Evaluation of Conventional and Novel Dietary Strategies to Promote Intake of Omega-3 Highly Unsaturated Fatty Acids

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Kinesiology

Waterloo, Ontario, Canada, 2012

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Intakes of the highly unsaturated fatty acids (HUFA, ≥20 Carbons, ≥3 double bonds) eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3, DHA) greater than 0.25 g/d are currently recommended for health benefits. Targets for omega-3 blood biomarkers have also been proposed based on associations with protection against coronary heart disease mortality. The relationship between diet intakes and blood biomarkers is not well defined, particularly differences between men and women. North American intakes and blood biomarkers of EPA and DHA are typically below recommendations and targets. To address this disparity, adherence to dietary advice strategies to increase EPA + DHA intake was investigated over one year. Adherence was sustained up to 12 weeks and long-term adherence was well characterized by the % of DHA in erythrocytes. For women, n-3 HUFA blood biomarkers increased following nutraceutical or combined strategy dietary advice but not seafood or functional food advice. To assist in the assessment of EPA + DHA intakes, food sources of EPA and DHA in Canada were incorporated into a semi-quantitative, nutrient-specific food frequency questionnaire (FFQ) and validated. The FFQ is an adequate tool for estimating habitual EPA and DHA intake and ranking Canadian adults by their intakes. The blood biomarker response to recommended intakes of 0.25, 0.5 and 1 g/d EPA + DHA was also characterized in adult men and women. Blood n-3 HUFA biomarkers increased in a dose-dependent manner and aligned with blood targets associated with primary cardiac arrest risk reduction. Sex differences in the DHA:EPA ratio in blood observed with low intakes at baseline disappeared following 0.25 g/d EPA + DHA. These findings are applicable towards informing achievable dietary guidelines for EPA + DHA intake and improving measurement of EPA + DHA intake in relation to blood n-3 HUFA biomarkers.

ACKNOWLEDGEMENTS

Sincerest thanks to my advisor, Dr. Ken Stark, for continually seeking opportunities in line with my interests and equipping me with practical skills for my future career. I am grateful to my committee members, Drs. Marina Mourtzakis, Rhona Hanning and Robin Duncan, for their guidance throughout this process and external examiner, Dr. Nimal Ratnayake, for his expertise. I extend my appreciation to Jing Ouyang and Marg Burnett for their always cheerful technical assistance and to the study participants for their dedicated effort and time. I also thank Ken for fostering a positive working environment within our group. It has truly been a pleasure to work with the many bright and amiable members of the Laboratory of Nutritional and Nutraceutical Research. I extend gratitude to the Canadian Institutes of Health and Research for supporting my studies.

My parents, Joanne and David Patterson, have reminded me to enjoy the journey, taught me that any goal is surmountable through determination and hard work, and provided me with a foundation of support. Lastly, I am tremendously lucky to have had (Dr.) Dan Courtney with whom to share in the ups and downs of our parallel scholarly paths. I am truly fortunate to have my family and Dan steadfastly behind me.

TABLE OF CONTENTS

AUTHOR'S DECLARATION ii

ABSTRACT iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS v

LIST OF FIGURES viii

LIST OF TABLES ix

LIST OF ABBREVIATIONS xi

CHAPTER 1. GENERAL INTRODUCTION 1

CHAPTER 2. SCIENTIFIC BACKGROUND

Fatty Acids and Simple Lipids 6
Fat Digestion and Absorption 8
Endogenous n-3 HUFA Synthesis 9
n-3 HUFA Biomarkers 10
Intakes of n-3 HUFA and Current Recommendations 15
Sources of n-3 HUFA in the Food Supply 18
Measuring Dietary n-3 HUFA Intake 21

CHAPTER 3. RATIONALE AND OBJECTIVES

Rationale 27
Objectives 28
Hypotheses 29

CHAPTER 4. GENERAL METHODS AND MATERIALS

Participants 30
Sample and Data Collection 30
Fatty Acid Composition Determinations 31
Statistical and Data Analyses 33

CHAPTER 5. DIETARY AND BIOMARKER VALIDATION OF A FOOD FREQUENCY QUESTIONNAIRE TO MEASURE EPA AND DHA INTAKES FROM CONVENTIONAL AND NOVEL CANADIAN DIETARY SOURCES

Introduction 34
Methods and Materials 35
Results 38
Discussion 40
Conclusion 46

CHAPTER 6. ONE YEAR ADHERENCE TO CONVENTIONAL AND NOVEL DIETARY ADVICE STRATEGIES TO INCREASE INTAKES OF EPA AND DHA

Introduction 53
Materials and Methods 53
Results 58
Discussion 62
Conclusion 68

CHAPTER 7. BLOOD BIOMARKER RESPONSES TO LOW DOSES OF EPA AND DHA IN MEN AND WOMEN

Introduction 75
Materials and Methods 75
Results 79
Discussion 83
Conclusion 89

CHAPTER 8. SUMMARY AND GENERAL DISCUSSION 98

REFERENCES 103

APPENDICES

APPENDIX A (CHAPTER 5)

Food Frequency Questionnaire 116

APPENDIX B (CHAPTER 6)

Take-Home Information for Dietary Advice Groups 123
Recruitment Materials 154
Information & Consent Form 156
Dietary Record 163
Health Screening Form 167

APPENDIX C (CHAPTER 7)

