.0%), other Asian (4.2%), Hispanic (2.5%), African (1.1%), Persian (1.1%), Jewish (0.7%), First Nations (0.4%), Egyptian (0.4%), and Mediterranean (0.4%) (ethnicity was self-reported). Thus, due to the small number of children of other ethnicities, subsequent analyses addressing a need for adjustment by ethnicity used only data for children of Caucasian and Chinese ethnicity.

Table 4.1 Family and Child Characteristics for all children and for the follow-up and cross-sectional groups.

	All	Follow-Up	Cross-sectional	*P*
	n = 285	n = 98	n = 187	
Caucasian/ Chinese/Other[1], %	66.7/15.8/17.5	74.5/11.2/14.3	62.6/18.2/19.2	0.131
Parental TONI IQ, $\bar{x}$ ± SD	34.8 ± 6.90	36.1 ± 6.86	34.2 ± 6.84	**0.014**
Adults in home, 2/1/>2,%	81.6/8.83/9.54	83.5/5.15/11.3	80.6/10.8/8.60	0.350
Children in home, 2/1/>2,%	62.5/19.4/18.0	64.9/11.3/23.7	61.3/23.6/15.1	**0.021**
Child sex, boys/girls, %	50.9/49.1	45.9/54.1	53.5/46.5	0.262
Child human milk-fed >3 months, %	85.6	89.8	83.4	0.159
Child BMI-for-age, z-score, $\bar{x}$ ± SD	0.15 ± 0.96	0.08 ± 0.99	0.18 ± 0.94	0.427

[1] Ethnicity, other is: East Indian/South Asian (n = 20), Other Asian (n = 12), Hispanic (n = 7), African (n = 3), Persian (n = 3), Jewish (n = 2) and n = 1 for each of First Nations, Egyptian, and Mediterranean.

There were no differences between the follow-up and cross-sectional groups' dietary intake assessed using the FFQ, 24 h recall prior to blood collection or average of three 24 h recalls for kcal, protein, carbohydrate, and total fat (Table B1.1). In addition, there were no differences in LA, ALA, EPA, or DHA intakes ($P > 0.05$) (Table 4.2). There was a small statistical difference in ARA intake assessed by FFQ, but not by one or three 24 h recalls, with a median (2.5-97.5 percentile) of 63.0 (14.4 – 218) and 77.8 (12.4 – 238) mg/d for the follow-up and cross-sectional groups, respectively, $P = 0.033$ (Table 4.2). Significant differences were found for relevant RBC fatty acids between the follow-up and cross-sectional groups of children, but not the RBC DHA (Table 4.3). Children in the follow-up cohort had higher RBC 22:4ω-6, 22:5ω-6, and 18:3ω-3 with lower 20:5ω-3 and DHA/22:4ω-6 + 22:5ω-6 and DHA/DHA + 22:5ω-6 compared to the cross-sectional group of children (Table 4.3). We also observed that the follow-up cohort achieved higher median neurodevelopmental test scores than the follow-up cohort, with a median (2.5-97.5 percentile) for the KABC scales of Learning 23.0 (13.4-34.0) and 21.0 (12.0-31.0), $P = 0.021$; MPI 81.0 (50.3-99.0) and 78.0 (54.2-98.8), $P = 0.026$; and Delayed Recall 22.0 (10.4-30.6) and 21.0 (14.0-29.7), $P = 0.047$ for the follow-up and cross-sectional groups, respectively (Table B1.2). There were no significant differences between the two groups for the KABC Sequential and Simultaneous scales, PPVT, Beery, or TOVA Response Time, Response Time Variability, Errors of Commission, and Errors of Omission.

Table 4.2 Dietary intake of ω-3 and ω-6 fatty acids for children in the follow-up or cross-sectional group, assessed using FFQ and one and three 24 h recalls.

		Follow-Up	Cross-Sectional	*P*
DHA, mg/d	FFQ	43.2 (1.73 – 300)	55.6 (4.86 – 336)	0.075
	1 x 24 h recall	11.0 (0.00– 215)	14.5 (0.00 – 478)	0.132
	3 x 24 h recalls	18.1 (0.85 – 274)	23.0 (1.15 – 381)	0.358
EPA, mg/d	FFQ	18.4 (0.58 – 180)	29.1 (0.82 – 262)	0.179
	1 x 24 h recall	2.20 (0.00 – 199)	3.00 (0.00 – 347)	0.265
	3 x 24 h recalls	3.85 (0.00 – 185)	4.30 (0.10 – 262)	0.343
ALA, g/d	FFQ	1.17 (0.48 – 2.59)	1.10 (0.53 – 2.97)	0.973
	1 x 24 h recall	0.85 (0.24 – 2.92)	0.90 (0.24 – 4.08)	0.218
	3 x 24 h recalls	0.87 (0.38 – 1.85)	0.98 (0.39 – 3.36)	0.180
ARA, mg/d	FFQ	63.0 (14.4 – 218)	77.8 (12.4 – 236)	**0.033**
	1 x 24 h recall	46.0 (2.22 – 229)	54.1 (0.60 – 276)	0.124
	3 x 24 h recalls	51.9 (8.61 – 209)	67.9 (7.00 – 227)	0.088
LA, g/d	FFQ	8.86 (3.64 – 22.3)	8.71 (3.64 – 19.5)	0.967
	1 x 24 h recall	7.57 (2.37 – 18.1)	7.21 (1.94 – 26.0)	0.865
	3 x 24 h recalls	6.91 (3.22 – 16.8)	7.34 (2.67 – 18.3)	0.587

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid

Data are median (2.5-97.5 percentile) and compared by Mann-Whitney *U* test. For the Follow-up and Cross-Sectional groups, respectively, n = 98, 182 for the FFQ, n = 92, 180 for 1 x 24 h recall, and n = 90, 169 for 3 x 24 h recalls.

Table 4.3 RBC fatty acids for all children and for the follow-up and cross-sectional groups.

	All n = 245	Follow-Up n = 73	Cross-sectional n = 172	*P*
Omega-3 fatty acids				
DHA	5.40 ± 1.56	5.26 ± 1.46	5.46 ± 1.61	0.346
22:5ω-3	2.50 ± 0.44	2.58 ± 0.47	2.46 ± 0.43	0.067
20:5ω-3[1]	0.86 ± 0.54	0.75 ± 0.30	0.91 ± 0.61	**0.006**
18:3ω-3[1]	0.19 ± 0.05	0.20 ± 0.04	0.18 ± 0.05	**0.014**
Omega-6 fatty acids				
22:5ω-6	0.64 ± 0.18	0.68 ± 0.18	0.62 ± 0.18	**0.009**
22:4ω-6[1]	3.34 ± 0.82	3.56 ± 0.74	3.25 ± 0.84	**<0.001**
20:4ω-6[1]	16.1 ± 1.92	16.3 ± 1.87	16.0 ± 1.93	0.100
18:2ω-6	11.8 ± 1.12	11.8 ± 0.97	11.8 ± 1.18	0.885
DHA/ DHA + 22:5ω-6	0.89 ± 0.04	0.88 ± 0.04	0.89 ± 0.04	**0.034**
DHA/ 22:4ω-6 + 22:5ω-6[1]	1.47 ± 0.69	1.32 ± 0.60	1.53 ± 0.72	**0.021**

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid
Data are mean ± SD and compared by student t-test
[1] Data are not normally distributed and compared by Mann-Whitney *U* test. For follow-up and cross-sectional group, respectively, median (2.5-97.5 percentile) RBC 20:5ω-3, 0.68 (0.43-1.55) and 0.73 (0.46-2.70); 18:3ω-3, 0.20 (0.12-0.30) and 0.18 (0.09-0.30); 22:4ω-6, 3.55 (1.90-4.91) and 3.25 (1.70-4.91); 20:4ω-6, 16.6 (10.1-18.7) and 16.3 (11.4-19.1); DHA/ 22:4ω-6 + 22:5ω-6, 1.21 (0.56-3.13) and 1.38 (0.64-3.49).

The dietary intake of energy, macronutrients, and ω-3 and ω-6 fatty acids for all children was higher when estimated by FFQ than with one or three 24 h recalls. For total energy, the median (2.5-97.5 percentile) intake assessed by FFQ was 1785 (1035-3033) kcal/d, but 1495 (848-2563) and 1489 (926-2289) kcal/d when using one and three 24 h recalls, respectively, ($P < 0.001$) (Table B1.3). Similarly, total fat, carbohydrate, and protein intake were also higher when assessed by FFQ than one or three day 24 h recall ($P < 0.05$), with no difference in macronutrient intake when intake was calculated per kcal (Table B1.3). Thus, the mean ranges as a percent total energy were 32.7-33.3 % total fat, 53.1-53.9 % carbohydrate, and 15.8-16.1 % protein for all three dietary intake approaches. The intakes of LA, ARA, and ALA were higher when assessed by FFQ as g or mg/d, again with no difference in intake among the methods when expressed as g/1000 kcal (Table 4.4). However, EPA and DHA intakes were higher when assessed by FFQ than the one and three 24 h recalls, expressed as either mg/d or mg/1000 kcal (Table 4.4).

The relationship between DHA intake and relevant RBC fatty acids was stronger using dietary intake data collected using the FFQ rather than one or three 24 h recalls (Table 4.5). DHA intake assessed by FFQ was positively related to the RBC DHA (rho = 0.383, $P < 0.001$) and EPA (rho = 0.457, $P < 0.001$), with an inverse relation to RBC 20:4ω-6 (rho = -0.244, $P < 0.001$), 22:4ω-6 (rho = -0.444, $P < 0.001$), 22:5ω-6 (rho = -0.432, $P < 0.001$). Notably, DHA intake showed the greatest association with RBC DHA/22:4ω-6 + 22:5ω-6 (rho = 0.517, $P < 0.001$). However, scatter plots of DHA intake and RBC ω-3 and ω-6 fatty acids show high individual variability, particularly at DHA intakes below 175 mg/d (Figure 4.1). For example, no child with a DHA intake ≥ 175 mg/d (n = 19) had an RBC DHA ≤ 4.0 % TFA, while 63.2% and 26.3% had an RBC DHA ≥ 6.0 or ≥ 8.0 %TFA, respectively (Table 4.6). In contrast, 34% of the children with DHA intakes ≤ 25 mg/d (n = 62) had an RBC DHA ≤ 4.0 %TFA, with 14.5% having an RBC DHA ≥ 6.0 %TFA, and no children having an RBC DHA ≥ 8.0 %TFA. Similarly, 28.8% of the children with DHA intakes ≤ 50 mg/d (n = 118) had an RBC DHA ≤ 4.0 %TFA, with only 2.54% having an RBC DHA ≥ 8.0 %TFA, respectively. Clearly, the group numbers fit a predicted pattern of higher DHA status with higher DHA intake, although individual children's status in relation to diet is varied.

Table 4.4 Children's ω-3 and ω-6 fatty acid intake assessed using FFQ and one or three 24 h recalls.

	FFQ, n = 280				1 x 24 h recall, n = 272				3 x 24 h recall, n = 259				*P*
	Mean	SD	Median	2.5-97.5	Mean	SD	Median	2.5-97.5	Mean	SD	Median	2.5-97.5	
Omega-3 fatty acids													
DHA, mg/d	77.7	80.6	52.9	4.61-327	48.2	112	12.2	0.00-367	57.7	108	19.5	1.20-328	**<0.001**
DHA, mg/1000 kcal	41.3	39.4	30.9	2.53-156	32.9	76.0	8.23	0.00-292	37.1	62.1	13.0	0.63-206	**<0.001**
EPA, mg/d	45.5	56.9	26.5	0.90-248	25.4	79.0	2.70	0.00-274	31.4	74.9	4.20	0.05-216	**<0.001**
EPA, mg/1000 kcal	24.1	27.8	16.4	0.43-108	17.6	54.3	1.90	0.00-219	20.3	44.4	2.57	0.00-146	**<0.001**
ALA, g/d	1.27	0.61	1.15	0.51-2.85	1.14	0.90	0.87	0.25-3.61	1.04	0.56	0.92	0.39-3.12	**<0.001**
ALA, g/1000 kcal	0.68	0.26	0.62	0.37-1.25	0.73	0.54	0.58	0.22-2.54	0.69	0.36	0.61	0.32-1.78	0.292
Omega-6 fatty acids													
LA, g/d	9.70	4.25	8.79	3.64-20.6	8.64	5.84	7.25	2.04-24.0	8.00	3.49	7.25	3.07-17.7	**<0.001**
LA, g/1000 kcal	5.14	1.43	4.88	2.91-8.72	5.42	2.66	4.91	1.88-11.6	3.29	1.35	3.03	1.47-7.09	0.888
ARA, mg/d	83.4	54.5	68.6	12.6-210	77.3	71.6	51.2	0.85-270	76.5	54.9	63.3	7.05-220	**0.002**
ARA, mg/1000 kcal	44.9	25.5	39.3	6.96-109	50.3	45.6	34.2	0.64-159	49.4	33.2	42.0	4.27-133	0.142

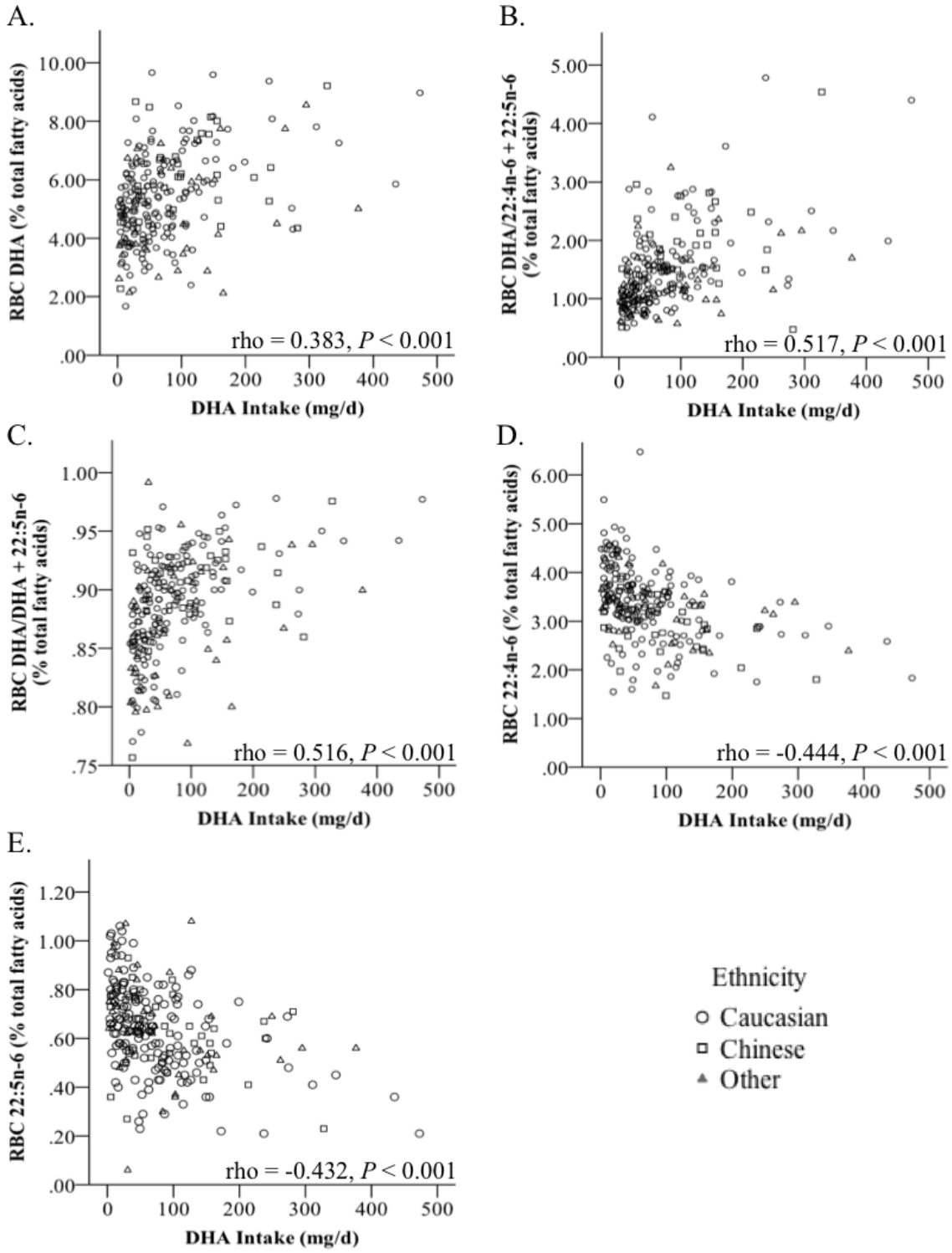
ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; IQR, inter-quartile range; LA, linoleic acid; 2.5-97.5, 2.5-97.5 percentile
Data by the three dietary approaches analyzed by Kruskal Wallis test for non-normal distributions.

Table 4.5 Associations between child DHA intake and RBC ω-3 and ω-6 fatty acids.

	DHA Intake					
	FFQ n = 239		1 x 24 h recall n = 235		3 x 24 h recall n = 224	
RBC	rho	*P*	rho	*P*	rho	*P*
ω-3 fatty acids						
DHA	0.383	**<0.001**	0.294	**<0.001**	0.357	**<0.001**
22:5ω-3	-0.190	**0.003**	-0.076	0.248	-0.036	0.595
20:5ω-3	0.457	**<0.001**	0.325	**<0.001**	0.371	**<0.001**
18:3ω-3	0.016	0.811	0.046	0.480	0.067	0.316
ω-6 fatty acids						
22:5ω-6	-0.432	**<0.001**	-0.229	**<0.001**	-0.286	**<0.001**
22:4ω-6	-0.444	**<0.001**	-0.243	**<0.001**	-0.342	**<0.001**
20:4ω-6	-0.244	**<0.001**	-0.088	0.177	-0.195	**0.003**
18:2ω-6	-0.021	0.747	0.055	0.399	0.072	0.286
DHA/ DHA + 22:5ω-6	0.516	**<0.001**	0.346	**<0.001**	0.406	**<0.001**
DHA/ 22:4ω-6 + 22:5ω-6	0.517	**<0.001**	0.363	**<0.001**	0.441	**<0.001**

DHA, docosahexaenoic acid; FFQ, food frequency questionnaire
Data are spearman's correlation coefficients.