Appendix Table C.1 169
Appendix Figure C.1 170

Recruitment Materials 171
Information & Consent Form 173
Dietary Record 180
Health Screening Form 185
Food Frequency Questionnaire 188
Appendix Tables C.2 - C.7 195
Appendix Figure C.2 207

LIST OF FIGURES

Figure 5.1	Agreement between food frequency questionnaire (FFQ) and 3-d dietary record estimates of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake assessed by the Bland-Altman method	52
Figure 6.1	Whole blood % n-3 HUFA in total HUFA by men (A) and women (B) completers in each advice group over time	70
Figure 6.2	Percent DHA (A) and EPA (B) over time in erythrocytes, plasma and whole blood by completers	71
Figure 6.3	Intake, by completers, of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from 3-d dietary records in Seafood, Functional Food, Nutraceutical and Combined advice groups	72
Figure 7.1	EPA, DHA and sum of n-3 HUFA in finger-tip prick whole blood at week 0 and following EPA + DHA intakes of 0.25g/d (weeks 1-4) and 0.5g/d (weeks 5-8)	91
Figure 7.2	Response of the % n-3 HUFA in total HUFA in whole blood (A) and the % EPA + DHA in erythrocytes (B) to intake of EPA + DHA	92
Figure 7.3	Relationship between the % n-3 HUFA in total HUFA in whole blood and the % EPA + DHA in erythrocytes	93
Figure 7.4	The % n-3 HUFA in total HUFA (**A**) and % EPA+ DHA (**B**) throughout fish oil supplementation (weeks 1-4: 0.25 g/d EPA + DHA, weeks 5-8: 0.5 g/d EPA + DHA)	95
Figure 7.5	DHA:EPA ratio for men and women in (A) finger-tip prick blood, (B) whole blood, (C) erythrocytes and (D) plasma phospholipids with supplementation	96
Appendix Figure C.1	Gas chromatogram of fish oil capsule fatty acid composition	170
Appendix Figure C.2	Gas chromatogram of whole blood fatty acid composition	207

LIST OF TABLES

Table 2.1	Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake recommendations for adults	26
Table 5.1	Characteristics of food frequency questionnaire (FFQ) study participants	47
Table 5.2	Food frequency questionnaire (FFQ) and dietary record intakes and blood biomarker fatty acid composition of FFQ validation study participants	48
Table 5.3	Agreement of quartile assignment by the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intakes by food frequency questionnaire (FFQ) and dietary record and n-3 HUFA blood biomarkers	49
Table 5.4	Pearson coefficients of correlation between food frequency questionnaire (FFQ), dietary record and blood biomarker fatty acid measurements	50
Table 5.5	Method of triads validity coefficients (VC) and VC ranges of food frequency questionnaire (FFQ), dietary record and blood biomarker fatty acid measurements with the true value	51
Table 6.1	Characteristics of dietary advice study participants	69
Table 6.2	EPA and DHA estimates by 3-d dietary records and 3-d food duplicate collections by completers	73
Table 6.3	Results of linear regression determining the association of sex, body mass index and n-3 HUFA intake on blood biomarkers for completers	74
Table 7.1	Dietary intake of EPA and DHA at baseline and week 8 by FFQ	90
Table 7.2	n-3 HUFA biomarkers in erythrocytes, plasma phospholipid and whole blood fractions with supplementation	94
Table 7.3	Results of linear regression models determining the association of sex, body mass or body mass index and n-3 HUFA intake on n-3 HUFA blood biomarkers	97
Appendix Table C.1	Fatty acid composition of fish oil capsules for dose-response study	169
Appendix Table C.2	Whole blood fatty acid composition in the dose-response study (n=20)	195
Appendix Table C.3	Erythrocyte fatty acid composition in the dose-response study (n=20)	197

Appendix Table C.4 Plasma phospholipid fatty acid composition in the dose-response study (n=20) 199

Appendix Table C.5 Subgroup whole blood fatty acid composition in the dose-response study (n=5) 201

Appendix Table C.6 Subgroup erythrocyte fatty acid composition in the dose-response study (n=5) 203

Appendix Table C.7 Subgroup plasma phospholipid fatty acid composition in the dose-response study (n=5) 205

LIST OF ABBREVIATIONS

HUFA	Highly Unsaturated Fatty Acids (≥20 Carbons, ≥3 double bonds)
EPA	Eicosapentaenoic Acid (20:5n-3)
DHA	Docosahexaenoic Acid (22:6n-3)
FFQ	Food Frequency Questionnaire

CHAPTER 1

GENERAL INTRODUCTION

The n-3 highly unsaturated fatty acids (HUFA; ≥20 carbons, ≥3 double bonds) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), prevalent in marine-based dietary sources are associated with cardio-protection. The effects of EPA and DHA on secondary cardiovascular disease prevention were first examined in the Diet and Reinfarction Trial where advice to consume EPA and DHA from two servings of oily fish per week resulted in a 29% reduction in all-cause mortality in patients with a history of myocardial infarction (Burr et al., 1989). The beneficial effects were further confirmed in a larger secondary prevention trial where fish oil supplementation at 0.85 g/d EPA + DHA was found to reduce the risk of sudden cardiac death by 45% in recent myocardial infarction patients (GISSI-Prevenzione Investigators, 1999).

Reduction in cardiovascular endpoints with EPA and DHA has not been replicated in recent trials. The Alpha-Omega (Kromhout, Giltay, and Geleijnse, 2010), Omega (Rauch et al., 2010) and SU.FOL.OM3 (Galan et al., 2010) trials did not demonstrate reduction in secondary coronary heart disease mortality following EPA + DHA doses between 0.38 and 0.85 g/d. Extensive use of antithrombotic, antihypertensive and statin drugs and low statistical power may contribute the lack of effect observed in these recent trials (Hu and Manson, 2012; Kromhout et al., 2012; Mozaffarian and Wu, 2011). Background fish intake increased during the Omega trial; approximately 45% of control and intervention participants reported consuming fish several times per week (Rauch et al., 2010). There is limited ability to discern the protective effects of EPA and DHA when the control group is already consuming substantial doses of n-3 HUFA

(Yokoyama et al., 2007). Adherence to study protocols in long-term, large-scale trials, such as maintaining background diets is challenging for participants to sustain and researchers to assess. Intention to treat analyses were performed in the Alpha-Omega, Omega and SU.FOL.OM3 trials, however relating clinical outcomes to blood levels of n-3 HUFA could have better represented the actual relationship between EPA and DHA status and cardiovascular risk reduction.

In spite of recent trials, general consensus is that EPA and DHA can reduce the risk of coronary heart disease mortality in populations with and without established coronary heart disease (He et al., 2004; Leon et al., 2008; Marik and Varon, 2009; Mozaffarian and Rimm, 2006; Musa-Veloso et al., 2011; Wang et al., 2006). Current intake recommendations for adults range from 0.25 to 4g/d with guidelines <1g/d aimed at healthy individuals and those ≥1g/d advised for secondary cardiovascular disease prevention and triacylglycerol lowering (Food and Agriculture Organization of the United Nations and World Health Organization, 2008; International Society for the Study of Fatty Acids and Lipids, 2004; Kris-Etherton, Harris, and Appel, 2003; Kris-Etherton et al., 2007; United States Department of Agriculture, 2010). A distinct dietary reference intake recommendation for EPA and DHA does not exist. Instead, a proportion of the dietary reference intake values for alpha-linolenic acid, which are based on median population-level intake, can be consumed as EPA and DHA (Institute of Medicine of the National Academies, 2005).

Re-evaluation of dietary reference intakes for EPA and DHA intake based on chronic disease endpoints has been proposed (Harris et al., 2009; Kris-Etherton, Grieger, and Etherton, 2009). While risk of primary cardiac arrest in relation to quartiles of blood n-3 HUFA status has been evaluated (Siscovick et al., 1995), the relationship between dietary intake of EPA and DHA and cardio-protection is often indirectly calculated (Mozaffarian and Rimm, 2006). Some

studies included in such computations collected insufficient intake data to accurately determine EPA and DHA consumption (Musa-Veloso et al., 2011; Osler, Andreasen, and Hoidrup, 2003). Establishing a relationship between dietary intake and blood levels of EPA and DHA which could in turn be related to cardiovascular disease risk, would facilitate establishing a dietary reference intake value based on cardiovascular disease as an endpoint. Dietary reference intake derivation for EPA and DHA has important financial and food policy implications related to dietary guidelines, food labeling, fortification and feeding programs. Furthermore, blood dose-response data for EPA and DHA are needed to better evaluate observational and clinical trials used to inform regulatory decisions, including product labelling and nutritional claims (Brownawell et al., 2009).

In contrast with current intake recommendations, median North American population-level intakes of EPA and DHA of 0.11g/d by women and 0.16g/d by men fall below existing guidelines (Institute of Medicine of the National Academies, 2005). As seafood provides appreciable amounts of EPA and DHA, it has traditionally been recommended as a whole food dietary source. Several health organizations, such as Dietitians of Canada, provide specific dietary advice suggesting the number of servings of fish to be consumed per week to meet EPA and DHA recommendations for healthy individuals (Kris-Etherton et al., 2007; United States Department of Agriculture, 2010) but acknowledge that achieving recommendations may not be sustainable through whole food sources alone (Kris-Etherton, Harris, and Appel, 2003). Novel functional food and nutraceutical sources provide additional options for consumers. Functional foods are food-based products enriched with EPA and DHA, such as omega-3 eggs, and nutraceuticals consist of isolated EPA and DHA sold in medicinal form, such as fish oil capsules. If recommendations to consume EPA and DHA are to be issued, the practical feasibility of

meeting EPA and DHA intake recommendations from the diverse dietary sources requires consideration.

The ability to accurately measure EPA and DHA intake is imperative for research and dietary counselling purposes; these measurements are needed to develop and evaluate the impact of dietary guidelines, assess diet-disease relationships and screen individuals for appropriate targeting of dietary advice. Specific challenges are associated with measurement of usual EPA and DHA intakes as these fatty acids are concentrated in sources not typically consumed daily in North America. Furthermore, the prevalence of EPA and DHA in novel functional food and nutraceutical products presents new considerations for traditional dietary assessment strategies. It has been observed that dietary intake of EPA and DHA estimated by 3-d dietary record does not always agree with biomarker-based assessment of EPA and DHA status (Fratesi et al., 2009). A FFQ may provide an alternative strategy to measure intake of EPA and DHA as this type of survey is aimed at measuring habitual dietary intake (Gibson, 2005).