Figure 4.1 Scatter plots of the relationship between DHA intake assessed by FFQ and RBC fatty acids.



The data were analyzed using Spearman's rank correlation coefficient between DHA intake and RBC DHA (A), DHA/22:4ω-6 + 22:5ω-6 ratio (B), DHA/DHA + 22:5ω-6 ratio (C), 22:4ω-6 (D), 22:5ω-6 (E) for children of Caucasian (O, n = 162), Chinese (□, n = 37), and other ethnicity (Δ, n = 40).

Table 4.6 Children with RBC DHA level grouped by DHA intake.

	Frequency of children (%)			
	RBC DHA, % total fatty acids			
DHA intake	≤ 4.0	≥ 6.0	≥ 7.0	≥ 8.0
≤ 25 mg/d, n = 62	33.9	14.5	1.61	0
≤ 50 mg/d, n = 118	28.8	19.5	5.93	2.54
≤ 100 mg/d, n = 174	23.0	25.9	7.47	2.87
≥ 100 mg/d, n = 65	9.23	53.8	40.0	15.4
≥ 150 mg/d, n = 30	3.33	63.3	40.0	20.0
≥ 175 mg/d, n = 19	0	63.2	42.1	26.3

DHA, docosahexaenoic acid; RBC, red blood cell

Children of Chinese ethnicity had significantly higher intakes of DHA (P = 0.005), EPA (P = 0.013), and ARA (P < 0.001) compared to Caucasian children (Table B1.4). There were no differences between the Chinese and Caucasian for dietary intakes of LA (P = 0.105) or ALA (P = 0.091). We found no other child or family characteristic collected in our study associated with ω-3 and ω-6 fatty acid intake (P > 0.05). Consistent with the dietary DHA intake, the RBC DHA was also highest in children of Chinese descent compared to Caucasian children, with a mean (SD) of 6.06 (1.42) % and 5.38 (1.52) %TFA, respectively, (P = 0.013) (Table B1.5). RBC LA was also higher, but 22:4ω-6 was lower in Chinese children compared to children of a Caucasian background. The relationship between DHA intake and RBC fatty acids also suggest possible differences by ethnicity (Table 4.7). For example, a significant positive relationship was found between DHA intake and RBC DHA for Caucasian children (rho = 0.389, P < 0.001, n = 162), but not for Chinese children (rho = 0.292, P = 0.080, n = 37). Similarly, DHA intake was positively associated with RBC DHA/ DHA + 22:5ω-6 for Caucasian children (rho = 0.578, P < 0.001), but not Chinese children (rho = 0.301, P = 0.070). However, these differences may reflect the different number of children, with about 4-fold higher number of children from Caucasian than Chinese backgrounds.

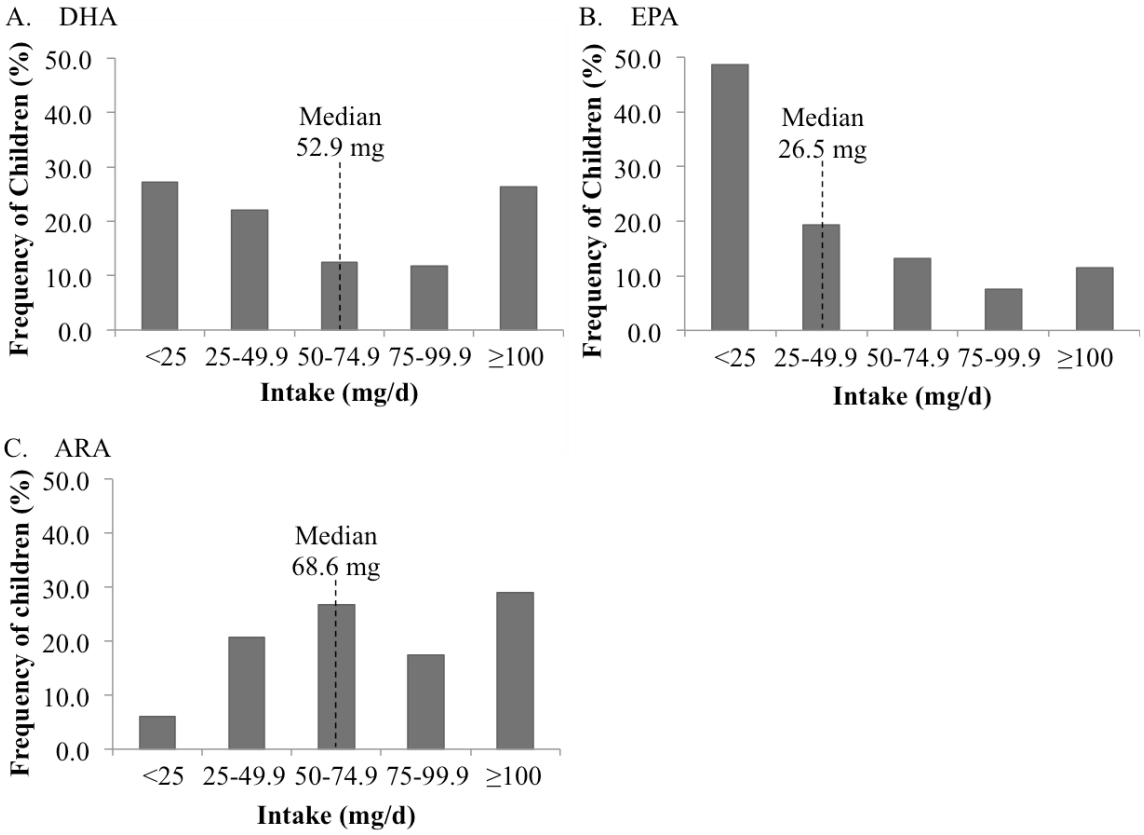
The intakes of DHA, EPA, and ARA for all children were highly skewed, with a median (2.5-97.5 percentile) of 52.9 (4.61-327), 26.5 (0.90-248), and 68.6 (12.6-210) mg/d assessed by FFQ. Figure 4.2 shows the frequency of intakes of DHA, EPA, and ARA in increments of 25 mg/d. The intake of DHA appears bi-modal, with more individuals at the tails of the distribution than clustered around the median. For example, 53.5% of the children had DHA intakes at the tails of the distribution, ≤ 25 and ≥ 100 mg/d, while 46.4% had intakes clustered within 25 mg above and below the median-containing interval. In contrast, for EPA and ARA intakes 81.1% and 65% of the children had intakes clustered within 25 mg above and below the median-containing interval. Figure 4.3 shows the frequency of DHA, EPA, and ARA intakes for children of Caucasian and Chinese backgrounds separately. Figure 4.3 highlights that the shape of the intake distributions differ between Caucasian and Chinese children, with the differences most pronounced for DHA intakes, with more Caucasian than Chinese children with DHA intakes ≤ 25 mg/d.

Table 4.7 Association between DHA intake and RBC fatty acids by child ethnicity.

		DHA Intake (FFQ)			
		All		Ethnicity	
		Unadjusted	Adjusted[1]	Caucasian	Chinese
RBC		n = 239	df = 196	n = 162	n = 37
ω-3 fatty acids					
DHA	Rho	0.383	0.394	0.389	0.292
	P	**<0.001**	**<0.001**	**<0.001**	0.080
22:5ω3	Rho	-0.190	-0.073	-0.076	-0.237
	P	**0.003**	0.308	0.336	0.158
20:5ω3	Rho	0.457	0.485	0.513	0.413
	P	**<0.001**	**<0.001**	**<0.001**	**0.011**
18:3ω3	Rho	0.016	0.065	0.066	0.152
	P	0.811	0.361	0.407	0.369
ω-6 fatty acids					
22:5ω6	Rho	-0.432	-0.423	-0.486	-0.285
	P	**<0.001**	**<0.001**	**<0.001**	0.087
22:4ω6	Rho	-0.444	-0.276	-0.477	-0.342
	P	**<0.001**	**<0.001**	**<0.001**	**0.038**
20:4ω6	Rho	-0.244	-0.261	-0.296	-0.356
	P	**<0.001**	**0.001**	**<0.001**	**0.031**
18:2ω6	Rho	-0.021	-0.095	-0.032	-0.222
	P	0.747	0.183	0.682	0.186
DHA/ DHA + 22:5ω6	Rho	0.516	0.475	0.578	0.301
	P	**<0.001**	**<0.001**	**<0.001**	0.070
DHA/ 22:4ω6 + 22:5ω6	Rho	0.517	0.510	0.556	0.354
	P	**<0.001**	**<0.001**	**<0.001**	**0.032**

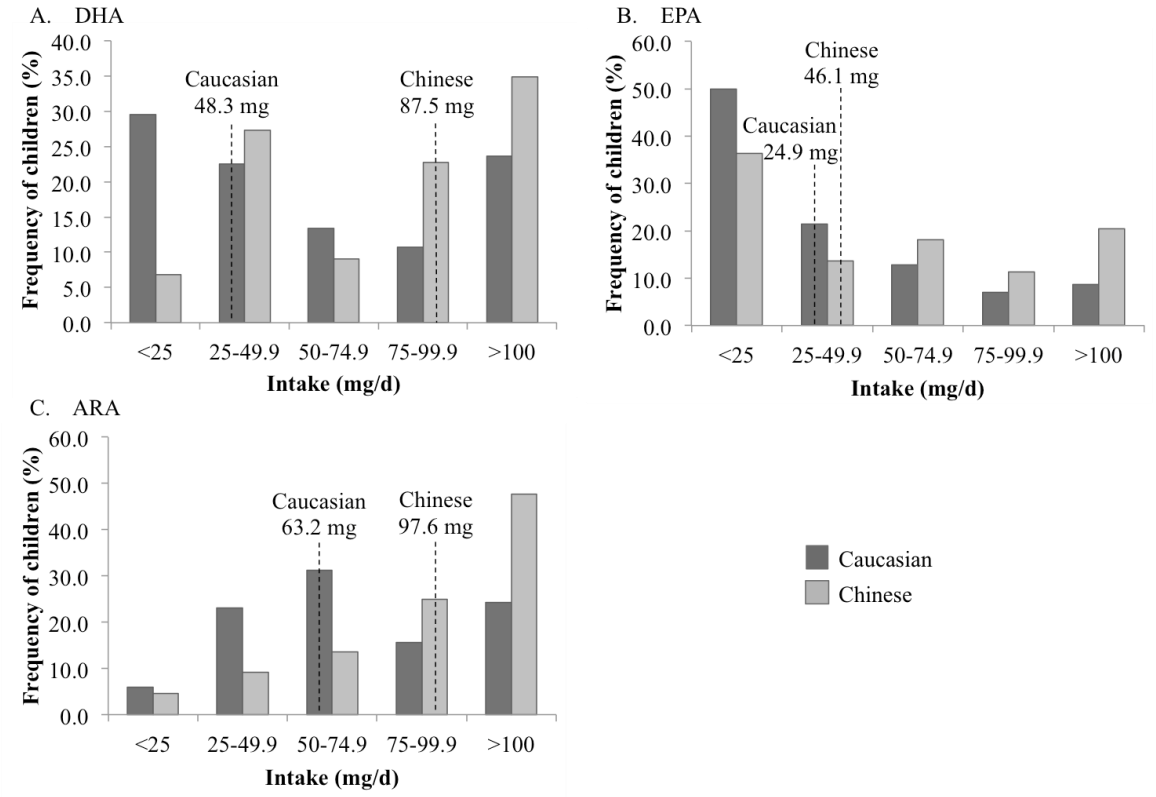
DHA, docosahexaenoic acid; FFQ, food frequency questionnaire
Data are spearman's correlation coefficients for the association between DHA intake and RBC fatty acids.
[1] Data are partial correlations adjusted for child ethnicity.

Figure 4.2 Frequency distributions of DHA, EPA, and ARA intake for all children.



ARA, Arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
Bars indicate the percent of children with intakes in increments of 25 mg/d for DHA (Panel A), EPA (Panel B), and ARA (Panel C) for all children, n = 280.

Figure 4.3 Frequency distributions of DHA, EPA, and ARA intake for Caucasian and Chinese children.



ARA, Arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
Bars indicate the percent of children with intakes in increments of 25 mg/d for DHA (Panel A), EPA (Panel B), and ARA (Panel C) for Caucasian (dark grey, n = 162) and Chinese (light grey, n = 37) children. The dashed line indicates the median intake of the respective fatty acid.

Next we sought to identify the food sources of DHA, and the contribution of different foods to DHA intake. For all children, the major food sources of DHA were fatty and lean fish and eggs, which accounted for 58.4, 13.1, and 9.17%, respectively, of the total mean DHA intake (Table 4.8). However, this does not reflect the 33.9, 32.1, and 18.2% of the children who did not eat these respective foods in the previous month, since it is a group average. Thus, to address the importance of dietary DHA source at the individual level, we determined the major contributing source to DHA intake for each child (Table 4.9). Fatty fish was the major source of DHA for 57.5% of the children, with eggs, lean fish, and poultry the major source for 12.1, 11.8, and 7.9%, respectively,

for the remaining children. A few had major sources of DHA from dairy (4.3%), shellfish (3.2%), baked goods (1.8%), fish roe (1.1%) and fortified foods (0.4%). Notably, children with fatty fish as their major DHA source had a median (IQR) DHA intake of 86.5 (53.6 – 137) mg/d compared to 13.6 (7.85 – 29.6), 27.6 (22.9 – 64.8), and 19.2 (10.8 – 31.2) mg/d for children with eggs, lean fish or poultry, respectively, as their major source of DHA. In addition, for children with fatty fish, eggs, lean fish, or poultry as their major dietary DHA source, the mean (SD) of the RBC DHA was 5.91 (1.55), 4.63 (1.12), 4.84 (1.38), and 4.37 (1.42) % DHA, respectively (Table B1.6).

Table 4.8 Dietary DHA sources as mg/g ranked by 75th percentile for all children.

	All, n = 280		Child consumers[1]		
	Mean ± SD	Median (2.5-97.5)	Mean ± SD	Median (2.5-97.5)	n %
Fatty fish	45.0 ± 64.9	21.3 (0.00-249)	67.4 ± 69.3	42.5 (4.36-281)	66.1
Lean fish	10.1 ± 15.6	4.70 (0.00-51.0)	14.7 ± 16.9	9.30 (0.47-73.2)	67.9
Eggs	7.06 ± 7.49	5.30 (0.00-27.9)	8.54 ± 7.43	6.20 (0.70-31.4)	81.8
Poultry	5.14 ± 5.93	2.90 (0.00-21.20)	5.51 ± 5.97	3.20 (0.34-21.9)	92.1
Dairy	2.26 ± 2.92	1.30 (0.00-9.37)	3.14 ± 3.02	2.50 (0.10-10.1)	71.1
Shellfish	3.33 ± 9.24	0.00 (0.00-23.7)	7.69 ± 12.8	3.20 (0.30-51.3)	42.5
Baked goods	1.58 ± 1.80	1.10 (0.19-4.71)	1.58 ± 1.80	1.10 (0.19-4.71)	98.9
Mixed Dishes	0.40 ± 1.64	0.10 (0.00-3.14)	0.72 ± 2.15	0.20 (0.10-3.98)	55.4
Fish Roe	1.72 ± 15.3	0.00 (0.00-0.00)	95.3 ± 70.4	86.8 (33.8-196)	1.78
Fortified foods	0.41 ± 3.82	0.00 (0.00-0.00)	22.8 ± 19.2	14.8 (2.74-44.3)	1.78
Sauce/dressing	0.06 ± 0.15	0.00 (0.00-0.00)	0.24 ± 0.23	0.20 (0.10-0.87)	24.3

2.5-97.5, 2.5-97.5 percentile

[1] Includes only children who consume the food category.

Table 4.9 Importance of different food categories as the major DHA source among children.

	DHA intake mg/d		
Food Category[1]	n (%)	Mean ± SD	Median (IQR)
Fatty fish	161 (57.5)	110 ± 86.3	86.5 (53.6 – 137)
Eggs	34 (12.1)	22.8 ± 28.0	13.6 (7.85 – 29.6)
Lean fish	33 (11.8)	45.7 ± 36.4	27.6 (22.9 – 64.8)
Poultry	22 (7.86)	23.0 ± 15.8	19.2 (10.8 – 31.2)
Dairy	12 (4.28)	9.29 ± 6.62	7.65 (5.00 – 11.5)
Shellfish	9 (3.21)	44.7 ± 29.5	28.1 (21.6 – 78.8)

DHA, docosahexaenoic acid; IQR, inter-quartile range; RBC, red blood cell
[1] Dietary DHA was not analyzed for food categories with < 2% of children having category as the major source, including baked goods (n = 5), fish roe (n = 3), fortified foods (n = 1), mixed dishes (n = 0), sauces/dressings (n = 0).

Differences in developmental test scores were also present among children of Caucasian and Chinese ethnicity (Table B1.7). Children of Caucasian descent had higher [median (IQR)] scores on the PPVT [121 (112-132)] compared to children of Chinese descent [108 (90.5-121)], $P < 0.001$. In contrast, children of Caucasian background had lower scores on the Beery ($P = 0.004$) and KABC Learning ($P = 0.022$), Simultaneous ($P = 0.009$), and MPI ($P = 0.050$) scales compared to Chinese children. No significant differences among ethnicities were found for scores on the KABC Sequential and Delayed Recall scales, or the TOVA ($P > 0.05$).