Biomarkers are a potential objective tool to evaluate EPA and DHA intakes. Blood levels of EPA and DHA are observed to respond to dietary intake and the response is characterized by a logarithmic relationship (Bjerve et al., 1993; Kobayashi et al., 2001; Vidgren et al., 1997). Measurement of blood fatty acid composition to assess omega-3 status has become increasingly feasible by recent methodological advances, but also by the breadth of information a complete fatty acid profile provides. Specifically, the combination of minimally invasive finger-tip prick blood samples (Armstrong, Metherel, and Stark, 2008; Marangoni, Colombo, and Galli, 2004) with rapid chemical preparation through direct transesterification (Armstrong, Metherel, and Stark, 2008) followed by fast gas chromatography result in cost efficient high throughput analyses (Stark and Salem, Jr., 2005).

The present thesis examines the relationship between the dietary intake of EPA and DHA and resulting blood levels. Specifically, adherence to dietary advice strategies based on seafood intake, nutraceuticals and/or functional foods were examined with dietary assessment tools and omega-3 blood biomarkers. The omega-3 blood biomarker responses to tightly controlled dietary intakes of 0.25, 0.5 and 1 g/d of EPA plus DHA were also examined. A novel FFQ for the determination of EPA and DHA intakes was also developed and validated to assist in dietary intake estimates.

CHAPTER 2

SCIENTIFIC BACKGROUND

Fatty Acids and Simple Lipids

Fatty acids play many important biological roles as energy-yielding substrates, structural membrane components, elements in cell signalling pathways and building blocks for lipid and lipid-derived compounds (Calder and Burdge, 2004). Structurally, fatty acids consist of a hydrophobic carbon chain with a methyl group at one end and a hydrophilic carboxyl group at the opposite end. Variation on this general structure leads to distinct fatty acids classes. Fatty acids with single bonds between each of the carbon atoms of the chain belong to the saturated family while those with one or multiple double bonds within the carbon chain belong to the monounsaturated and polyunsaturated families, respectively. To illustrate, the polyunsaturated fatty acid, docosahexaenoic acid (DHA; 22:6n-3) consists of a 22-carbon fatty acid chain with six methylene interrupted, *cis*-double bonds. The first double bond is located at the third carbon from the methyl or "omega" carbon and accordingly, DHA is referred to as an omega-3 (n-3) polyunsaturated fatty acid. Polyunsaturated fatty acids such as DHA with ≥20 carbons and ≥3 double-bonds can be further sub-classified as highly unsaturated fatty acids (HUFA). HUFA, such as DHA and eicosapentaenoic acid (EPA; 20:5n-3), have distinctive structural and signalling functions in pathways related to health and disease (Lands, 2005).

Fatty acids may be found in free, unesterified form or bound in lipid complexes. Various classes of lipid complexes exist including triacylglycerols, glycerophospholipids (phospholipids) and cholesteryl esters. The fatty acid composition of these lipids is influenced by dietary intake and the specificity of acyltransferases and transacylases involved in the initial synthesis or

subsequent remodelling in various tissues (Yamashita, Sugiura, and Waku, 1997; Yeo and Holub, 1990). Fatty acid composition varies between classes of lipids and accordingly has a relationship to function (Christie, 2003). The triacylglycerol class consists of three fatty acids bound to a glycerol molecule. These simple lipids are the primary storage form of fatty acids in adipose tissue and the predominant form of dietary fat (Frayn, 2010). Triacylglycerols of terrestrial animal origin are mainly composed of C_{16} and C_{18} fatty acids while those of marine origin also contain amounts of C_{20} and C_{22} fatty acids (Christie, 2003). Consequently, the predominant dietary fatty acids in human triacylglycerols are 16:0, 18:0, 18:1n-9 and 18:2n-6.

Phospholipids are structurally distinct from triacylglycerols with a phosphate and polar head group, such as choline, serine, ethanolamine or inositol, in place of one of the fatty acids on the glycerol backbone (Ratnayake and Galli, 2009). Functionally, these amphipathic phospholipids form the lipid bilayer of cell membranes, such as erythrocytes and cardiac myocytes. The various phospholipids have characteristic fatty acid compositions but typically consist of a saturated fatty acid at the first position (*sn*-1), an unsaturated fatty acid at the second position (*sn*-2) and the polar head group at the third position (*sn*-3). HUFA are commonly selectively incorporated into the *sn*-2 position of phospholipids by acyl transferases and are therefore typically low in triacylglycerols. The localization of HUFA in the *sn*-2 position of phospholipids influences membrane fluidity, but also makes HUFA readily available for cellular functions through specific phospholipases, such as serving as substrates for the production of lipid mediators such as eicosanoids and docosanoids. Conversely, the predominance of saturated fatty acids, with no double bonds and straight carbon chains in triacylglycerol structures allows for tighter packing in adipose tissue storage depots.

Fatty acids can also be esterified to cholesterol in the liver for transport in the plasma or storage (Christie, 2003). In the plasma, lecithin cholesterol acyltransferase catalyzes fatty acid esterification of cholesterol from the *sn*-2 position of the phospholipid phosphatidylcholine. The substrate preference of this acyltransferase and the fatty acid composition of phosphatidylcholine can also dictate the fatty acid composition of cholesteryl esters, with linoleic acid (18:2n-6) being preferentially incorporated (Holub, Bakker, and Skeaff, 1987).