Due to the lower sample size of Chinese and other ethnicities and the potential interaction with diet, RBC fatty acids and test scores, we assessed the relationship between the dietary and RBC relevant fatty acids and test scores for Caucasian children only. For Caucasian children, only the KABC Sequential scale was associated with dietary fatty acid intake, with a positive relationship with DHA (rho = 0.227, $P = 0.002$) and EPA (rho = 0.258, $P = 0.001$) (Table B1.8). Similarly, there were no differences in dietary DHA, EPA or ARA between children in the highest and lowest quintiles for the PPVT, Beery, KABC Learning, Simultaneous, MPI and Delayed Recall scales, or the TOVA scores (Table B1.10-1.20). DHA intake was related to the KABC Sequential

scale, with a higher [median (IQR)] intake among children in the highest quintile [69.9 (13.1-48.9) mg/d] compared to lowest quintile of scores [24.1 (13.1-48.9) mg/d], $P<0.001$ (Table B.1.13). EPA intake was also higher in the highest quintile of KABC Sequential scale scores ($P < 0.001$). Notably, dietary EPA and DHA are tightly correlated in our group (rho = 0.969, $P < 0.001$) because of fish intake, which is high in both EPA and DHA.

For Caucasian children, the RBC DHA was weakly, but positively related to scores on the PPVT (rho = 0.211, $P = 0.008$) and the KABC Sequential (rho = 0.182, $P = 0.022$) scale, with a non-significant trend for the MPI scale (rho = 0.149, $P = 0.067$, n = 152) (Table B1.9). A significant positive relationship was found between RBC DHA/DHA + 22:5ω-6 and scores on the PPVT (rho = 0.237, $P = 0.003$), KABC Sequential scale (rho = 0.237, $P = 0.003$), and the Beery (rho = 0.168, $P = 0.031$). Next we compared RBC fatty acids by quintile of test score for the Caucasian children (Table 4.10-4.20). Children with test scores in the highest compared to the lowest quintile had RBC with higher (mean ± SD %TFA) DHA for the KABC Sequential (4.95 ± 1.32 and 5.80 ± 1.51, $P = 0.013$) and MPI (5.07 ± 1.54 and 5.94 ± 1.50, $P = 0.036$) scales, with a close trend for the PPVT (5.26 ± 1.41 and 5.99 ± 1.41, $P = 0.053$). Similarly, the highest quintile compared to the lowest quintile had RBC with higher [median, (IQR)] DHA/22:4ω-6 + 22:5ω-6 for the PPVT [1.58 (1.08-2.12) and 1.24 (0.96-1.48), $P = 0.007$] and KABC sequential scale [1.46 (1.14-1.93) and 1.02 (0.83-1.35), $P = 0.002$], respectively. Lower RBC 22:5ω-6 was also found in the highest, compared to the lowest, quintile of children for the PPVT (0.70 ± 0.19 and 0.57 ± 0.17, $P = 0.008$) and KABC Sequential scale (0.72 ± 0.18 and 0.62 ± 0.20, $P = 0.035$) scores. There were no differences in RBC DHA and ω-6 fatty acids between children in the highest and lowest quintiles for the KABC Learning and Delayed Recall scales, or TOVA scores.

Table 4.10 RBC fatty acids by quintiles of PPVT scores for Caucasian children.

		Quintiles of PPVT Score					*P*
	min-max	73-109	111-115	116-125	126-133	134-159	
RBC Fatty acids		n = 28	n = 30	n = 35	n = 31	n = 30	
DHA		5.26 ± 1.41	4.98 ± 1.52	5.31 ± 1.46	5.56 ± 1.66	5.99 ± 1.41	0.053
22:5ω6		0.70 ± 0.19	0.64 ± 0.18	0.63 ± 0.16	0.66 ± 0.19	0.57 ± 0.17	**0.008**
22:4ω6		3.44 ± 0.71	3.94 ± 0.86	3.46 ± 0.89	3.52 ± 0.77	3.20 ± 0.66	0.204
DHA/DHA + 22:5ω6		0.88 ± 0.04	0.88 ± 0.04	0.89 ± 0.04	0.89 ± 0.04	0.91 ± 0.04	**0.004**
DHA/22:4ω6 + 22:5ω6[1]		1.38 ± 0.77	1.29 ± 0.64	1.39 ± 0.58	1.43 ± 0.74	1.69 ± 0.77	**0.007**

DHA, docosahexaenoic acid; PPVT, Picture Peabody Vocabulary Test; RBC, red blood cell
Data are mean ± SD; quintile 1 and 5 compared by student's t-test.
[1] DHA/22:4ω6 + 22:5ω6 was not normally distributed, compared by Mann-Whitney *U* test; for the quintiles median (IQR) was 1.24 (0.96-1.48), 1.20 (0.92-1.53), 1.18 (0.95-1.69), 1.21 (1.01-1.62), 1.58 (1.08-2.12), respectively.

Table 4.11 RBC fatty acids by quintiles of Beery scores for Caucasian children.

	Quintiles of Beery Score					
min-max	11-14	15	16-17	18	19-23	
RBC Fatty acids	n = 28	n = 25	n = 63	n = 20	n = 30	*P*
DHA	5.24 ± 1.54	5.31 ± 1.58	5.29 ± 1.60	5.45 ± 1.22	5.74 ± 1.51	0.221
22:5ω6	0.66 ± 0.16	0.70 ± 0.21	0.63 ± 0.17	0.64 ± 0.19	0.59 ± 0.17	0.121
22:4ω6	3.37 ± 0.69	3.46 ± 0.91	3.42 ± 0.72	3.38 ± 0.67	3.45 ± 0.97	0.718
DHA/DHA + 22:5ω6	0.88 ± 0.04	0.88 ± 0.05	0.89 ± 0.04	0.89 ± 0.04	0.90 ± 0.04	**0.048**
DHA/22:4ω6 + 22:5ω6[1]	1.41 ± 0.68	1.41 ± 0.74	1.38 ± 0.61	1.40 ± 0.40	1.60 ± 0.92	0.304

DHA, docosahexaenoic acid; Beery, Beery-Buktenica Developmental Test of Visual-Motor Integration; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.

[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.31 (0.90-1.63), 1.20 (0.88-1.76), 1.18 (0.98-1.64), 1.38 (1.10-1.57), 1.47 (1.04-1.70), respectively.

Table 4.12 RBC fatty acids by quintiles of KABC Sequential scale scores for Caucasian children.

	Quintiles of KABC Sequential Scale Score					
min-max	15-17	18-19	21-22	23-24	25-34	
RBC Fatty acids	n = 36	n = 23	n = 33	n = 30	n = 37	*P*
DHA	4.95 ± 1.32	5.62 ± 1.18	5.49 ± 1.76	5.31 ± 1.69	5.80 ± 1.51	**0.013**
22:5ω6	0.72 ± 0.18	0.64 ± 0.14	0.58 ± 0.15	0.61 ± 0.18	0.62 ± 0.20	**0.035**
22:4ω6	3.65 ± 0.77	3.50 ± 0.63	3.23 ± 0.75	3.26 ± 0.77	3.33 ± 0.76	0.083
DHA/DHA + 22:5ω6	0.87 ±0.04	0.89 ± 0.03	0.90 ± 0.04	0.89 ± 0.04	0.90 ± 0.04	**0.003**
DHA/22:4ω6 + 22:5ω6[1]	1.24 ± 0.73	1.42 ± 0.48	1.54 ± 0.77	1.48 ± 0.76	1.56 ± 0.62	**0.002**

DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.02 (0.83-1.35), 1.30 (1.04-1.58), 1.29 (1.02-1.70), 1.39 (1.02-1.65), 1.46 (1.14-1.93), respectively.

Table 4.13 RBC fatty acids by quintiles of KABC Learning scale scores for Caucasian children.

	Quintiles of KABC Learning Scale Score					
min-max	11-17	18-20	21-23	24-26	27-35	
RBC Fatty acids	n = 32	n = 37	n = 29	n = 29	n = 29	*P*
DHA	5.10 ± 1.58	5.68 ± 1.71	5.37 ± 1.42	5.03 ± 1.46	5.71 ± 1.36	0.112
22:5ω6	0.65 ± 0.18	0.62 ± 0.17	0.59 ± 0.20	0.64 ± 0.17	0.68 ± 0.18	0.533
22:4ω6	3.30 ± 0.79	3.31 ± 0.69	3.23 ± 0.75	3.54 ± 0.78	3.66 ± 0.71	0.066
DHA/DHA + 22:5ω6	0.88 ± 0.04	0.89 ± 0.04	0.89 ± 0.04	0.88 ± 0.04	0.89 ± 0.04	0.393
DHA/22:4ω6 + 22:5ω6[1]	1.37 ± 0.66	1.55 ± 0.76	1.56 ± 0.86	1.28 ± 0.55	1.39 ± 0.54	0.707

DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.22 (0.95-1.56), 1.48 (1.04-1.80), 1.24 (1.07-1.66), 1.14 (0.83-1.61), 1.22 (1.00-1.60), respectively.

Table 4.14 RBC fatty acids by quintiles of KABC Simultaneous scale scores for Caucasian children.

		Quintiles of KABC Simultaneous Scale Score					
	min-max	13-30	31-32	33-35	36-38	39-51	
RBC Fatty acids		n = 34	n = 24	n = 36	n = 32	n = 38	*P*
DHA		5.27 ± 1.32	5.26 ± 1.70	5.31 ± 1.92	5.39 ± 1.43	5.63 ± 1.30	0.248
22:5ω6		0.63 ± 0.15	0.65 ± 0.17	0.63 ± 0.22	0.63 ± 0.20	0.66 ± 0.15	0.420
22:4ω6		3.48 ± 0.72	3.28 ± 0.73	3.29 ± 0.83	3.36 ± 0.71	3.61 ± 0.87	0.492
DHA/DHA + 22:5ω6		0.89 ± 0.03	0.88 ± 0.05	0.88 ± 0.05	0.89 ± 0.04	0.89 ± 0.04	0.897
DHA/22:4ω6 + 22:5ω6[1]		1.32 ± 0.40	1.50 ± 0.95	1.48 ± 0.82	1.46 ± 0.72	1.41 ± 0.55	0.710

DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.28 (1.00-1.60), 1.11 (0.92-1.66), 1.32 (0.95-1.64), 1.21 (1.04-1.80), 1.33 (0.97-1.71), respectively.

Table 4.15 RBC fatty acids by quintiles of KABC Mental Performance Index scale scores for Caucasian children.

	Quintiles of KABC MPI Scale Score					
min-max	39-68	69-73	74-82	83-86	87-113	
RBC Fatty acids	n = 29	n = 28	n = 42	n = 26	n = 27	*P*
DHA	5.07 ± 1.54	5.52 ± 1.71	5.53 ± 1.55	4.94 ± 1.23	5.94 ± 1.50	**0.036**
22:5ω6	0.64 ± 0.16	0.63 ± 0.17	0.61 ± 0.19	0.66 ± 0.18	0.65 ± 0.18	0.931
22:4ω6	3.36 ± 0.73	3.37 ± 0.85	3.30 ± 0.72	3.46 ± 0.74	3.55 ± 0.75	0.347
DHA/DHA + 22:5ω6	0.88 ± 0.04	0.89 ± 0.05	0.90 ± 0.04	0.88 ± 0.04	0.90 ± 0.04	0.116
DHA/22:4ω6 + 22:5ω6[1]	1.37 ± 0.79	1.49 ± 0.75	1.52 ± 0.75	1.30 ± 0.59	1.49 ± 0.54	0.147

DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.

[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.13 (0.91-1.62), 1.32 (0.98-1.64), 1.39 (1.13-1.55), 1.11 (0.87-1.60), 1.42 (0.98-1.99), respectively.

Table 4.16 RBC fatty acids by quintiles of KABC Delayed Recall scale scores for Caucasian children.

		Quintiles of KABC Delayed Recall Scale Score					
	min-max	9-17	18-19	20-22	23-25	26-32	
RBC Fatty acids		n = 24	n = 28	n = 38	n = 33	n = 29	*P*
DHA		5.11 ± 1.36	5.46 ± 1.66	5.48 ± 1.78	5.58 ± 1.33	5.00 ± 1.40	0.765
22:5ω6		0.65 ± 0.13	0.58 ± 0.19	0.62 ± 0.18	0.66 ± 0.18	0.69 ± 0.17	0.290
22:4ω6		3.44 ± 0.71	3.27 ± 0.81	3.49 ± 0.93	3.44 ± 0.74	3.56 ± 0.58	0.502
DHA/DHA + 22:5ω6		0.88 ± 0.04	0.90 ± 0.04	0.89 ± 0.04	0.89 ± 0.04	0.87 ± 0.04	0.412
DHA/22:4ω6 + 22:5ω6[1]		1.31 ± 0.51	1.56 ± 0.80	1.50 ± 0.94	1.44 ± 0.53	1.22 ± 0.46	0.500

DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.18 (0.96-1.55), 1.42 (1.01-1.88), 1.26 (0.85-1.75), 1.39 (0.98-1.76), 1.07 (0.93-1.49), respectively.

Table 4.17 RBC fatty acids by quintiles of TOVA Response Time Variability scores for Caucasian children.

		Quintiles of TOVA Response Time Variability[1]					
	min-max	135-199	203-232	234-258	259-290	291-382	
RBC Fatty acids		n = 26	n = 26	n = 27	n = 25	n = 26	*P*
DHA		5.49 ± 1.20	5.45 ± 1.79	5.27 ± 1.37	5.21 ± 1.81	5.47 ± 1.48	0.956
22:5ω6		0.69 ± 0.18	0.61 ± 0.16	0.62 ± 0.18	0.62 ± 0.20	0.61 ± 0.19	0.087
22:4ω6		3.54 ± 0.63	3.25 ± 0.62	3.44 ± 0.88	3.48 ± 0.71	3.41 ± 0.84	0.524
DHA/DHA + 22:5ω6		0.88 ± 0.04	0.89 ± 0.04	0.89 ± 0.04	0.88 ± 0.05	0.89 ± 0.04	0.298
DHA/22:4ω6 + 22:5ω6[2]		1.37 ± 0.51	1.47 ± 0.62	1.40 ± 0.62	1.39 ± 0.82	1.49 ± 0.74	0.840

DHA, docosahexaenoic acid; TOVA, Test of Variables of Attention; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] For TOVA scores, better performance is the achievement of a lower score.
[2] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.27 (0.99-1.64), 1.42 (1.00-1.86), 1.34 (0.95-1.66), 1.18 (0.92-1.53), 1.31 (1.00-1.72), respectively.

Table 4.18 RBC fatty acids by quintiles of TOVA Response Time scores for Caucasian children.

		Quintiles of TOVA Response Time[1]					
	min-max	6.29-596	603-660	661-706	709-771	772-1026	
RBC Fatty acids		n = 26	n = 25	n = 26	n = 26	n = 27	*P*
DHA		5.63 ± 1.24	5.65 ± 1.58	4.94 ± 1.72	4.96 ± 1.24	5.71 ± 1.66	0.838
22:5ω6		0.67 ± 0.17	0.68 ± 0.19	0.59 ± 0.14	0.61 ± 0.21	0.60 ± 0.19	0.155
22:4ω6		3.55 ± 0.68	3.50 ± 0.66	3.20 ± 0.68	3.40 ± 0.82	3.47 ± 0.85	0.712
DHA/DHA + 22:5ω6		0.89 ± 0.03	0.88 ± 0.05	0.88 ± 0.04	0.89 ± 0.04	0.90 ± 0.04	0.486
DHA/22:4ω6 + 22:5ω6[2]		1.39 ± 0.44	1.46 ± 0.66	1.41 ± 0.81	1.31 ± 0.51	1.54 ± 0.81	0.901

DHA, docosahexaenoic acid; TOVA, Test of Variables of Attention; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] For TOVA scores, better performance is the achievement of a lower score
[2] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.30 (1.02-1.72), 1.22 (1.02-1.92), 1.17 (0.94-1.64), 1.16 (0.93-1.52), 1.46 (0.94-1.69), respectively.

Table 4.19 RBC fatty acids by quintiles of TOVA Errors of Commission for Caucasian children.

	Quintiles of TOVA Errors of Commission[1]					
min-max	0-4.32	4.94-6.88	7.41-11.1	11.2-14.2	14.6-32.5	
RBC Fatty acids	n = 24	n = 25	n = 32	n = 24	n = 25	*P*
DHA	5.29 ± 1.88	5.66 ± 1.54	5.51 ± 1.36	4.80 ± 1.37	5.57 ± 1.41	0.554
22:5ω6	0.60 ± 0.22	0.61 ± 0.19	0.63 ± 0.17	0.68 ± 0.14	0.62 ± 0.18	0.640
22:4ω6	3.35 ± 0.84	3.40 ± 0.91	3.29 ± 0.52	3.62 ± 0.71	3.52 ± 0.74	0.454
DHA/DHA + 22:5ω6	1.45 ± 0.75	1.55 ± 0.78	1.48 ± 0.65	1.16 ± 0.42	1.44 ± 0.62	0.984
DHA/22:4ω6 + 22:5ω6[2]	0.89 ± 0.05	0.90 ± 0.04	0.89 ± 0.04	0.87 ± 0.04	0.89 ± 0.04	0.629

DHA, docosahexaenoic acid; TOVA, Test of Variables of Attention; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] For TOVA scores, better performance is the achievement of a lower score.
[2] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.34 (0.84-1.88), 1.32 (1.01-1.82), 1.43 (1.05-1.63), 1.05 (0.81-1.51), 1.17 (1.02-1.80), respectively.

Table 4.20 RBC fatty acids by quintiles of TOVA Errors of Omission for Caucasian children.

		Quintiles of TOVA Errors of Omission[1]					
	min-max	0-4.97	5.13-9.88	10.0-18.4	19.2-31.5	32.7-71.8	
RBC Fatty acids		n = 26	n = 25	n = 28	n = 25	n = 26	*P*
DHA		5.60 ± 1.66	5.42 ± 1.37	4.98 ± 1.59	5.37 ± 1.53	5.54 ± 1.47	0.908
22:5ω6		0.62 ± 0.18	0.69 ± 0.16	0.68 ± 0.20	0.56 ± 0.18	0.59 ± 0.16	0.545
22:4ω6		3.36 ± 0.72	3.50 ± 0.62	3.52 ± 0.74	3.26 ± 0.80	3.47 ± 0.84	0.624
DHA/DHA + 22:5ω6		1.49 ± 0.65	1.35 ± 0.49	1.29 ± 0.69	1.52 ± 0.73	1.44 ± 0.62	0.798
DHA/22:4ω6 + 22:5ω6[2]		0.89 ± 0.05	0.88 ± 0.04	0.87 ± 0.05	0.90 ± 0.04	0.90 ± 0.03	0.520

DHA, docosahexaenoic acid; TOVA, Test of Variables of Attention; RBC, red blood cell
Data are mean ± SD and quintile 1 and 5 were compared by student's t-test.
[1] For TOVA scores, better performance is the achievement of a lower score.
[2] 22:6ω3/22:4ω6 + 22:5ω6 was not normally distributed and compared by Mann-Whitney *U* test. For quintile 1-5 median (IQR) was 1.26 (0.99-1.96), 1.34 (1.06-1.61), 1.03 (0.84-1.46), 1.26 (1.03-1.61), 1.47 (1.02-1.70), respectively.

4.5 Discussion

In the present study, we addressed DHA intake and RBC DHA, and their potential relationship to neurodevelopment at 5.75 y. Although some family and child characteristics differed between those in the prospective study lasting over 6 years, and those in the cross-sectional group, there were no differences in DHA intake or RBC DHA. Thus, when the follow-up and cross-sectional data were combined, the results suggest that child RBC DHA is associated with scores on neurodevelopment tests assessed at 5.75 y. Dietary DHA was only associated to the KABC Sequential scale, which is a measure short-term memory. Notably, variability in DHA intake and RBC DHA among the children was high, with a complex relationship between DHA intake and RBC fatty acids. The association between DHA intake and RBC fatty acids was stronger with DHA intake assessed by FFQ than one or three 24 h recalls. Perhaps more important, stronger associations were found between dietary DHA and RBC indices of potential DHA sufficiency, including DHA/DHA + 22:5n6 and DHA/22:4n6 + 22:5n6.

The DHA intakes of all children were skewed and bimodal, with a mean (median) intake of 77.7 (52.9) mg/d assessed by FFQ, with 48.2 (12.2) and 57.7 (19.5) mg/d assessed by one or three 24 h recalls, respectively. Two smaller studies of Canadian children 4-8 y in Ontario (n = 41) and Alberta (n = 91) assessed DHA intakes over three days and reported mean (SD) intakes of 54.1 (72.7) and 37 (63) mg/d DHA, respectively [106,107]. A US study reported children 4-5 y had a mean (SD) DHA intake of 21 (80) mg/d, assessed by 24 h recall, and that 30% of the children had not consumed any DHA [99]. Similarly, a national dietary survey of children 4-8 y in Australia (n = 1216) used a 24 h recall and reported a mean (SD) and median (IQR) of 35.9 (92.7) and 5.1 (0.9-26.5) mg/d DHA, respectively, indicating that at least 75% of the children had DHA intakes below the mean [110]. Clearly, the usefulness of dietary assessment of one or three days may be limited for nutrients that may not be eaten daily, such as DHA.

In our study, macronutrient and ALA, LA, and ARA intakes were not different among the three dietary approaches when controlled for total energy intakes, but the mg/1000 kcal EPA and DHA was higher when assessed by FFQ than one or three 24 h recalls. In the present study, DHA intake assessed by FFQ was more strongly associated with the RBC DHA, than DHA intakes assessed by a 24 h recall for one or three days. Although this study suggests that an FFQ may provide a more accurate estimate of DHA intake than one or three day records, as illustrated

in Figure 4.2, the mean or median DHA intake assessed using an FFQ may not reflect the intakes of the majority of individuals in a group. However, in our group 27.1% consumed < 25 mg/d DHA, 22.1% consumed 25-49.9 mg/d DHA, 12.5% consumed 50-74.9 mg/d DHA, 11.8% consumed 75-99.9 mg/d DHA, and 26.4% consumed ≥ 100 mg/d DHA. Thus, it appears that an FFQ provides a more accurate estimate of DHA intake for individuals enabling better interpretation of DHA and a health outcome. In addition, reporting DHA intakes beyond mean and SD or SE data to include distributions or ranges may enable better comparison of DHA intakes within and across populations.

Associations between DHA intake and RBC fatty acids among the children in the present report were variable, with some children with low intakes having RBC DHA levels equivalent to some children with high intakes (Figure 4.1). Thus, high DHA intakes may protect children from having a low RBC DHA, but an apparent low dietary DHA intake does not necessarily imply low RBC DHA. Other studies have reported moderate relationships between dietary DHA and RBC DHA (rho = 0.3-0.6) [102,439-441]. Notably, we found stronger associations between dietary DHA and RBC indices of potential DHA sufficiency, including DHA/DHA + 22:5ω-6 and DHA/22:4ω-6 + 22:5ω-6, than RBC DHA. However, at low DHA intakes considerable variability was still apparent in the associations between DHA intake and RBC DHA/DHA + 22:5ω-6 and DHA/22:4ω-6 + 22:5ω-6 among the children. This suggests that the majority of variability in RBC DHA status may not be due to dietary DHA alone, highlighting implications for studies using DHA intake as a marker of DHA status.

Analysis of the major dietary sources of DHA for the children showed that fish and other seafood was the major DHA source for 72.5% of the children. Although children with fish or other seafood as their major dietary DHA source had higher DHA intakes than children obtaining most of their DHA from other foods, we found considerable overlap in the children's RBC DHA. For example, the median (IQR) DHA intakes were 19.2 (10.8-31.2) and 86.5 (53.6-137) mg/d for children with poultry (n = 22) or fatty fish (n = 161) as their major DHA source, respectively. Although the mean RBC DHA was 26.1% higher in children with fatty fish as the major DHA source compared to poultry, the range of ± 2SD of the mean RBC DHA was 2.81-9.01 % and 1.53-7.21%, for those with fatty fish or poultry as their major dietary DHA source, respectively.

Dietary DHA intakes were significantly higher for children of Chinese descent compared to children of other ethnic backgrounds, with a median (IQR) of 87.5 (36.4 – 146) and 48.3 (22.5 – 95.3) mg/d DHA for Chinese and Caucasian children, respectively. Although there is substantial overlap in DHA intakes of Chinese and Caucasian children, the frequency distribution shows that approximately 7% and 30% of Chinese and Caucasian children have DHA intakes < 25 mg/d, with 34% and 24%, respectively, consuming ≥100 mg/d. The higher DHA intakes of the Chinese children group likely led to their significantly higher RBC DHA compared to the Caucasian children. However, DHA intake and RBC DHA was significantly related in Caucasian, but not Chinese children. While this may be attributed to the small number of Chinese children (n = 37) in this study, there were significant associations between DHA intake and RBC 20:5ω-3 (rho = 0.413, $P = 0.011$), 22:4ω-6 (rho = -0.342, $P = 0.038$), 20:4ω-6 (rho = -0.356, $P = 0.031$), and DHA/22:4ω-6 + 22:5ω-6 (rho = 0.354, $P = 0.032$) for Chinese children. Whether or not these differences may be explained by other dietary factors is unknown, but a recent study has suggested ethnic-specific differences in fatty acid desaturase activity [442]. Notably, the major allele of three FADS SNPs differed between young adults of Asian and Caucasian backgrounds, with differences by ethnicity also observed in the minor allele frequency for several SNPs [442]. In addition, whereas a SNP in FADS1 was associated with altered fatty acid desaturase activity in all participants, a SNP in FADS2 was additionally associated with altered desaturase activity in Asians, but not Caucasians [442]. However, the difference in plasma fatty acids was small, with no difference in plasma EPA between Caucasians and Asians, and plasma DHA data was not provided [442]. Thus, further work is required to determine whether genetic variability may place some individuals at risk for low circulating levels of DHA, specifically in individuals who do not consume DHA, and also if genetic variations in fatty acid metabolism genes are related to ethnicity.

Differences between Chinese and Caucasian children were also found for the neurodevelopment test scores. These differences in test scores are challenging to interpret, and may at least in part be due to cultural factors related to child development [443,444]. For example, studies in the US have reported that Asian-American children perform better than Caucasian children on academic tests, explained in part by differing cultural beliefs on achievement [444,445]. In the present study, Chinese children achieved higher scores on

measures of visual-motor integration (Beery) and long-term storage and retrieval (KABC Learning), visual processing (KABC Simultaneous), and general mental processing ability (KABC MPI) than the Caucasian children. In contrast, Chinese children had lower scores on the language test (PPVT) than Caucasian children. However, it is possible that Chinese children had been primarily exposed to a language other than English, potentially explaining their lower scores on the vocabulary test.

For Caucasian children, there were significant, but weak, associations between RBC fatty acids and neurodevelopmental tests. The RBC fatty acid with strongest association with test scores was RBC DHA/DHA + 22:5ω-6, which was associated with scores on the measure of language (PPVT) (rho = 0.237, $P = 0.003$), short-term memory (KABC Sequential) (rho = 0.237, $P = 0.003$) and visual-motor integration (Beery) (rho = 0.168, $P = 0.018$). Notably, in a US multi-site study, a positive association between child RBC DHA and language (PPVT) scores at 4 y ($r^2 = 0.14$, $P = 0.018$) was reported, with a stronger association when Hispanic/Latino children (major minority group), were removed from the analyses ($r^2 = 0.21$, $P = 0.018$). In another study, cheek cell DHA was positively associated with non-verbal IQ (r = 0.10, $P < 0.05$) in children 8-10 y [27]. The latter study also reported positive associations between cheek cell 22:4ω-6, 22:5ω-6 and ω-6 metabolites/ω-3 metabolites with measures of child behaviour, including inattention and impulsivity [27].

In the present study, dietary DHA was not associated with neurodevelopment test scores with the exception of short-term memory (KABC Sequential), which was positively associated with EPA (rho = 0.258, $P = 0.001$) and DHA (rho = 0.227, $P = 0.002$). Similarly, children with the highest, compared to the lowest, short-term memory (KABC Sequential) scores had higher intakes of EPA and DHA. However, in the present study, long-term memory, assessed by the KABC Delayed Recall scale, was not related to dietary or RBC fatty acids. Similarly, a study in the US of children 7-9 y reported that total ω-3 fatty acid intake was positively associated with accuracy on hippocampal-dependent relational memory (r = 0.28, $P = 0.04$), but not hippocampal-independent memory (r = 0.21, $P = 0.14$) [26]. Hippocampal-dependent relational memory was also positively associated with DHA intakes after adjustment for BMI z-score (r = 0.32, $P < 0.03$), but neither DHA intake nor memory was related to BMI z-score and the bivariate analysis was not reported [26].

Notably, the hippocampus has been suggested to be involved in both short- and long-term memory as well as learning [180], and experimental studies have shown that the structure and function of the hippocampus is altered by an ω-3 fatty acid deficient diet in development [211,437,446,447]. In the present study, the KABC learning scale, which assesses long-term information storage and retrieval, was not related to dietary DHA or RBC fatty acids. In contrast, experimental studies have shown inferior learning ability in ω-3 fatty acid deficient rats [9,307,308,310,312]. Importantly, in animal studies assessing learning ability and ω-3 fatty acids, an ω-3 fatty acid deficient diet is often given for two or more generations, thus, it may be that DHA was not low enough in our population to have an effect on learning. One study in healthy humans reported improved verbal learning ability in children 7-9 y after supplementation with DHA [371]. However, it is possible that the verbal learning assessment was more related to language development, which has previously been associated with the DHA supply during pregnancy [14,16,17,398], infancy [363] and childhood [369].

Although we found a weak but positive association between the RBC DHA/DHA + 22:5ω-6 and the measure of visual-motor integration (Beery), visual processing (KABC Simultaneous) was not associated with dietary DHA or RBC fatty acids. In contrast, a positive association has been reported for infant plasma PL DHA at 4 wk and visual processing (KABC Simultaneous) at 4 y [342]. One study reported improved visual attention among children 8-10 y following 16 wk of fish oil supplementation [27], but whether the improved score of children in the fish oil group was due to visual processing or attention is not known. In the present study, we found no differences in DHA intake or RBC fatty acids among the quintiles of children for the TOVA, which provides a score for processing speed (response time), consistency (response time variability), impulsivity (errors of commission), and attention (errors of omission). However, the need to sit and concentrate on a computer screen for nine minutes for this test may have led to the high number of incomplete assessments (n = 56), and thus affected the validity of these results in our study. Results of other studies have shown that DHA supplementation improved teacher-rated impulsivity [27,370], and one study reporting an inverse association between RBC DHA and reaction time on a sustained attention test (r^2 = -0.18, P = 0.01) in 8-10 yr old boys [28].

To summarize, the results of the present study suggest that children's DHA intake and status at 5.75 y is related to neurological performance, particularly short-term memory, and is consistent with continued brain growth, including synthesis of DHA rich synapses, throughout the first 6-7 years of life [21]. However, the lack of association of dietary DHA with measures of language (PPVT), visual-motor integration (Beery), and general mental processing ability (KABC MPI) suggest that the associations between RBC fatty acids and scores on these measures may not be explained by DHA intake. In addition, this study emphasized the importance of dietary methodology to assess DHA intake in a group of children, while also illustrating that mean and median DHA intakes do not reflect the individual intakes of the majority in the group. Although our data suggests that the FFQ provided a better estimate of DHA intake than one or three day recalls, the RBC DHA may not be explained by DHA intake alone, with potential ethnic-specific interactions also noted. This raises the possibility that potential interactions with ethnicity may be overlooked if sample sizes are inadequate to detect significant differences between ethnic groups. Notably, stronger associations of both dietary DHA and scores on neurodevelopment tests with RBC DHA/DHA + 22:5ω-6 and DHA/22:4ω-6 + 22:5ω-6, than RBC DHA were found, but variability at low DHA intakes was high. Thus, whether RBC DHA/DHA + 22:5ω-6 and DHA/22:4ω-6 + 22:5ω-6 are more sensitive indices of DHA sufficiency in the CNS requires further investigation. Finally, although the critical time period(s) when the developing brain is most vulnerable to inadequate DHA is not yet known, this study supports evidence that the child's own DHA status may have a role in neural function.

Chapter 5: Conclusion

5.1 Summary

The ω-3 fatty acid DHA accumulates in neural tissue during development, but its roles in neural lipids and neurodevelopment are not completely understood. Importantly, human brain development begins early during gestation and continues well after birth. It is unknown whether DHA inadequacy *in utero* or after birth has implications for CNS development. The results of this dissertation have shown that fetal DHA inadequacy low enough to constrain infant neurodevelopment to 18 mo of age occurred in our population (Chapter 2), although no evidence of persisting effects to 5.75 y was found (Chapter 3). The apparent effect of fetal DHA inadequacy found in infancy may have been lost or masked by postnatal dietary exposures in the child. However, associations were found between the mothers' dietary and RBC DHA during gestation and the children's diet and RBC DHA at 5.75 y (Chapter 3). Further investigation showed associations of child dietary and RBC DHA with neurodevelopment at 5.75 y (Chapter 4), adding complexity to understanding the potential pre- and post-natal effects of DHA in CNS development. Notably, the variability in RBC DHA among the mothers and children was high (Chapters 2-4), raising the question about the relationship of DHA intake and other dietary fatty acids, DHA status and transfer to the brain, and neural function. This chapter begins with a summary of the hypotheses and outcomes for Chapters 2, 3 and 4 (Tables 5.1-5.3). This is followed by a general discussion of the results, including specific contributions of this work, study strengths and limitations, and concludes with future directions of the research.

Table 5.1 Hypotheses and summary of major findings for Chapter 2.

Null hypothesis # 1: The level of RBC DHA among pregnant women will not differ between 16 and 36 wk gestation, or between women assigned to 400 mg/d DHA or placebo at 36 wk gestation. *Outcome:* Gestation from 16 to 36 wk led to an increase in RBC DHA among all pregnant women with 400 mg/d DHA leading to an increase of approximately 42% higher RBC DHA than that in the placebo group.
Null hypothesis # 2: The distributions of scores on tests of infant neurodevelopment will not differ between infants born to women assigned to 400 mg/d DHA or a placebo. *Outcome:* There were no differences in mean scores on any test from 2-18 mo for infants born to mothers in the placebo and DHA groups. However, infants in the placebo group were at increased risk of failing to be among infants achieving a high score for visual acuity at 2 mo, and language development at 14 and 18 mo. There was no difference in risk of failure to achieve a high score for visual acuity at 12 mo, or cognitive and motor skill tests at 18 mo.
Null hypothesis # 3: Fetal DHA inadequacy sufficient to constrain infant neurodevelopment does not occur in our population *Outcome:* Increased risk of lower developmental scores among infants in the placebo group, compared to the DHA group was found, suggesting that fetal DHA inadequacy sufficient to constrain infant neurodevelopment was present in this group of pregnant women.
Conclusion: Despite ~20 weeks of intervention with 400 mg/d DHA leading to higher RBC DHA in the DHA group, varying amounts of DHA in the diet led to considerable overlap of RBC DHA between women in the DHA group and the placebo group. Using a risk-reduction model, this study suggests that inadequate DHA supplies during pregnancy occurs and limits infant developmental potential to 18 mo. Our results suggest that language development was more sensitive to the *in utero* DHA supply than other developmental tests used.

Table 5.2 Hypotheses and summary of major findings for Chapter 3.

Null hypothesis # 1: Fetal DHA inadequacy sufficient to constrain neurodevelopment will not be detected in children assessed at 5.75 y. *Outcome:* There was no difference in risk of infants in the placebo group achieving performance in the upper quartile of performance on any test. This may suggest no lasting effects of fetal DHA inadequacy or that effects were lost or masked by postnatal variables by 5.75 y. However, children of mothers with RBC DHA at 36 wk gestation in the highest quartile had higher scores on measures of language (PPVT), short-term memory (KABC Sequential), and visual processing (KABC Simultaneous) compared to the children of mothers in the lowest quartile.
Null hypothesis # 2: Maternal intake and RBC DHA during gestation will show no relationship to child DHA intake and RBC DHA at 5.75 y. *Outcome:* Child DHA intake was positively related to the maternal DHA intake at 16 and 36 wk gestation, as well as with maternal RBC PE and PC DHA/22:4ω-6+22:5ω-6 at 16 wk gestation. Child RBC DHA was related to maternal DHA intake at 36 wk gestation, as well as to maternal RBC PE DHA at 16 wk gestation.
Conclusion: The apparent effects of DHA insufficiency during gestation on CNS development up to 18 mo may have been lost or masked by postnatal dietary variables in children at 5.75 y. Associations between the children's dietary and RBC DHA status and that of the mother's add complexity to separating the pre- and post-natal effects of DHA supplies on brain development.