Fat Digestion and Absorption

Following emulsification through the churning action of the stomach, dietary lipids are predominantly digested in the small intestine through the action of lipases. These enzymatic cleavage products then diffuse into the intestinal enterocytes where they are re-assembled and packaged into chylomicrons. The more non-polar triacylglycerols and cholesterol esters form the chylomicron core and are surrounded by apo-proteins and the more polar phospholipids and cholesterol molecules (Ratnayake and Galli, 2009). Chylomicrons are then transported through the lymphatic system, bypassing the liver, and enter the bloodstream at the thoracic duct. The chylomicrons travelling in the plasma are then directed to various tissues, such as skeletal muscle or adipose tissue. In the capillaries of these tissues, lipoprotein lipase cleaves fatty acids from the glycerol backbone of triacylglycerols and these components enter the cell for immediate use, for instance as energy-yielding substrates (Ratnayake and Galli, 2009). The chylomicron particle shrinks as triacylglycerols are removed and some of the phospholipids, cholesterol and proteins comprising the outer coat dissociate and are scavenged by other particles such as high density lipoprotein (Frayn, 2010). The chylomicron remnants and any free fatty acids not taken up by the tissues are bound to albumin and transported to the liver. The liver then repackages the

remnant components along with endogenous lipids into lipoprotein particles which are released back into circulation to supply tissues (Gropper, Smith, and Groff, 2009).

In the post-prandial period, plasma fatty acid composition is therefore largely influenced by the circulating chylomicrons. Triacylglycerols account for approximately 85% of the total lipids by weight in chylomicrons and chylomicron fatty acid composition reflects the diet (Christie, 2003). Upon clearance of chylomicrons from the plasma, the fatty acid composition of plasma increasingly reflects the supply of fatty acids from endogenous synthesis or adipose tissue release. Indeed, in the immediate post-prandial period, measurements of plasma total, triacylglycerol and cholesteryl ester fatty acids reflect recent dietary intake while measurements of phospholipid-rich blood fractions, including erythrocytes and plasma phospholipids, remain unaffected by a recent meal (Burdge, Powell, and Calder, 2006; Polozova and Salem, Jr., 2007; Sadou et al., 1995). The n-3 HUFA are predominantly incorporated into phospholipids and in the time immediately following a meal containing n-3 HUFA, corresponding blood measurements of these fatty acids are not altered (Metherel et al., 2012).

Endogenous n-3 HUFA Synthesis

In addition to structural classification, fatty acids can also be categorized by origin. Essential fatty acids cannot be synthesized endogenously by humans to meet physiological need and therefore must be obtained through the diet. Conversely, non-essential fatty acids can be synthesized from an essential fatty acid precursor. Within the family of n-3 polyunsaturated fatty acids, α-linolenic acid (18:3n-3) serves as the parent essential fatty acid for synthesis of n-3 HUFA. Specifically, the n-3 HUFA are synthesized from α-linolenic acid in the liver in limited amounts through a pathway which involves repeated steps of fatty acid chain desaturation and

elongation with a final step of peroxisomal β-oxidation (Sprecher et al., 1995; Su et al., 2001). The n-3 HUFA can be considered conditionally essential fatty acids as there are certain conditions, such as an absence of dietary precursor, which would make dietary inclusion of n-3 HUFA essential (Cunnane, 2003). The n-3 HUFA may also be essential metabolites in pathways related to health and disease. Endogenous synthesis is sufficient to prevent overt n-3 HUFA deficiency in the absence of dietary intake. However, overall conversion of α-linolenic acid to n-3 HUFA is low with conversion to EPA and n-3 docosapentaenoic acid (22:5n-3) exceeding conversion to DHA. Conversion to DHA is particularly limited as delta 6 desaturase is required for the insertion of the 6^{th} carbon-carbon double bond, but linoleic acid and α-linolenic acid are also competing delta 6 desaturase substrates (Kitson, Stroud, and Stark, 2010). A kinetic stable isotope study determined that 0.2%, 0.13% and 0.05% of plasma α-linolenic acid is converted to EPA, n-3 docosapentaenoic acid and DHA respectively in individuals consuming a beef-based diet (Pawlosky et al., 2003b). At very low intakes of EPA and DHA (0.06 g/d), the rate of conversion of n-3 docosapentaenoic acid to DHA is higher in women than men however, when the diet includes moderate intakes of EPA and DHA (0.5 g/d), these sex differences in endogenous synthesis are not observed (Pawlosky et al., 2003a; Pawlosky et al., 2003b).

n-3 HUFA Biomarkers

Levels of n-3 HUFA in the body reflect both endogenous synthesis and dietary intake; synthesis makes an important contribution at very low n-3 HUFA intakes while diet becomes a greater determinant as n-3 HUFA intakes increase. Measurement of n-3 HUFA status can be determined by collecting a tissue or blood sample and determining the fatty acid composition. Blood is often selected as a marker of fatty acid status as it is minimally invasive to collect and

can reflect tissue status (Stark, 2008b). The blood dose-response curves for EPA and DHA in response to dietary intake demonstrate a logarithmic relationship; blood levels rise with intake before reaching a plateau as saturation occurs (Vidgren et al., 1997). This curve does not begin at a zero value due to background endogenous HUFA synthesis. Various blood fractions, including whole blood, erythrocytes, plasma and serum, have all been employed as markers of fatty acid status. These pools can be further subdivided into the various lipid classes including phospholipids, triacylglycerols, cholesteryl esters and non-esterified fatty acids. Additional analytical steps can be performed to further examine these specific lipid classes, for example specific phospholipids including phosphatidyl choline, ethanolamine, serine and inositol can be isolated. Selection of a blood fraction for fatty acid analysis is influenced by the unique information it provides and analytical and other practical considerations.