Table 5.3 Hypotheses and summary of major findings for Chapter 4.

Null hypothesis # 1: Dietary and RBC fatty acids and neurodevelopment test scores will not differ between a prospective cohort of children and a cross-sectional group of children. *Outcome:* Dietary fatty acid intake did not differ between children in the follow-up and cross-sectional groups when assessed by FFQ, one or three 24h recalls, except for higher ARA intake assessed by FFQ in the cross-sectional than follow-up group. There was no difference in RBC DHA between the two groups of children. The follow up children had higher RBC ALA, 22:5ω-6, 22:4ω-6, and lower RBC EPA, and DHA/ 22:4ω-6 + 22:5ω-6 compared to the cross-sectional children. Children in the follow-up group also had higher scores on measures of long-term storage and retrieval, general mental processing ability, and long-term memory than the cross-sectional group.
Null hypothesis # 2: Child DHA intake will show no relationship to neurodevelopment test score. *Outcome:* The relationship of dietary and RBC fatty acids with neurodevelopment test scores was analyzed for Caucasian children only, due to the small number of children from other backgrounds and the potential interaction of diet, RBC fatty acids, and test scores with ethnicity. For Caucasian children, dietary DHA as well as EPA were positively associated with scores on a measure of short-term memory. In addition, the quintile of children with the highest short-term memory scores had significantly higher intakes of DHA and EPA than the lowest quintile. Dietary DHA was not associated with scores on any other test.
Null hypothesis # 3: Child RBC DHA will show no relationship to neurodevelopment test score. *Outcome:* For Caucasian children, RBC DHA was positively associated with scores on measures of language and short-term memory, with a trend for general mental processing ability. A positive association was observed between RBC DHA/DHA + 22:5ω-6 and scores on tests of language, visual-motor integration, and short-term memory. The RBC DHA was higher in the quintile of children with the highest scores on the test of short-term memory and general mental processing, with a trend for language scores, compared to the lowest quintile. The children's RBC DHA/ 22:4ω-6 + 22:5ω-6 was higher in the quintile of children with the highest scores, than the lowest quintile for the measure of language and short-term memory.
Conclusion: Child RBC DHA is associated with neurodevelopment at 5.75 y, but the association on some tests may not be explained by DHA intake alone. Variability in RBC DHA was high, raising important questions on the association between DHA intake and other fatty acids, DHA transfer to the brain and CNS function. Exploratory analyses in this chapter raise further questions on potential interactions with ethnicity, suggesting further investigation of non-Caucasian children to determine the associations between dietary and RBC ω-3 and ω-6 fatty acids, and neurodevelopment. Given that the child's RBC DHA was associated with neurodevelopment test performance, this adds further implications for studies assessing DHA status *in utero* or infancy and neurodevelopment in later childhood.

5.2 General Discussion

5.2.1 Specific Contributions

The overall goal of my research was to address whether ω-3 fatty acid inadequacy among pregnant women is sufficient to influence early infant and child CNS development, and also if child dietary and RBC DHA impacts neurodevelopment at 5.75 y. The specific contributions of this work are listed below.

1. The study in Chapter 2 identified fetal DHA inadequacy in a group of pregnant women and that increasing DHA intakes during pregnancy reduced risk of lower infant neurodevelopment.

The unique enrichment of DHA in neural and retinal membrane lipids has led to considerable interest into the role of DHA in the developing CNS. Despite reported positive associations between fish intake and/or DHA in maternal blood during gestation and visual and neurodevelopment in the infants, many of the randomized DHA intervention studies during pregnancy have reported no effect of DHA on infant/child neurodevelopment. Our study in Chapter 2 was designed with the expectation that increasing maternal DHA intake should reduce the risk of insufficient fetal DHA, enabling infants to achieve their developmental potential. The risk-reduction model showed that fewer infants in the placebo than DHA supplement group achieved neurodevelopmental test scores in the highest quartile, illustrated by the frequency distributions of test scores for the DHA and placebo groups in Figure 2.5. Furthermore, the distributions show the considerable overlap in test scores of the DHA and placebo groups, highlighting that the subtle differences may be missed in mean score comparisons between the two groups. Thus, as the prevalence of DHA deficiency in a population is not known, null findings of a DHA intervention may be explained by a low prevalence of DHA deficiency in a population, rather than no effect of DHA on the developing CNS.

2. The study in Chapter 3 was the first to show a relationship between maternal DHA intake and status during pregnancy and their children's DHA intake and status at 5.75 y.

Several studies have addressed the role of the family food environment on child intake, with most of the information in this field focused on macronutrient intake. Little is known about

family and child DHA intake, although one small Italian study (n = 37) reported no significant difference in fish and seafood intake between mothers and children 8-11 y [434]. A positive association between maternal and child DHA intake is a reasonable assumption, and if correct would have implications for studies of maternal DHA during gestation with neurodevelopment outcomes in children, since both brain development and DHA accretion begin *in utero* and continue after birth. Remarkably, our data showed a positive association between maternal DHA intake during gestation and the child's DHA intake six years later. Perhaps of greater importance were the positive associations between RBC fatty acids of the mother at 16 wk gestation and the child at 5.75 y, with stronger associations for RBC markers of DHA sufficiency than for RBC DHA. While understanding the pre- and post-natal effects of DHA on the developing human CNS remains unclear, this work suggests that a clear separation is complex, with interaction of the family diet and continued CNS development.

3. Potential markers of DHA sufficiency, including RBC 22:5ω-6, DHA/DHA + 22:5ω-6 and DHA/22:4ω-6+22:5ω-6 may be more sensitive than RBC DHA.

As described, low brain DHA is accompanied by increased brain 22:5ω-6 and sometimes 22:4ω-6, reducing the ratio of DHA to 22:4ω-6 + 22:5ω-6. The degree to which this change in the brain is reflected in RBC lipids is less clear. Regardless, the potential relationship between markers of DHA sufficiency in RBC with DHA intake has not been previously reported. The studies presented in this dissertation (Chapters 3 and 4) report that RBC 22:5ω-6, DHA/DHA + 22:5ω-6, and DHA/ 22:4ω-6+22:5ω-6 were more strongly related to DHA intake, and some child test scores, than RBC DHA. Thus, whether RBC 22:5ω-6, DHA/DHA + 22:5ω-6, or DHA/ 22:4ω-6+22:5ω-6 reflects DHA in the brain and can serve as sensitive markers of DHA sufficiency, or if they simply reflect DHA in the diet, warrants further investigation.

4. Results of studies on infants and children suggest that language development is sensitive to DHA supply.

One of the major challenges in understanding the role of DHA in CNS development is the developmental time-course and absence of a specific outcome measurement indicative of adequate or delayed development. Although methodologies more indicative of brain function,

such as the functional MRI, are becoming more accessible, studies to date of DHA on infant or child outcome have used multiple approaches assessing different domains of neurodevelopment. Many studies, including those described here, have used standardized clinical tools designed to identify infants and children with developmental delay. Further problematic, comparability of the same domain, cognition for example, assessed by different tests and at different ages is unclear. The results presented in this dissertation, however, show that DHA is related to language development assessed with the CDI infant scale at 14 mo, the CDI infant and toddler scale and the BSID-III at 18 mo, and the PPVT at 5.75 y. Although receptive (words understood) language at 14 mo, 18 mo, and 5.75 y was associated with maternal DHA during gestation (Chapter 2 and 3), it was also associated with the child's own RBC DHA at 5.75 y (Chapter 4). Observational studies have reported positive associations of language development with maternal and/or infant fish intake [14,16,17], whole blood DHA/22:5n6 [363], and whole blood DHA [369]. Whether DHA has a role in the learning or processing of language, or has a role in the auditory system requires further study.

5.2.2 General Comments

This research has also raised several important implications related to work towards the establishment of dietary recommendations for ω-3 and ω-6 fatty acids.

1. Future research on ω-3 and ω-6 fatty acids will be limited by dietary assessment methodology and food composition databases.

The Canadian food supply has changed dramatically in the past 100 years, adding complexity to current dietary assessment methods, and raising the question of how to accurately assess the intake of some nutrients despite continued and rapid changes to food supply. As noted in section 1.2.2.2, obtaining accurate dietary information for nutrients that may not be consumed daily, such as DHA from fish, is a challenge. However, several studies, including large population surveys used to guide dietary recommendations, assess intake for only 1 to 3 days [99,100,104,107,110]. The study in Chapter 4 suggests that an FFQ provides a better estimate of DHA intake than a 24 h recall for one or three days. In addition, the publication of daily average intake may limit interpretation and comparison of DHA intakes across and within populations, as

the average data is poorly reflective of the intake of most individuals in a group. This suggests that future studies should describe intakes that reflect the majority in a group to enable the development of future guidelines based on the lowest association of risk. Also important, studies of ω-3 and ω-6 fatty acid intake are limited by food composition databases, which may not always reflect the nutrient composition of specific foods eaten by a study participant [70]. Incomplete nutrition information on product labels, as well as new or modified products, add further limitations to the accuracy of food intake data, and therefore studies relating dietary intakes to specific health outcomes.

Further relevant to the study of DHA intake is that dietary methodology is typically used to assess food intake over a period of weeks to years, with the resulting nutrient analysis reported as an average daily intake. For example, one 75g serving/wk of wild Atlantic salmon providing a single dose of approximately 840 mg DHA which would be collapsed to 120 mg/d, whereas two eggs/d would provide approximately 60 mg DHA every day [70]. Whether or not a large amount of DHA, or other ω-3 and ω-6 fatty acids, consumed infrequently is functionally equivalent to a small amount eaten daily deserves further attention.

2. Dietary DHA intake should not be used as a proxy to infer DHA status.

Several observational studies have reported associations between fish and/or DHA intake during gestation, infancy or childhood and measures of infant and child neurodevelopment [14-19,26,326]. In Chapter 4 we showed considerable individual variability in the association between DHA intake and RBC DHA, at least at DHA intakes below 175 mg/d. Although high DHA intakes may reduce the risk of low RBC DHA, many children with lower DHA intakes have RBC DHA levels equivalent to those with high DHA intakes. Thus, the usefulness of DHA intake as a proxy for DHA status is limited to children with high intakes, and further work is required to explain the dietary or other factors that may influence RBC DHA in individuals with lower intakes.

3. Dietary recommendations for ω-3 and ω-6 fatty acids should be based on evidence and not observed dietary intakes.

A major question remaining is whether or not the current food supply, dietary recommendations, and thus the resulting food intakes, limit the synthesis and/or circulating levels of DHA. Until 1977, dietary recommendations in Canada and the US were 1-3 % energy from LA for infants and adults, with the guidelines based on preventing essential fatty acid deficiency. The first Canadian recommendation, which preceded the US, for ALA of 0.5 % energy was set in 1990 [448-450]. Despite a lack of evidence, the controversial "Dietary Goals for the United States" report, published in 1977 by the US Senate Select Committee on Nutrition and Human Needs, recommended an increase in polyunsaturated fat intake to 10 % energy, a small increase from the estimated American intake of 7 % energy [451]. However, this estimate of polyunsaturated fat intake was based on food disappearance data, which in addition to wastage, does not account for vegetable oil used in deep-frying but is not actually consumed, thus the actual intake of polyunsaturated fatty acids may have been much lower. Further problematic was a lack of support from experts in the field of nutrition and lipid metabolism, with multiple objections including caution of potential adverse effects of increased polyunsaturated fat intakes [452]. Indeed, evidence suggests that intakes of LA of 10-12 % energy with 1-2 % energy from ALA may be too high to support endogenous synthesis of DHA [25,53]. Regardless, the current (2002) dietary recommendation in Canada and the US for LA and ALA is 8-10 and 0.6-1.2 % energy, respectively, and was based on the median intake of Americans in 1994-1996. In the present studies, the intake of LA was estimated as an approximate median (2.5-97.5 %) of 5 (2.7-9.4) % energy and for ALA of 0.6 (0.3-1.7) % energy for pregnant women, and 4 (1.7-10.5) % energy LA and 0.3 (0.1-1.5) % energy ALA for children 5.75 y. Thus, while most individuals in our study in Vancouver have LA and ALA intakes below the American intakes used to set the current DRI for LA, the high LA and ALA intakes of some in our study may place them at risk for low circulating DHA.

4. Potential ethnic-specific differences in diet and/or requirements should be considered in dietary recommendations.

The Canadian population is made up of people of many ethnicities, and ethnic differences in diet and metabolism may exist. For each nutrient, there is a single DRI for healthy individuals in a given life-stage. In Chapter 4, the data show that children of Chinese background had higher

intakes of DHA and higher RBC DHA than Caucasian children. Similarly, the NHANES 2011-2012 data is also suggestive of ethnic differences in DHA intake, with intakes 3-fold and 2-fold higher in 'non-Hispanic Asian' adults and children, respectively, than 'non-Hispanic white' adults and children [100]. Although evidence of ethnic-specific variability in FADS and ELOVL genes is limited [442], work on other nutrients suggests that ethnic-specific differences may alter metabolism of some nutrients. For example, lactase persistence, caused by a SNP upstream of the lactase gene conferring the ability to digest lactose into adulthood, shows a high frequency (50-90%) in European populations, but is low (<10%) in some Asian populations [453,454]. This suggests that further work is required to determine if genetic variability may place some individuals at risk for low DHA status, as well as the potential implications for dietary recommendations.

5.3 Strengths and Limitations

As noted, the study in Chapter 2 was the first to prospectively assess fetal DHA inadequacy using a randomized placebo controlled DHA supplementation trial and CNS outcomes until 18 mo of age. The intervention of 400 mg/d DHA was designed to create one group of pregnant women as unlikely to be DHA deficient in a group where the presence of DHA deficiency is unknown and a biomarker indicative of DHA deficiency or sufficiency is currently unavailable. The study design recognized that the placebo group is not a 'non-exposure' group, and thus both groups would contain individuals that would not benefit from intervention. This is an important distinction from efficacy studies, which carry the underlying assumption that all individuals in the intervention group have the ability to respond to the intervention.

The results of these studies may not be reflective of other populations, including across Canada. The wide availability of fish in Vancouver may have influenced the amount consumed by our population, with fish and other seafood providing the major source of DHA to 74% of the children in our study (Chapter 4). Similarly, the volunteer nature of this study likely impacted the population that enrolled, with parents interested in nutrition or child development more likely to participate. Despite our attempts to recruit a sample reflective of the Vancouver population, requirements of our studies, including study visits at the Child and Family Research Institute and

the ability to understand English, may have limited enrollment. Importantly, although subject and family characteristics were similar among the participants, variability of dietary intake and RBC DHA among the pregnant women and children was high. Thus, the homogenous population in these studies of predominantly Caucasian background, with a large proportion being highly educated and with a high household income, may have reduced the effect of some environmental variables of infant and child development.

The challenges of obtaining accurate dietary information, including reporting bias and over or under-estimation of dietary intake, may have affected the accuracy of the usual dietary intake estimation. Although trained interviewers and data crosschecking methods were used, the parents' report on the child's intake provided additional limitations. As introduced in section 1.2.2.2, accurate estimation of dietary nutrient intake is in part limited by the available food composition databases. Relevant to DHA intake, while the dietary assessments included questions specific to fish type, the amount of DHA varies among sub-species, e.g. Atlantic or Coho salmon, and may also vary by other factors including age and diet of the fish. This causes the possibility of potential variations in DHA content in foods consumed by the children, which was analyzed using the average composition of each food. Similarly, differences or changes to formulations of prepared foods and products containing vegetable oils may have limited the accuracy of dietary LA and ALA, for example when the product label states "vegetable oil" or "soy/canola oil," or products purchased from the many food service establishments in the city.

The developmental assessments used in these studies were chosen to minimize cultural content. However, there were differences in neurodevelopment test scores between children of Caucasian and Chinese ethnicity (Chapter 4), with separate analyses not possible for the small number of children of other ethnicities. Regardless, the differences between Caucasian and Chinese children are challenging to interpret, and may be due to differing cultural social and children rearing norms. For example, Caucasian children achieved higher scores on the PPVT (a measure of receptive vocabulary) than Chinese children. Although English comprehension was required for study participation, it is possible that some Chinese children had less exposure to English and may not have been exposed to words used in the test. However, because ethnicity was collected by the parent report, interpretation of data related to ethnicity was not possible.

Infants and children with the highest neurodevelopmental scores were defined by an arbitrary definition of the highest quantile of children stratified by test score. However, it is possible that alternate definitions would yield different results. Equally important, the developmental assessments were chosen to reflect different cognitive and behavioural abilities, but they may not be sensitive to detect effects related to DHA. Although brain development continues rapidly in early childhood, our studies in chapters 3 and 4 were also limited by neurodevelopment assessment and DHA status at a single-time point at 5.75 y, with DHA intake assessed over the previous month. Whether or not current DHA intake and status reflects DHA intake and status at earlier child ages is not known.

5.4 Future Directions

The results of this research have raised important considerations for future research. Although the study in Chapter 2 suggests that DHA inadequacy sufficient to limit infant development to 18 mo of age occurs among some pregnant women in Vancouver, it did not address the cause of the inadequacy. In addition, this study did not identify dietary ω-3 fatty acid requirements, nor the level of RBC DHA associated with DHA inadequacy. An important next step would be to better identify the determinants of DHA status, such as diet or genetic variation, as well as DHA transfer to the fetus. Better understanding of these factors would enable targeted enrolment of individuals at risk for DHA inadequacy, providing a group more likely to be able to respond to a potential DHA intervention.