The incorporation of fatty acids into erythrocytes reflects the cell lifespan, remodelling during the cell lifespan, and partitioning of particular fatty acids into specific classes of phospholipids within the inner and outer cell membrane. Erythrocytes have generally been regarded as the preferred blood marker of n-3 HUFA intake over the preceding months, reflecting the cell lifespan of roughly 120 days (Ebaugh, Jr., Emerson, and Ross, 1953). However, erythrocyte fatty acid composition will also respond to dietary change within days as the outer bilayer of erythrocyte membranes is in direct contact with the plasma for fatty acid exchange (Skeaff, Hodson, and McKenzie, 2006). On a gram-per gram basis of dietary intake, EPA and DHA incorporate by the same magnitude into erythrocytes however; the rate of DHA incorporation and washout is slower (Katan et al., 1997). Various transport mechanisms result in an asymmetric distribution of phospholipid classes across the erythrocyte membrane; phosphatidyl choline largely remains in the outer membrane and phosphatidyl ethanolamine and

serine are predominantly moved to the inner membrane (Connor et al., 1992; Seigneuret and Devaux, 1984). DHA preferentially accumulates in phosphatidyl ethanolamine over phosphatidyl choline (Lemaitre-Delaunay et al., 1999). As a result, DHA accumulates in the inner erythrocyte membrane which accounts for its slower response to dietary change relative to EPA. In general, the erythrocyte phosphatidyl choline pool equilibrates to dietary change within weeks (Skeaff, Hodson, and McKenzie, 2006) while complete equilibration of the fatty acid composition of phospholipids on the interior erythrocyte membrane occurs within months, but specific times may depend on the dietary dose of n-3 HUFA (Von Schacky C., Fischer, and Weber, 1985).

Plasma and serum fatty acid composition is largely influenced by lipoproteins with fatty acids incorporated into triacylglycerols (49%), phospholipids, specifically phosphatidyl choline (24%), and cholesteryl esters (16%) (Christie, 1985). As HUFA are most preferentially esterified into the *sn*-2 position of phospholipids, this pool is often selected to examine EPA and DHA. Overall, the fatty acid composition of plasma responds to dietary change within days and reaches an equilibrium state within weeks (Metherel et al., 2009; Skeaff, Hodson, and McKenzie, 2006).

Whole blood fatty acid composition has been examined to a greater extent in recent years. Incorporating both plasma and erythrocytes, whole blood has been observed to respond to changes in dietary intake in a manner intermediate between these two pools (Metherel et al., 2009). Whole blood measurements of EPA and DHA have also been inversely associated with risk of sudden cardiac death (Albert et al., 2002). A whole blood sample can easily be collected through finger-tip prick sampling. This technique provides a measure of whole blood n-3 HUFA composition equivalent to samples collected by conventional venous puncture (Armstrong,

Metherel, and Stark, 2008) and reflects dietary fatty acid intake (Metherel et al., 2009). Finger-tip prick sampling does not require collection by a trained phlebotomist and eliminates the need for fatty acid extraction prior to preparation of fatty acids for gas chromatography analysis (Marangoni, Colombo, and Galli, 2004). Consequently, this technique reduces the time and resources required for blood fatty acid determinations, and has the potential to enable screening of fatty acid profiles.

In addition to determining the individual fatty acid composition of various blood fractions, blood biomarkers incorporating multiple fatty acids can also be expressed. For n-3 HUFA, such composite measurements have been related to tissue status and disease risk. The relative percentage of EPA + DHA in erythrocytes has been proposed as a risk factor for coronary heart disease mortality (Harris and Von Schacky, 2004) due to its' strong correlation with the EPA and DHA composition of cardiac tissue (Harris et al., 2004). It has been suggested that ≥8% EPA + DHA in erythrocytes is associated with the greatest level of protection against coronary heart disease mortality while <4% is associated with the greatest risk (Harris and Von Schacky, 2004). This range is an estimate based on measured and predicted erythrocyte % EPA + DHA values associated with coronary heart disease mortality risk in several studies examining individuals with and without existing cardiovascular disease. Only one case-control study has directly evaluated erythrocyte % EPA + DHA and cardiac outcomes (Siscovick et al., 1995). From this direct assessment, a 90% lower risk of primary cardiac arrest risk was observed at 6.5% erythrocyte % EPA + DHA compared with 3.3%. Further work is needed to clearly establish blood levels of EPA and DHA associated with cardio-protection, but blood biomarkers of n-3 HUFA status have the potential to serve as modifiable risk factors.