The CNS begins to develop as early as the third week post-conception, with the major scaffolding of the brain complete by eight weeks gestation. However, intervention studies of DHA during gestation, including ours, usually begin supplementation between 15-25 wk gestation. It is possible that interventions beginning in the second trimester miss potential early effects of the DHA supply *in utero,* which may explain the discrepancy in findings between intervention and observational studies of DHA during gestation. Although methodologically challenging, a future study beginning intervention prior to conception is required to determine if the DHA supply early during gestation is important for the structural development of the embryonic CNS.

Brain development continues after birth, with a 160 % increase in brain weight occurring by the end of the first year of life [177]. However, very little is known about current feeding and weaning practices of Canadian infants. Thus, whether current feeding practices place infants at risk of inadequate DHA to support rapid CNS development occurring in the first year is also unknown. Although available studies of DHA during gestation include assessments of infant neurodevelopment in the first two years after birth or beyond, consideration of the infants' and/or children's ω-3 fatty acid intake has not been addressed. The association between maternal DHA intake and status during gestation and child DHA intake and status at 5.75 y shown in Chapter 3 highlights important implications for studies assessing long-term outcomes of DHA during gestation. Further work is required to determine if ω-3 fatty acids provided after birth have the ability to overcome potential effects of inadequate DHA *in utero*. Alternately, it may be that the reversal is possible, that inadequate DHA after birth may negate effects of adequate DHA *in utero.*

Further studies are also needed to determine what transfers DHA to the brain, which may enable identification of a biomarker that best reflects brain DHA. Although RBC DHA has been shown to correlate with brain DHA in animals fed diets with or without DHA [51,144,167-170], the association in humans is less clear [163,171]. Some experimental studies have suggested that DHA esterified to the *sn*-2 position of LPC is preferentially incorporated into the brain compared to unesterified DHA [241,251,253], but it is plausible that other pathways are present.

Significant differences in DHA intake, RBC DHA and neurodevelopmental test scores between Caucasian and Chinese children were reported in Chapter 4. Notably, differences in the association of dietary DHA intake and RBC fatty acids were also found between the Caucasian and Chinese children. It is very possible that the differences are due to the smaller number of Chinese children, but ethnic-specific differences in genetic variability in FADS genes have been reported [442]. Whether or not differences are also present between other ethnicities should be considered in future investigations. It would also be important to determine if outcomes that differ by ethnicity are explained by cultural or biological factors, either alone or in combination. This would then have implications for how to collect data on ethnicity and culture, especially for individuals that identify with one ethnicity and adopt the culture of another. Potential ethnic-specific differences in ω-3 fatty acid requirement and metabolism would therefore have

important implications for dietary recommendations in Canada and the US, which do not currently differentiate by ethnicity or cultural dietary patterns.

Notably, several international expert groups have made recommendations for fish and/or DHA intake for pregnant and lactating women and infants to support infant CNS development. Although DHA in RBC, infant cord blood, and human milk of vegetarian and vegans has been shown to be lower than in omnivores [262,274], no published studies have investigated the neurodevelopment of infants and/or children born to vegetarian women. Thus, future work is warranted to specifically address if neurodevelopment differs among infants/children born to vegetarian and omnivore women. In addition, DHA intervention studies in vegetarians are also required to determine if infants/children in this population are at risk of inadequate DHA to compromise CNS development.

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Appendices

Appendix A Study Documents

A.1 Food Frequency Questionnaire

Food Frequency Questionnaire
Nutrition Research and Metabolism Program, University of British Columbia

Date: _____ Subject Number: _____

Does the child eat or drink the food? If yes, how often (per day, week, or month), and how much? If no, continue on to the next food or drink.

MILK PRODUCTS:				
Cow Milk	□ yes □ no	□ Whole □ 2% □ 1% □ skim	___ □ oz □ cup/ml	___ □ Day □ Week □ Month
Chocolate Milk/ Flavored Milks *e.g.Nestle Quik, choc,straw,banana*	□ yes □ no	□ Ready-to-drink □ Powder added to milk □ Syrup added to milk	___ □ oz □ cup/ml	___ □ Day □ Week □ Month
Other Milks/Milk Alternates (What type?)	□ yes □ no	□ Goat □ Whip Cream □ Rice □ Soy □ Half-N-Half/Creamo □ Other Milk products	___ □ oz □ cup/ml	___ □ Day □ Week □ Month
CHEESE, EGGS, YOGURT:				
Hard Cheeses □ Regular □ Low Fat □ Skim (skim mozza) **(What type?)**	□ yes □ no	□ cheddar □ mozzarella □ gouda □ swiss □ havarti □ edam □ other: ----------	___ total all types □ oz/g □ tbsp □ 1 inch cube	___ □ Day □ Week □ Month
Soft cheeses (What type?)	□ yes □ no	□ brie □ camembert □ feta □ goat □ other: ----------	___ total all types □ oz/g □ tbsp □ 1 inch cube	___ □ Day □ Week □ Month
Processed cheese slices or Cheese spread *used on toast, bread, hamburgers, sandwiches?* □ Regular □ Low Fat □ Skim	□ yes □ no		___ □ slice □ tbsp	___ □ Day □ Week □ Month
Soy cheese	□ yes □ no		___ □ oz/g □ tbsp □ 1 inch cube	___ □ Day □ Week □ Month
Paneer	□ yes □ no		___ □ oz/g □ tbsp □ 1 inch	___ □ Day □ Week □ Month

			cube	
Cottage Cheese	□ yes □ no	□ Regular 4% □1 or 2 %	___ □ oz/g □ tbsp □ cup/ml	___ □ Day □ Week □ Month
Cream cheese □ regular □ low fat "lite"	□ yes □ no	□ spread	___ □ oz/g □ tbsp	___ □ Day □ Week □ Month
Yogurt □ regular/ full fat >4% □ 1% or 2% □ fat free/Skim/ 0% □ DHA/omega-3	□ yes □ no	□ Mini-go □ Tubes □ Balkan Style *Most Common Type:* _____	___ □ ¼ C./ 60g □ 125g □ 175g □ each	___ □ Day □ Week □ Month
Sour Cream □ regular 14% □ low fat 7% □ fat free 0%	□ yes □ no		___ □ tsp □ tbsp □ cup/ml	___ □ Day □ Week □ Month
Eggs □ regular □ omega-3 □ egg yolk only □ egg white only □ egg beaters	□ yes □ no	□ whole, all forms □ scrambled □ hard boiled	___ □ each S M L XL ___ □ oz/g □ tbsp □ cup/ml	___ □ Day □ Week □ Month
SNACK ITEMS:				
Which 3 types of crackers most often eaten: *Please describe: soda, Ritz, Wheat Thins, Gold Fish, Cheddar Bunnies*	□ yes □ no	**Cracker #1:_____** **Cracker #2:_____** **Cracker #3:_____**	___ □ piece(s)	___ □ Day □ Week □ Month
Which 3 types of cookies most often eaten: *Please describe:* *Choc. Chip, Oreos, Peanut Butter, gingersnap, choc/marshmallow*	□ yes □ no	**Cookie #1:_____** **Cookie #2:_____** **Cookie #3:_____**	___ □ piece(s)	___ □ Day □ Week □ Month
Granola Bars & Cereal Bars *hard, chewy, Nutrigrain, omega-3*	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ pieces	___ □ Day □ Week □ Month
Popcorn **What type?** **Added ?** □ butter □ margarine □ oil (specify): _____	□ yes □ no	□ air □ microwave plain □ microwave butter flavr □ oil popped □ light	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month

Pudding or Custard RTE pudding cup	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Cake or Cupcakes *Yellow/Choc Cake, cupcakes with icing*	□ yes □ no	*Most Common Type:* _____ □ with icing □ no icing	___ □ oz/g □ piece	___ □ Day □ Week □ Month
Pop Tarts/Breakfast Pastry	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Pies/ Fruit Crisps/ Fruit Crumbles: fruit, pumpkin	□ yes □ no	*Most Common Type:* _____ □ added cream □ added whip cream	___ □ oz/ □ cup/ml □ piece	___ □ Day □ Week □ Month
BREADS, CEREALS, BAKED GOODS:				
Bread Slices	□ yes □ no	□ White □ Whole Wheat □ Other: _____	___ □ slices	___ □ Day □ Week □ Month
Dinner rolls/buns	□ yes □ no	□ White □ Whole Wheat	___ □ each	□ Day □ Week ___ □ Month
Bagels: *Brand: _____* *(cinnamon-raisin; oat bran, whole wheat)*	□ yes □ no	*Most Common Type:* □ White □ Whole Wheat □ Flax □ Sesame □ Other: _____	___ □ small □ med □ large	___ □ Day □ Week □ Month
English muffin, crumpet	□ yes □ no	□ White □ Whole Wheat	___ □ each	___ □ Day □ Week □ Month
Muffin **Usual type or source?**	□ yes □ no	Most Common Type: □ Homemade □ Tim Hortons □ Starbucks □ McDonalds □ Blueberry/fruit □ Chocolate Chip □ Bran/whole grain □ Carrot □ Other_____	___ □ each	___ □ Day □ Week □ Month
Scones *Regular* *Whole wheat*		*Most Common Type:* _____	___ □ each	___ □ Day □ Week □ Month
Quick Breads Banana Bread, Lemon loaf Homemade / store bought		*Most Common Type:* _____	___ □ each	___ □ Day □ Week □ Month

What is added to breads/bagels/crumpets/ muffins/ scones?	□ yes □ no	□ Jam/jelly	___ □ tsp □ Tbsp	___ □ Day □ Week □ Month
	□ yes □ no	□ peanut butter Regular/ low fat/ Natural	___ □ tsp □ Tbsp	___ □ Day □ Week □ Month
	□ yes □ no	□ Nut butters almond butter, seed butter, nutella	___ □ tsp □ Tbsp	___ □ Day □ Week □ Month
	□ yes □ no	□ Butter □ Margarine *Brand*_____	___ □ tsp □ Tbsp	___ □ Day □ Week □ Month
	□ yes □ no	□ Mayo (regular, low fat, olive oil)/ Miracle Whip(regular/low fat)	___ □ tsp □ Tbsp	___ □ Day □ W □ Month
Donuts, pastries *(Chocolate cake; glazed donut; jelly-filled donut; danishes, Timbits)*	□ yes □ no	*Most Common Type/Source:* _____	___ □ each	___ □ Day □ Week □ Month
Croissants	□ yes □ no	*Most Common Type/Source:* _____	___ □ each	___ □ Day □ Week □ Month
Rice cakes (What type?)	□ yes □ no	*Most Common Type:* □ plain □ with topping □ mini □ other:_____	___ □ each	___ □ Day □ Week □ Month
Chappati/Roti/Pita	□ yes □ no	*Most Common Type/Source:* _____	___ □ each	___ □ Day □ Week □ Month
Steamed buns	□ yes □ no	*Specify filling*: _____	___ □ each	___ □ Day □ Week □ Month
Tortillas □ 6-8" □ 10-12 "	□ yes □ no	□ Corn □ Whole Wheat □ White	___ □ each	___ □ Day □ Week □ Month
Breakfast Cereal Most Common Type?	□ yes □ no	**breakfast cereal #1** _____ **breakfast cereal #2** _____ **breakfast cereal #3** _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Milk added to cold cereal?	□ yes □ no	□ Whole □ 2% □ 1% □ Skim □ Other_____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Anything else added to cold cereal? *Please describe:*	□ yes □ no	□ fruit □ dried fruit □ sugar Other: _____	___ □ tsp □ tbsp	___ □ Day □ Week □ Month

Cooked Cereal/Oatmeal What type?	□ yes □ no	□ Oatmeal □ Plain Inst □ Flavored □ Cream of Wheat □ Red River □ Other_____	___	□ oz/g □ cup/ml □ Pkg ea	___	□ Day □ Week □ Month
Milk added to cooked cereal/Oatmeal?	□ yes □ no	□ Whole □ 2% □ 1% □ Skim □ Other_____	___	□ oz/g □ cup/ml □ tsp □ tbsp	___	□ Day □ Week □ Month
Anything else added to hot cereal? *Please describe:*	□ yes □ no	□ fruit □ dried fruit □ sugar □ butter □ margarine □ Other_____	___	□ oz/g □ cup/ml □ tsp □ tbsp	___	□ Day □ Week □ Month
Pancakes/Waffles/French Toast (includes dining out) What type?	□ yes □ no	□ homemade from scratch □ mix add eggs & milk □ mix add water □ frozen □ restaurant: (specify) _____	___	□ pieces	___	□ Day □ Week □ Month
Are the following added to pancakes, waffles, french toast? *Please describe:*	□ yes □ no	□ fruit □ whipped cream □ sugar □ syrup □ artificial sweetener □ butter □ margarine □ other:_____	___	□ oz/g □ cup/ml	___	□ Day □ Week □ Month
Breakfast Sandwiches (e.g McDonalds Egg McMuffin, Tim Hortons, Subway)	□ yes □ no	Most Common Type/Source: _____	___	□ piece □ oz/g □ cup/ml	___	□ Day □ Week □ Month
Rice, cooked	□ yes □ no	□ white □ brown	___	□ oz/g □ cup/ml	___	□ Day □ Week □ Month
PASTAS/ SAUCES						
Macaroni and Cheese	□ yes □ no	□ boxed (eg. Kraft Dinner, Amy's Organic) □ homemade □ ketchup added	___ ___	□ oz/g □ cup/ml □ tsp □ tbsp	___	□ Day □ Week □ Month
Cooked pastas and noodles: (e.g: spaghetti, linguini, fettuccini, macaroni, orzo)	□ yes □ no	□ white □ whole wheat	___	□ oz/g □ cup/ml	___	□ Day □ Week □ Month
Are the following added to pasta? *Please describe:*	□ yes □ no	□ oil: _____ □ butter □ margarine □ cheese: _____ □ other:_____	___	□ tsp □ tbsp	___	□ Day □ Week □ Month

Tomato-based Sauces added to pasta	□ yes □ no	□ tomato based, no meat □ tomato based w meat Type of meat: _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Other Sauces added to pasta (What type?)	□ yes □ no	□ Alfredo □ Pesto □ Other	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Canned Pasta (eg. Alphagetti, Chef Boyardee)	□ yes □ no	□ tomato based □ tomato based w meat *Most Common Type:* _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Frozen Pasta Dishes (eg. Michaelina's, Lean Cuisine)	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ cup/ml □ serving	___ □ Day □ Week □ Month
Lasagna	□ yes □ no	□ w/ meat □ w/ cheese/ vegetables	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Instant Noodles (e.g. Mr. Noodles, Ichiban)	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ cup/ml □ serving	___ □ Day □ Week □ Month
Stuffed Pastas (e.g. tortellini, ravioli)	□ yes □ no	□ meat □ cheese	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Other cereal grain products	□ yes □ no	□ couscous □ bulgar □ Quinoa □ other_____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
COMBINATION FOOD/MEALS				
Pasta, rice, potato, or noodle dishes: (no meat added) prepared from dry mixes	□ yes □ no	□ Scalloped/Mash Potato □ Lipton Side kicks □ Rice-a-Roni □ Other: _____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Meat Pies	□ yes □ no	□ beef □ chicken □ other:_____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Shepherd's Pie	□ yes □ no		___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Sausage rolls	□ yes □ no		___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Quiche What Type?	□ yes □ no	□ with meat:_____ □ vegetable □ with seafood □ other:_____	___ □ piece	___ □ Day □ Week □ Month
Potstickers/gyoza	□ yes □ no	□ pork □ beef □ shrimp □ other:_____	___ □ piece	___ □ Day □ Week □ Month

Perogies **What Type?** **Anything Added to Perogies?**	□ yes □ no	□ potato □ potato w/ cheese □ potato w/ other □ sour cream added □ bacon added □ w/ butter/margarine □ onions added □ other:_____	___ □ piece ___ □ tsp □ Tbsp	□ Day ___ □ Week □ Month
Tacos **(includes dining out)**	□ yes □ no	□ Beef □ Chicken □ Bean	___ □ piece	□ Day ___ □ Week □ Month
Burritos/Enchiladas	□ yes □ no	□ Beef (w/ bean/chs) □ Chicken □ Bean & cheese	___ □ piece	□ Day ___ □ Week □ Month
Pizza **What Type?** **Take-out/delivery** **Homemade**	□ yes □ no	Most Common Type: □ Cheese □ Vegetable □ Ham/Pineapple □ Pepperoni □ Chicken □ Other: _____	___ □ piece	□ Day ___ □ Week □ Month
Pizza rolls/Pizza Pockets	□ yes □ no	□ Cheese □ Pepperoni □ Other:_____	___ □ piece	□ Day ___ □ Week □ Month
Chili	□ yes □ no	□ Chili with meat (type?) □ Chili - no meat □ Other _____	___ □ oz/g □ cup/ml	□ Day ___ □ Week □ Month
Any other mixed dishes (e.g Cabbage Rolls, Casseroles)	□ yes □ no		___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month
MEATS, POULTRY & ALTERNATES				
Chicken – Not fried □ white □ dark thigh □ drumstick	□ yes □ no	*Most Common Type:* _____ *Cooking Method?* _____	___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month
Turkey – Not fried	□ yes □ no	*Most Common Type:* _____	___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month
Chicken nuggets, chicken fingers, or fried chicken	□ yes □ no	*Most Common Type:* _____ Dip?	___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month
Beef- roast beef or steak *Cooking Method?* _____	□ yes □ no	□ roast □ steak □ ground (lean, regular)	___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month
Hamburgers **(includes dining out)**	□ yes □ no	*Most Common Type:* _____ (eg. restaurant/ homemade/ brand)	___ □ oz/g □ cup/ml □ piece	□ Day ___ □ Week □ Month

Veggie Burgers	□ yes □ no	*Most Common Type:* _____ (eg. restaurant/ homemade/ brand)	___ □ piece	___ □ Day □ Week □ Month
Hamburger Buns	□ yes □ no	□ white □ whole wheat	___ □ piece	___ □ Day □ Week □ Month
Hamburger/Veggie Burger Condiments	□ yes □ no	□ mayo/Miracle Whip □ butter □ margarine □ ketchup □ mustard □ other	___ □ tsp □ tbsp	___ □ Da □ Week □ Month
Hot Dogs	□ yes □ no	*Most Common Type:* _____	___ □ piece	___ □ Day □ Week □ Month
Veggie Hot Dogs	□ yes □ no	*Most Common Type:* _____ (eg. brand)	___ □ piece	___ □ Day □ Week □ Month
Hot Dog Buns	□ yes □ no	□ white □ whole wheat	___ □ piece	___ □ Day □ Week □ Month
Hot Dog/Veggie Hot Dog Condiments	□ yes □ no	□ mayo/Miracle Whip □ butter □ margarine □ ketchup □ mustard □ other	___ □ tsp □ tbsp	___ □ Day □ Week □ Month
Pork- roast or chops *Cooking Method?* _____	□ yes □ no	□ roast, steak, chops □ tenderloin □ deli sliced or smoked	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Lamb - roast or chops *Cooking Method?* _____	□ yes □ no	□ roast, steak □ chops □ ground	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Deli meats/ Processed meats	□ yes □ no	□ bologna □ bratwursts □ canned meats □ salami □ ham □ turkey □ liverwurst □ other	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Sausages	□ yes □ no	□ beef □ pork □ Italian sausage	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Bacon **(includes dining out)**	□ yes □ no		___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Tofu □ med/firm □ soft *Cooking Method?* _____	□ yes □ no	□ soybean curd fi □ dessert/soft □ other:_____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Other meats (e.g. duck, deer, buffalo)	□ yes □ no	*Cooking Method?* _____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month

FISH & SEAFOOD				
Salmon – fresh or frozen *Cooking Method?* _____	□ yes □ no		___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
White Fish – fresh or frozen **What Type?** *Cooking Method?* _____	□ yes □ no	□ sole □ halibut □ cod □ snapper □ pollock □ tilapia □ other:_____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Canned Salmon	□ yes □ no	□ water pack □ oil pack	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Canned Tuna	□ yes □ no	□ water pack □ oil pack □ With mayo _____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Battered or breaded fish (e.g. fish sticks, fish fillets)	□ yes □ no	□ Tarter Sauce □ Ketchup	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Shellfish - as fresh or frozen **What Types?**	□ yes □ no	□ prawns □ crab □ mussels □ oysters □ shrimp □ lobster □ clams □ other_____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Sushi/Sashimi *please specify type*	□ yes □ no		___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
VEG & FRUITS				
Potatoes	□ yes □ no	□ boiled □ baked □ mashed w/ milk/butter □ mashed w/ milk/marg	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Potato Salad	□ yes □ no		___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
French Fries (frozen/restaurant/) If homemade, how?	□ yes □ no	Most Common: _____ Ketchup?	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Other Fried Potatoes (eg: Hashbrowns, potato pancakes, potato nuggets)	□ yes □ no	Most Common Type/Source: _____	___ □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month
Salads	□ yes □ no	□ bean □ coleslaw □ lettuce □ pasta □ caesar □ greek □ spinach □ other:_____	___ □ serving □ oz/g □ cup/ml □ piece	___ □ Day □ Week □ Month

Salad Dressings/Dips *vinaigrette; Italian; ranch; Fat-free; Low-Cal;Caesar*	□ yes □ no	**Dressing/Dip #1:** _____ **Dressing/Dip #2:** _____ **Dressing/Dip #3:** _____	___ □ tsp □ tbsp	___ □ Day □ Week □ Month
SOUPS: **Indicate if canned, from package or homemade**				
Soups *Chicken Noodles; Tomato*	□ yes □ no	Most Common Types: #1_____ #2_____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
BEVERAGES				
Orange juice and other citrus juices	□ yes □ no	□ reg □ added Calcium □ added omega-3	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Apple juice, pure	□ yes □ no	□ reg	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Other fruit juices **What Type?**	□ yes □ no	□ Carrot □ Five Alive □ Tomato □ Cranberry □ Lemonade □ V8 □ Carbonated Fruit Drinks □ other:_____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Sweetened beverages, non pop, not diet **What type?**	□ yes □ no	□ Kool-Aide □ Snapple □ Sunny D □ other:_____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Smoothies	□ yes □ no	*Most Common Source/Type:* _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Milkshakes and Blended Drinks (eg Frappaccinos, Ice Caps -includes dining out)	□ yes □ no	Most Common Type: _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Soda – Not Diet (includes dining out)	□ yes □ no	Most Common Type: _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Soda –Diet (includes dining out)	□ yes □ no	Most Common Type: _____	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month
Other Beverages	□ yes □ no	□ Coffee □ Tea □ Green Tea □ Hot Chocolate (whip cream/marshmallows, milk/ water?)	___ □ oz/g □ cup/ml	___ □ Day □ Week □ Month

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Fruits											
Apple (each)											
Orange (each)											
Banana (each)											
Grapes (1/2 Cup)											
Grapefruit (1/2 Grapefruit)											
Melon (1/2 Cup)											
Lychee (each)											
Kiwi (each)											
Pear (each)											
Peach/ Nectarine (each)											
Plum (each)											
Apricot (each)											
Pineapple (1/2 Cup)											
Dried fruit/ raisins (1/4 Cup)											
Fruit Cocktail (1/2 Cup)											
Applesauce (1/2 Cup)											
Strawberries (1/2 Cup)											
Blueberries (1/2 Cup)											
Raspberries (1/2 Cup)											
Blackberries (1/2 Cup)											

Any other fruits?

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Vegetables											
Avocado (1/4 Cup)											
Broccoli (1/2 Cup)											
Carrots (1/2 Cup)											
Celery (1 stalk)											
Lettuce (1/2 Cup)											
Radish (1/2 Cup)											
Cabbage (1/2 Cup)											
Cucumber (1/2 Cup)											
Pepper (1/2 Cup)											
Tomato (1/2 Cup)											
Green Beans (1/2 Cup)											
Yellow Beans (1/2 Cup)											
Spinach (1/2 Cup)											
Brussel Sprouts (1/2 Cup)											
Green Peas (1/2 Cup)											
Cauliflower (1/2 Cup)											
Corn (1/2 Cup)											
Squash (1/2 Cup)											
Lentils (1/2 Cup)											
Dried peas (1/2 Cup)											
Canned beans (1/2 Cup)											
Black beans (1/2 Cup)											
Baked Beans (1/2 Cup)											

Hummus? (store bought/homemade(oil?))

Any other Vegetable? (asparagus, kale, mushrooms, bok choy?)

Any butter/sauce added to vegetables?

Food	Not Eaten	Times Eaten in an Average Week							or Month		
		7	6	5	4	3	2	1	3	2	1
Snacks											
Potato Chips (1/2 Cup)											
Cheezies (1/2 Cup)											
Corn Chips (1/2 Cup)											
Tortilla Chips/ Nacho Chips (1/2 Cup)											
Pretzels (1/2 Cup)											
Peanuts (1/4 Cup)											
Almonds (1/4 Cup)											
Walnuts? (1/4 Cup)											
Other nuts/ Mixed Nuts (1/4 Cup)											
Trail Mix (1/4 Cup)											
Chocolate Bars											
Chocolate Candies (1/4 Cup)											
Jelly Beans (1/4 Cup)											
Hard Candies (1/4 Cup)											
Chewy Candies/ Gummies (1/4 Cup))											
Freezies/ Popsicles											
Regular Ice Cream (1/2 Cup)											
Low-Fat/Skim Ice Cream (1/2 Cup)											
Frozen Yogurt/ Sherbet (1/2 Cup)											
Jello (1/2 Cup)											

Any other snacks?(fruit leather, fruit snacks, rice krispie squares etc.)

A.2 24 Hour Recall Telephone Script

24 hour Recall by Telephone

During the two weeks following the participant visit, (2) 24 hour recalls will be completed, ensuring that one of the recalls (including the study visit) captures a weekend day (or holiday). Each participant will have a preferred time and phone number to call written on the flow sheet in their chart.

Phone Calls:

If you reach a voicemail or need to leave a message:

Hello, this is a message for _____.
My name is _____ and I'm calling from the UBC Nutrition Research Program at Children's Hospital. I am calling to follow up with you about the study that you and _child's name_ participated in. The phone call will take approximately 15 minutes. Please give us a call back at 604-875-2345, ext. 4896 at your earliest convenience. Again, we really appreciate your participation. Thank you, _Your Name_

If you reach a person:

"Hi there, my name is _____ and I'm calling from the UBC Nutrition Research Program at Children's Hospital. I am calling to follow up with _child's name's participation in our study. Is this a convenient time to talk for about 15 minutes?" *(If No, ask about a more convenient time. If yes, continue.)*

1. "I am calling to find out what _child's name_ had to eat yesterday. I would like to start out by asking you to tell me everything that _child's name_ ate yesterday, from the time he/she woke up, until he/she went to sleep." *(Have them list the foods without interruption).*
2. *Now ask about foods frequently forgotten. Go through foods and ask about water, other beverages, margarine, mayonnaise (or miracle whip) and other spreads etc. Eg. If they had toast for breakfast, ask what was on the toast. Remember to clarify if it was margarine or butter, as some people call it all the same.*
3. *Now ask about details about the meals. Time, location, description and quantity. (ie. Child's name had toast, what time was that? Can you tell me how many pieces he/she had? What kind of bread was it? Can you tell me the brand? How much peanut butter was on the toast? (see bottom for portion size estimation guide using hands.) Did he/she eat the entire banana? What percent of milk did he/she drink). Also be sure to ask about brands of foods, and about foods that might be fortified. (ie. If the child had orange juice, what kind was it? Was it fortified with calcium or omega 3?) What brand and kind of yogurt (fat free? 2% whole? Anything added (probiotics, DHA)? As much detail as possible.*
4. *Finally, review the foods.* "I am now going to repeat the food list with you to make sure I have everything correct. "For breakfast child's name had toast with peanut butter, a banana and a glass of milk. He/she had crackers with cheese for a snack. For lunch he/she had a turkey sandwich with carrot sticks and a glass of milk. Child's name had 2 cookies with orange juice for a snack. Dinner was rice, fish, green beans and a glass of milk and child's name didn't have anything else after dinner."
5. "Finally, did child's name take any supplements yesterday?" *(If yes, obtain the type and brand and how many they took. If No, write no supplements).*
6. "Thank you so much for your participation, again, we really appreciate it. (*If this is the first phone call, remind them that you will call one more time and ask if the time you are calling is convenient. If not, change the time in their chart to their specified time (not date).* Have a great day."

1 cup ½ cup 3 oz ½ cup Thumb tip = 1 tsp
Whole Thumb = 1 tbsp

Appendix B Supplemental Data for Chapter 4

Table B.1.1 Energy and macronutrient intake for children in the follow-up or cross-sectional group.

	Follow-Up n = 98	Cross-Sectional n = 182	*P*
Total Energy			
FFQ	1828 (1020 – 3331)	1748 (1038 – 2962)	0.495
1 x 24 h recall	1495 (798 – 2644)	1490 (855 – 2593)	0.973
3 x 24 h recalls	1481 (958 – 2308)	1497 (884 – 2400)	0.686
Carbohydrate, g/d			
FFQ	251 (134 – 406)	236 (128 – 425)	0.280
1 x 24 h recall	194 (99.4 – 355)	195 (91.8 – 384)	0.866
3 x 24 h recalls	199 (132 – 300)	195 (108 – 319)	0.238
Protein, g/d			
FFQ	69.9 (40.6 – 141)	71.1 (40.4 – 135)	0.984
1 x 24 h recall	56.4 (26.7 – 114)	55.4 (24.2 – 112)	0.626
3 x 24 h recalls	59.4 (36.5 – 102)	58.9 (32.9-114)	0.907
Total Fat, mg/d			
FFQ	65.1 (33.8 – 134)	62.4 (34.5 – 118)	0.610
1 x 24 h recall	56.4 (18.6 – 110)	55.4 (18.2 – 117)	0.762
3 x 24 h recalls	52.6 (29.3 – 106)	54.6 (27.7 – 94.3)	0.540

FFQ, food frequency questionnaire

Data are median (2.5-97.5 percentile) and compared by Mann-Whitney *U* test.

Table B.1.2 Development test scores for children in the follow-up or cross-sectional group.

	n	Follow-Up	Cross-sectional	P
PPVT	97,174	117 (73.9 – 144)	115 (73.8 – 146)	0.185
Beery	98,186	17.0 (12.5 – 21.0)	17.0 (12.7 – 21.0)	0.456
KABC				
Sequential	95,180	22.0 (12.4 – 32.0)	22.0 (12.5 – 29.0)	0.082
Learning	94,176	23.0 (13.4 – 34.0)	21.0 (12.0 – 31.0)	**0.021**
Simultaneous	98,182	35.0 (18.5 – 44.0)	35.0 (22.2 – 44.0)	0.875
MPI	92,169	81.0 (50.3 – 99.0)	78.0 (54.2 – 98.8)	**0.026**
Delayed Recall	94,170	22.0 (10.4 – 30.6)	21.0 (14.0 – 29.7)	**0.047**
TOVA				
RT Variability, ms	81,148	232 (140 – 337)	247 (161 – 345)	0.130
Response Time, ms	81,148	667 (480 – 903)	684 (451 – 986)	0.085
Errors of Commission, %	81,148	9.26 (1.26 – 26.0)	9.29 (1.24 – 31.6)	0.883
Errors of Omission, %	81,148	12.0 (0.65 – 61.3)	14.2 (1.06 – 70.5)	0.296

PPVT, Peabody Picture Vocabulary Test; Beery VMI, Beery-Buktenica Developmental Test of Visual-Motor Integration; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; ms, miliseconds; TOVA, Test of Variables of Attention; RT, Response Time
Data are median (2.5-97.5 percentile) and compared by Mann-Whitney *U* test.

Table B.1.3 Macronutrient intake for all children, assessed by FFQ and one and three 24h recalls.

	FFQ, n = 280		1 x 24 h recall, n = 272		3 x 24 h recall, n = 259		
	Median	2.5 – 97.5	Median	2.5 – 97.5	Median	2.5 – 97.5	*P*
Total Energy, kcal/d	1785	1035-3033	1495	848-2563	1489	926-2289	**<0.001**
Protein, g/d	70.6	41.3-132	58.9	25.8-113	59.4	33.7-103	**<0.001**
Protein, %energy	16.0	12.0-22.0	15.6	9.27-24.0	15.9	10.9-22.3	0.444
Carbohydrate, g/d	244	132-417	195	92.7-364	196	113-307	**<0.001**
Carbohydrate, %energy[1]	54.0	42.3-65.5	52.5	35.5-70.6	53.2	39.2-66.3	0.326
Total Fat, g/d	63.4	34.7-119	55.8	18.4-114	54.0	26.6-96.9	**<0.001**
Total Fat, %energy[1]	32.6	24.3-42.1	33.6	15.6-50.0	33.1	21.7-44.9	0.580

FFQ, food frequency questionnaire; IQR, interquartile range; 2.5 – 97.5, 2.5 – 97.5 percentile
Data was non-normally distributed and analyzed by Kruskal Wallis test.
[1]Data are normally distributed and analyzed by ANOVA. For FFQ, one and three 24h recalls, mean ± SD for carbohydrate, %energy was 53.9 ± 5.57, 53.2 ± 9.48, and 53.1 ± 6.72, and for total fat, %energy was 32.7 ± 4.47, 33.3 ± 8.52, and 33.2 ± 5.62, respectively.

Table B.1.4 Dietary intake of ω-3 and ω-6 fatty acids for Caucasian and Chinese children.

	Caucasian n = 186	Chinese n = 44	*P*
mg/d			
DHA	48.3 (4.97 – 322)	87.5 (4.62 – 323)	**0.005**
EPA	24.9 (0.90 – 252)	46.1 (0.39 – 264)	**0.013**
ARA	63.2 (19.0 – 217)	97.6 (8.38 – 413)	**<0.001**
g/d			
ALA	1.15 (0.61 – 2.96)	1.00 (0.33 – 2.01)	0.091
LA	8.72 (4.58 – 21.1)	8.65 (2.78 – 21.1)	0.105

ALA, alpha-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFQ, food frequency questionnaire; LA, linoleic acid
Data are median (2.5-97.5 percentile) and compared by Mann-Whitney *U* test.

Table B.1.5 The RBC ω-3 and ω-6 fatty acids in Caucasian and Chinese children.

	Caucasian n = 166	Chinese n = 39	*P*
Omega-3 fatty acids			
DHA	5.38 ± 1.52	6.06 ± 1.42	**0.013**
22:5ω-3	2.55 ± 0.44	2.40 ± 0.42	0.060
20:5ω-3[1]	0.82 ± 0.41	1.02 ± 0.80	**0.041**
18:3ω-3[1]	0.19 ± 0.05	0.19 ± 0.06	0.720
Omega-6 fatty acids			
22:5ω-6	0.64 ± 0.18	0.61 ± 0.16	0.378
22:4ω-6[1]	3.42 ± 0.78	3.07 ± 1.07	**0.001**
20:4ω-6[1]	16.2 ± 1.95	15.7 ± 1.48	**0.014**
18:2ω-6	11.7 ± 1.16	12.3 ± 1.06	**0.004**
DHA/ DHA + 22:5ω-6	0.89 ± 0.04	0.90 ± 0.04	**0.028**
DHA/ 22:4ω-6 + 22:5ω-6[1]	1.43 ± 0.68	1.81 ± 0.74	**<0.001**

DHA, docosahexaenoic acid

Data are mean ± SD, compared by student t-test.

[1] Data are not normally distributed, compared by Mann-Whitney *U* test. For Caucasian and Chinese children, respectively, median (IQR) RBC 20:5ω-3, 0.72 (0.60-0.94) and 0.76 (0.67-1.00); 18:3ω-3, 0.19 (0.16-0.22) and 0.18 (0.15-0.22); 22:4ω-6, 3.42 (2.96-3.88) and 3.08 (2.47-3.32); 20:4ω-6, 16.6 (15.5-17.4) and 16.0 (15.1-16.7); DHA/ 22:4ω-6 + 22:5ω-6, 1.25 (0.97-1.64) and 1.73 (1.31-2.13).