The percentage of n-3 HUFA in total HUFA has also been examined as a marker of n-3 HUFA status as it represents the competition between HUFA for incorporation into lipid structures, particularly esterification at the *sn*-2 position of phospholipids where HUFA are concentrated (Lands, 2008; Metherel et al., 2009; Stark, 2008b). The % n-3 HUFA in total HUFA is consistent between various tissues and blood fractions when dietary intake and other metabolic factors are constant (Stark, 2008b). As a result, the % n-3 HUFA in total HUFA can be determined for any available blood fraction, including whole blood, and does not require additional isolation of erythrocytes. While a % n-3 HUFA in total HUFA of 20% is typical of North American populations, % n-3 HUFA in total HUFA of 40% is roughly equivalent to 8% EPA + DHA in erythrocytes (Armstrong, Metherel, and Stark, 2008; Lands, 2008). Additional data is required to confirm the relationship between the % n-3 HUFA in total HUFA and erythrocyte % EPA + DHA biomarkers. Further clinical validation is also necessary to verify the relationship of the % n-3 HUFA in total HUFA biomarker with disease risk and be considered as one of the many risk factors contributing to coronary heart disease risk. The purpose of the % n-3 HUFA in total HUFA biomarker is to model HUFA competition for phospholipid incorporation, not to stress a reduction in the dietary n-6:n-3 ratio. Both the n-3 and n-6 series HUFA are implicated in pathways associated with reducing disease risk, such as resolution of inflammation (Serhan, 2010).

Biological and analytical variability are both important considerations when examining blood fatty acid status. The analytical coefficient of variation has been observed to be <4% for the EPA and DHA content in total lipids of various blood fractions analyzed in quadruplicate from the blood of a single individual collected on a single occasion (Armstrong, Metherel, and Stark, 2008). Isolation of phospholipid classes introduces some additional analytical variability

with coefficients of variation <10%. The coefficient of variation from finger-tip prick samples collected from the same individual on one occasion is 1.5 - 6.4% for whole blood % EPA + DHA and 0.46 - 1.14 % for whole blood % n-3 HUFA in total HUFA (unpublished observations).

Intakes of n-3 HUFA and Current Recommendations

Recommendations for n-3 HUFA intakes have been made by several North American and global groups. While no formal recommendation for n-3 HUFA intakes has been made within the Dietary Reference Intakes, up to 10% of the Acceptable Macronutrient Distribution Range for 18:3n-3 may be consumed as EPA and DHA (Institute of Medicine of the National Academies, 2005). This results in an Acceptable Macronutrient Distribution Range of 0.06-0.12% of energy for EPA + DHA, with the lower bound reflecting median North American population-level intakes and the upper bound representing the highest intakes from foods consumed by the population. Accordingly, for a healthy female between the ages of 19 and 70, consuming a typical North American diet of approximately 1800 kcal/d (Institute of Medicine of the National Academies, 2005), the Acceptable Macronutrient Distribution Range would be 0.120 - 0.240g/d EPA + DHA and the male counterpart consuming roughly 2500 kcal/d would be 0.167 - 0.333 g/d. These ranges reflect the upper ~50% of the distribution of usual intakes by North Americans.

Current North American and global recommendations for EPA and DHA intakes by healthy individuals are generally between 0.25 and 0.5g/d (Kris-Etherton et al., 2007; United States Department of Agriculture, 2010). These recommendations are based on evidence from prospective studies and randomized trials which taken together, suggest that an EPA + DHA intake between 0.25 and 0.5 g/d lowers relative risk of coronary heart disease death and sudden

death compared with little or no intake (Mozaffarian and Rimm, 2006). In addition, this intake goal could theoretically be achieved using whole food strategies through two, 4 oz servings of oily fish per week. For secondary coronary heart disease prevention, 1 g/d of EPA and DHA is recommended by the American Heart Association and 2-4 g/d are recommended for triacylglycerol lowering (Kris-Etherton, Harris, and Appel, 2003; Lichtenstein et al., 2006). These recommendations are based on observations that EPA and DHA act through several cardio-protective mechanisms in a heterogeneous manner with the anti-arrhythmic effect levelling at intakes of 0.75 to 1g/d while the triacylglycerol-lowering effect continues linearly at intakes above 2 g/d (Mozaffarian and Rimm, 2006). The distinct pathophysiologies of primary and secondary coronary heart disease likely account for the need for higher doses of EPA and DHA in secondary prevention. The acceptable macronutrient distribution range for EPA + DHA provided by the World Health Organization is 0.25-2 g/d, emphasizing more consistent benefits with dosages between 1 and 2 g/d, particularly for secondary disease prevention (Elmadfa and Kornsteiner, 2009). North American and global recommendations for EPA and DHA intakes are summarized in **Table 2.1**.

In formulating the Dietary Reference Intakes, the Institute of Medicine concluded that there was insufficient evidence to establish a Tolerable Upper Level of Intake for EPA and DHA based on adverse effects related to immune function, bleeding times, risk of hemorrhagic stroke and oxidative damage (Institute of Medicine of the National Academies, 2005). Health Canada limits EPA and DHA intake from fish oil supplementation to 3 g/d (Health Canada, 2009) based on a report from the United States Food and Drug Administration which found insufficient evidence to determine the effect of EPA and DHA supplementation above this level on glycemic control in non-insulin dependent diabetes, bleeding times and circulating levels of low density

lipoprotein (Food and Drug Administration, 1997). A Maximum Level for EPA and DHA has also been set at 3 g/d by the World Health Organization based on observations of reduced cytokine production and increased lipid peroxidation at high intakes of EPA and DHA from dietary supplements (Elmadfa and Kornsteiner, 2009). Based on recent evidence showing that high blood n-3 HUFA status or n-3 fatty acid supplementation is not associated with serious bleeding in coronary heart disease patients or perioperative blood loss, bleeding concerns may warrant re-evaluation (Meredith et al., 2012; Salisbury et al., 2012; Watson et al., 2009).