Table B.1.6 RBC DHA, 22:5ω-6 and 22:4ω-6 in children with different food categories as major dietary DHA source.

RBC fatty acids[1]	Fatty fish n = 137	Lean fish n = 30	Shellfish n = 8	Eggs n = 23	Dairy n = 10	Poultry n = 20
DHA	5.91 ± 1.55	4.84 ± 1.38	4.65 ± 1.22	4.63 ± 1.12	4.50 ± 1.08	4.37 ± 1.42
22:5ω6	0.57 ± 0.17	0.71 ± 0.17	0.70 ± 0.21	0.78 ± 0.15	0.71 ± 0.21	0.70 ± 0.12
22:4ω6	3.07 ± 0.69	3.57 ± 0.60	3.70 ± 0.74	3.64 ± 0.52	3.63 ± 0.74	3.73 ± 0.82
DHA/DHA + 22:5ω6	0.91 ± 0.04	0.86 ± 0.04	0.87 ± 0.02	0.85 ± 0.04	0.86 ± 0.05	0.85 ± 0.04
DHA/22:4ω6 + 22:5ω6	1.73 ± 0.75	1.18 ± 0.48	1.06 ± 0.22	1.08 ± 0.35	1.08 ± 0.36	1.00 ± 0.31

DHA, docosahexaenoic acid; RBC, red blood cell
Data are mean ± SD.
[1] RBC fatty acids were not analyzed for children with major source of DHA from baked goods (n = 5), fish roe (n = 1), fortified foods (n = 1), mixed dishes (n = 0) or sauces/dressings (n = 0).

Table B.1.7 Developmental test scores for Caucasian and Chinese children.

	Caucasian	Chinese	n	*P*
PPVT	121 (112-132)	108 (90.5-121)	178, 44	**<0.001**
Beery	16.0 (15.0-18.0)	17.0 (16.0-19.0)	190, 44	**0.004**
KABC				
Sequential	22.0 (18.0-24.0)	22.0 (19.0-24.0)	182, 43	0.439
Learning	21.0 (18.0-25.0)	23.0 (20.2-27.8)	179, 44	**0.022**
Simultaneous	35.0 (32.0-38.0)	37.5 (34.0-39.8)	188, 44	**0.009**
MPI	80.0 (71.0-86.0)	82.5 (75.0-88.0)	174, 42	**0.050**
Delayed Recall	21.0 (18.2-24.0)	22.0 (19.0-24.0)	176, 43	0.859
TOVA				
Response Time Variability, ms	242 (205-277)	230 (205-269)	147, 39	0.373
Response Time, ms	674 (614-745)	670 (594-756)	147, 39	0.832
Errors of Commission, %	9.32 (5.56-13.6)	8.07 (4.94-13.3)	147, 39	0.709
Errors of Omission, %	13.1 (6.08-26.2)	12.0 (5.23-24.7)	147, 39	0.631

Beery VMI, Beery-Buktenica Developmental Test of Visual-Motor Integration; IQR, inter-quartile range; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; ms, milliseconds; PPVT, Peabody Picture Vocabulary Test; TOVA, Test of Variables of Attention; RT, Response Time
Data are median (IQR), analyzed by Mann-Whitney *U* Test.

Table B.1.8 Associations between dietary DHA, EPA and ARA and neurodevelopment test scores for Caucasian children.

		DHA, mg/d		EPA, mg/d		ARA, mg/d	
	n	rho	*P*	rho	*P*	rho	*P*
PPVT	175	0.136	0.072	0.127	0.093	-0.012	0.878
Beery	186	0.061	0.410	0.063	0.396	-0.033	0.653
KABC							
Sequential	178	0.227	**0.002**	0.258	**0.001**	0.058	0.440
Learning	175	0.036	0.633	0.022	0.777	0.055	0.471
Simultaneous	184	-0.012	0.868	-0.005	0.947	-0.047	0.524
MPI	170	0.048	0.533	0.064	0.410	-0.013	0.864
Delayed Recall	173	-0.030	0.693	-0.054	0.483	0.015	0.848
TOVA							
RT Variability, ms	144	0.069	0.413	0.053	0.532	0.066	0.433
Response Time, ms	144	-0.014	0.871	-0.005	0.955	-0.057	0.498
Errors of Commission, %	144	0.010	0.901	-0.012	0.891	0.046	0.588
Errors of Omission, %	144	0.032	0.707	0.037	0.663	-0.008	0.921

ARA, arachidonic acid; Beery, Beery-Buktenica Developmental Test of Visual-Motor Integration; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; ms, milliseconds; PPVT, Peabody Picture Vocabulary Test; TOVA, Test of Variables of Attention; RT, Response Time

Data are spearman's correlation coefficients for the association between dietary fatty acids and neurodevelopmental test score for Caucasian Children.

Table B.1.9 Associations between RBC DHA, 22:5ω6 and 22:4ω6 and neurodevelopment test scores for Caucasian children.

		RBC Fatty Acids, % total fatty acids									
		DHA		22:5ω6		22:4ω6		DHA/ DHA + 22:5ω6		DHA/ 22:4ω6 + 22:5ω6	
	n	rho	*P*	rho	*P*	rho	*P*	rho	*P*	rho	*P*
PPVT	154	0.211	**0.008**	-0.162	**0.045**	-0.078	0.337	0.237	**0.003**	0.190	**0.018**
Beery	166	0.097	0.212	-0.102	0.193	0.006	0.940	0.168	**0.031**	0.103	0.187
KABC											
Sequential	159	0.192	**0.022**	-0.163	**0.041**	-0.159	**0.046**	0.237	**0.003**	0.231	**0.003**
Learning	156	0.067	0.402	0.025	0.760	0.139	0.083	0.023	0.779	-0.042	0.603
Simultaneous	164	0.083	0.293	0.019	0.810	0.045	0.567	0.052	0.508	0.044	0.575
MPI	174	0.149	0.067	0.017	0.839	0.032	0.692	0.116	0.156	0.093	0.253
Delayed Recall	152	-0.003	0.976	0.118	0.146	0.072	0.376	-0.082	0.318	-0.049	0.552
TOVA											
RT Variability, ms	130	-0.066	0.454	-0.107	0.227	0.009	0.922	0.033	0.708	-0.041	0.642
Response Time, ms	130	-0.029	0.747	-0.143	0.105	-0.027	0.761	0.080	0.365	-0.009	0.920
Errors of Commission, %	130	-0.088	0.322	0.109	0.216	0.108	0.223	-0.085	0.339	-0.085	0.339
Errors of Omission, %	130	-0.038	0.671	-0.124	0.160	0.014	0.874	0.081	0.361	0.000	0.999

Beery VMI, Beery-Buktenica Developmental Test of Visual-Motor Integration; DHA, docosahexaenoic acid; KABC, Kaufman Assessment Battery for Children, 2nd edition; MPI, Mental Performance Index; ms, milliseconds; PPVT, Peabody Picture Vocabulary Test; TOVA, Test of Variables of Attention; RT, Response Time

Data are spearman's correlation coefficients for the association between RBC fatty acids and neurodevelopmental test score for Caucasian children.

Table B.1.10 Dietary ω-3 and ω-6 fatty acids by quintiles of PPVT scores for Caucasian children.

	Quintiles of PPVT Score					
min-max	73-110	111-116	117-124	125-132	133-159	
Dietary Fatty acids	n = 34	n = 36	n = 32	n = 36	n = 37	*P*
DHA, mg/d	42.4 (19.9-96.5)	48.2 (15.1-67.0)	47.1 (25.0-79.2)	53.3 (24.7-99.9)	56.5 (27.9-121)	0.205
EPA, mg/d	22.6 (5.65-54.3)	18.8 (3.72-41.6)	18.4 (9.65-44.9)	29.4 (9.52-65.1)	35.2 (8.55-72.1)	0.295
ARA, mg/d	68.6 (51.8-108)	58.4 (38.9-80.5)	64.2 (49.1-93.0)	69.8 (48.6-95.2)	65.8 (48.2-94.8)	0.604

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PPVT, Peabody Picture Vocabulary Test
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.11 Dietary ω-3 and ω-6 fatty acids by quintiles of Beery scores for Caucasian children.

	Quintiles of Beery Score					
min-max	11-14	15	16-17	18	19-23	
Dietary Fatty acids	n = 30	n = 28	n = 70	n = 24	n = 34	*P*
DHA, mg/d	41.1 (18.7-108)	39.4 (14.4-101)	50.9 (28.2-84.3)	69.8 (25.6-113)	46.8 (23.8-90.2)	0.930
EPA, mg/d	17.8 (5.40-70.4)	16.6 (3.72-65.5)	26.2 (10.8-48.7)	35.4 (6.52-73.1)	25.8 (7.25-53.0)	0.643
ARA, mg/d	65.8 (51.4-118)	60.4 (35.4-85.9)	68.8 (48.6-94.2)	65.2 (47.3-90.8)	58.8 (42.8-108)	0.300

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Beery, Beery Buktenica Developmental Test of Visual-Motor integration
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.12 Dietary ω-3 and ω-6 fatty acids by quintiles of KABC Sequential Scale scores for Caucasian children.

	Quintiles of KABC Sequential Scale Score					
min-max	9-17	18-20	21-22	23-24	25-34	
Dietary Fatty acids	n = 39	n = 27	n = 37	n = 40	n = 39	*P*
DHA, mg/d	23.9 (13.4-48.9)	60.2 (27.8-88.3)	62.3 (24.4-106)	41.9 (22.1-93.7)	69.8 (37.9-134)	<0.001
EPA, mg/d	6.90 (2.60-29.5)	31.7 (10.2-54.3)	32.1 (9.90-68.4)	14.6 (4.70-64.4)	40.6 (18.2-82.1)	<0.001
ARA, mg/d	59.0 (43.5-91.4)	66.6 (51.1-84.6)	70.8 (49.7-104)	55.7 (41.8-88.5)	65.4 (52.6-105)	0.164

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children 2nd edition
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.13 Dietary ω-3 and ω-6 fatty acids by quintiles of KABC Learning Scale scores for Caucasian children.

	Quintiles of KABC Learning Scale Score					
min-max	11-17	18-20	21-23	24-26	27-35	
Dietary Fatty acids	n = 36	n = 39	n = 35	n = 33	n = 32	*P*
DHA, mg/d	42.7 (15.0-64.6)	62.7 (31.7-101)	76.4 (28.6-107)	26.6 (16.2-70.8)	48.4 (24.9-113)	0.208
EPA, mg/d	18.8 (5.12-38.1)	35.9 (9.60-53.9)	39.0 (10.8-69.4)	13.1 (4.45-35.2)	17.0 (5.92-72.4)	0.347
ARA, mg/d	58.4 (43.8-78.8)	67.9 (49.8-105)	77.1 (56.3-96.4)	60.9 (42.4-95.2)	70.8 (46.0-106)	0.215

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children 2nd edition
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.14 Dietary ω-3 and ω-6 fatty acids by quintiles of KABC Simultaneous Scale scores for Caucasian children.

	Quintiles of KABC Simultaneous Scale Score					
min-max	13-30	31-33	34-36	37-39	40-51	
Dietary Fatty acids	n = 35	n = 42	n = 42	n = 36	n = 29	*P*
DHA, mg/d	55.9 (13.6-104)	45.9 (28.7-90.5)	49.1 (22.1-94.0)	40.2 (18.2-82.8)	47.2 (26.4-106)	0.813
EPA, mg/d	35.2 (5.00-59.7)	22.6 (8.55-57.8)	23.4 (6.40-55.5)	17.0 (4.60-48.8)	29.2 (8.30-68.6)	0.762
ARA, mg/d	78.4 (43.5-116)	58.6 (33.3-90.8)	67.2 (53.0-89.6)	54.9 (45.1-98.3)	65.7 (44.2-94.1)	0.613

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children 2nd edition
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.15 Dietary ω-3 and ω-6 fatty acids by quintiles of KABC Mental Performance Index Scale scores for Caucasian children.

	Quintiles of KABC MPI Scale Score					
min-max	39-69	70-75	76-81	82-86	87-113	
Dietary Fatty acids	n = 36	n = 33	n = 30	n = 38	n = 33	*P*
DHA, mg/d	40.2 (13.5-76.6)	62.6 (36.4-110)	69.4 (23.9-121)	43.1 (23.0-79.4)	41.8 (22.8-111)	0.290
EPA, mg/d	18.8 (3.58-38.9)	36.8 (14.6-71.0)	37.2 (9.00-75.9)	14.3 (5.40-44.6)	21.3 (7.25-73.8)	0.136
ARA, mg/d	59.8 (43.1-96.4)	66.8 (48.8-101)	67.8 (51.9-88.0)	76.8 (54.4-115)	54.1 (42.2-84.9)	0.509

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children 2nd edition; MPI, mental performance index
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.

Table B.1.16 Dietary ω-3 and ω-6 fatty acids by quintiles of KABC Delayed Recall Scale scores for Caucasian children.

	Quintiles of KABC Delayed Recall Scale Score					
min-max	9-18	19-20	21-22	23-25	26-32	
Dietary Fatty acids	n = 43	n = 34	n = 26	n = 36	n = 34	*P*
DHA, mg/d	41.9 (17.3-73.0)	54.3 (24.6-89.2)	65.4 (42.2-124)	43.3 (22.0-113)	35.6 (20.4-79.0)	0.674
EPA, mg/d	19.3 (7.50-42.6)	25.4 (8.20-49.5)	37.6 (17.0-69.2)	20.6 (5.18-76.1)	14.2 (4.62-38.0)	0.433
ARA, mg/d	57.5 (46.4-84.2)	68.4 (52.4-112)	67.1 (48.6-100)	66.3 (47.9-112)	60.1 (47.4-81.6)	0.833

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KABC, Kaufman Assessment Battery for Children 2nd edition
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U t*est.

Table B.1.17 Dietary ω-3 and ω-6 fatty acids by quintiles of TOVA Response Time scores for Caucasian children.

	Quintiles of TOVA Response Time[1]					
min-max	6.29-596	599-655	660-700	704-760	766-1026	
Dietary Fatty acids	n = 29	n = 30	n = 26	n = 29	n = 30	*P*
DHA, mg/d	47.8 (22.7-99.2)	40.6 (16.3-110)	48.1 (15.2-116)	66.4 (35.2-86.5)	47.9 (16.5-87.6)	0.524
EPA, mg/d	28.9 (9.05-53.4)	18.2 (5.30-72.1)	23.2 (5.38-72.8)	37.3 (13.7-53.3)	20.7 (3.72-56.0)	0.519
ARA, mg/d	62.8 (45.6-105)	57.4 (43.9-100)	61.6 (44.2-106)	62.0 (46.8-88.1)	59.2 (32.8-83.4)	0.448

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TOVA, test of variables of attention
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.
[1] For TOVA scores, better performance is the achievement of a lower score.

Table B.1.18 Dietary ω-3 and ω-6 fatty acids by quintiles of TOVA Response Time Variability scores for Caucasian children.

	Quintiles of TOVA Response Time Variability[1]					
min-max	135-197	199-229	231-256	257-287	290-382	
Dietary Fatty acids	n = 29	n = 29	n = 27	n = 30	n = 29	*P*
DHA, mg/d	39.4 (14.3-69.8)	52.5 (20.8-106)	74.7 (22.6-127)	47.0 (22.1-92.9)	53.8 (24.0-84.4)	0.186
EPA, mg/d	16.0 (2.95-42.8)	28.2 (7.90-64.8)	38.5 (6.30-82.0)	20.4 (8.32-61.1)	25.2 (7.55-56.6)	0.240
ARA, mg/d	59.0 (42.3-100)	57.5 (45.1-105)	59.1 (43.9-103)	73.0 (45.0-100)	69.1 (52.8-84.4)	0.652

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TOVA, test of variables of attention
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.
[1] For TOVA scores, better performance is the achievement of a lower score.

Table B.1.19 Dietary ω-3 and ω-6 fatty acids by quintiles of TOVA Errors of Commission scores for Caucasian children.

	Quintiles of TOVA Errors of Commission[1]					
min-max	0-4.32	4.94-7.41	7.50-11.1	11.2-14.6	14.8-32.5	
Dietary Fatty acids	n = 24	n = 29	n = 35	n = 27	n = 29	*P*
DHA, mg/d	50.9 (21.6-97.6)	37.0 (15.5-77.6)	60.3 (25.9-127)	53.3 (16.2-106)	47.5 (15.8-91.5)	0.694
EPA, mg/d	32.6 (7.12-63.9)	18.1 (4.10-44.6)	36.8 (13.1-88.4)	35.2 (7.50-69.2)	23.2 (4.25-46.9)	0.376
ARA, mg/d	61.8 (43.0-83.7)	58.5 (43.6-89.2)	60.1 (42.8-107)	60.3 (44.0-90.3)	69.1 (52.3-96.8)	0.707

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TOVA, test of variables of attention
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.
[1] For TOVA scores, better performance is the achievement of a lower score.

Table B.1.20 Dietary ω-3 and ω-6 fatty acids by quintiles of TOVA Errors of Omission scores for Caucasian children.

	Quintiles of TOVA Errors of Omission[1]					
min-max	0-4.35	4.38-9.88	10.0-17.8	17.9-31.4	31.5-71.8	
Dietary Fatty acids	n = 27	n = 31	n = 28	n = 29	n = 29	*P*
DHA, mg/d	41.8 (16.3-115)	47.2 (17.3-86.7)	41.5 (13.5-111)	49.3 (31.4-86.5)	55.9 (21.9-107)	0.718
EPA, mg/d	27.2 (4.80-92.3)	20.9 (6.10-53.9)	20.1 (3.88-70.5)	29.5 (17.2-51.2)	28.9 (7.50-62.8)	0.731
ARA, mg/d	55.7 (42.8-101)	62.8 (48.9-101)	62.0 (44.7-91.6)	67.9 (46.1-90.8)	60.1 (43.2-79.8)	0.762

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TOVA, test of variables of attention
Data are median (IQR) and quintile 1 and 5 were compared by Mann-Whitney *U* test.
[1] For TOVA scores, better performance is the achievement of a lower score.