Sources of n-3 HUFA in the Food Supply

In order to meet dietary recommendations, EPA and DHA can be obtained through whole foods, functional foods and nutraceutical sources (Patterson and Stark, 2008). Whole food sources are those which inherently contain EPA and DHA. Traditionally, seafood particularly fish, is the primary whole food dietary source of EPA and DHA (Gebauer et al., 2006; National Cancer Institute, 2009). Oily fish such as sardines, mackerel and salmon provide up to several grams of EPA and DHA per serving, while shellfish, such as shrimp, and lean fish, such as tilapia, cod and haddock, supply less than one gram of EPA and DHA per serving. Limited amounts of EPA and DHA occur naturally in other whole foods such as poultry and eggs. These non-marine foods become important sources of EPA and DHA for individuals who do not consume seafood (Welch et al., 2010). Whole food sources of n-3 HUFA are predominantly animal-derived (Novak and Innis, 2012; Stark and Patterson, 2012), with the exception of marine-based plants like seaweed and algae, and supply these fatty acids in triacylglycerol and phospholipid form. Two, 4oz servings of fish per week are recommended to achieve current EPA and DHA intake recommendations for healthy adults (Kris-Etherton, Harris, and Appel, 2003; Kris-Etherton et al., 2007; Lichtenstein et al., 2006; United States Department of Agriculture, 2010) and at this level of intake, the benefits of consuming a variety of fish outweigh the risks associated with environmental contaminants such as methylmercury (Mozaffarian and Rimm, 2006). Adults with high fish intakes (≥5 servings/week) and pregnant and breast feeding women should limit intake of specific species with higher levels of methylmercury, such as swordfish (Health Canada, 2008).

Novel nutrient sources provide alternatives to the traditional whole food sources. Nutraceuticals provide EPA and DHA typically derived from fish or algal oils in pharmaceutical form. A recent survey of nutraceutical products available in the Canadian retail market found

that 80% of products were in capsule, 17% in liquid and 3% in soft-chew form (Chalil, Patterson, and Stark, 2011). Nutraceutical products supply EPA and DHA in varying concentrations. In general, preparations of polyunsaturated fatty acid blends and products for children supply the lowest amount of EPA and DHA per serving, followed by fish body oils composed of 18% EPA and 12% DHA and concentrated oil preparations composed of 30-40% EPA and 20% DHA. Designer preparations supplying a higher concentration of either EPA or DHA, products derived from microalgae suitable for vegetarian and vegan consumers, and concentrated prescription products are also available. EPA and DHA in concentrated products are typically in ethyl ester or re-esterified triacylglycerol form. Evidence examining equal bioavailability of EPA and DHA in different chemical forms is mixed with some reports of greater blood biomarker response to n-3 HUFA in triacylgylcerol form as compared with ethyl esters (Dyerberg et al., 2010; Neubronner et al., 2010) and others reports showing no difference (Harris et al., 2007; Krokan, Bjerve, and Mork, 1993; Nordoy et al., 1991). Blood biomarker response to n-3 HUFA in triacylglycerol and ethyl ester form may be similar in blood fractions less responsive to recent dietary intake, like plasma phospholipids, as compared with blood fractions sensitive to recent dietary intake, like cholesterol esters (Hansen et al., 1993). Levels of organic pollutants, like polychlorinated biphenyls, are typically lowest in Canadian nutraceutical products derived from lower rungs of the marine food chain, such as anchovy, mackerel and sardine oils, compared with those derived from larger fish, such as shark, seal and menhaden, and farmed fish such as salmon which may bio-accumulate pollutants (Rawn et al., 2009b; Rawn et al., 2009a).

Functional foods are foods or beverages which contain EPA and/or DHA through either direct addition or manipulation of production conditions. For example, the DHA content of cow's milk can be increased by supplying feed material, such as fish meal, that contains

preformed DHA. Microencapsulated oils, including those derived from algal and fish body oil sources, can be directly added to various food vehicles to enhance the EPA and DHA content. Although the functional foods available to consumers changes over time, Canadian products enriched with EPA and DHA have included cow's/soy milk, yogurt, cheese, margarine, eggs, peanut butter, bread, pork and juice.

Various studies comparing blood levels of EPA and DHA following supplementation from fish, nutraceutical or functional food sources suggest equal bioavailability from the various food matrices (Arterburn et al., 2007; Arterburn et al., 2008; Harris et al., 2007; Wallace et al., 2000). Conventionally, dietetic practice emphasizes food-based approaches to achieve nutritional guidelines. Fish intake advice, for example, can also provide intake of a variety of important nutrients in addition to EPA and DHA including protein, vitamins A, B_3, B_6, B_{12}, E and D and minerals including iron, calcium, selenium and zinc. It has been acknowledged, however, that meeting EPA + DHA intake recommendations from a seafood-based whole food approach alone may not be feasible for many North Americans (Kris-Etherton, Harris, and Appel, 2003) and that fish is not well tolerated by all individuals (Burr et al., 1989). Nutraceuticals and functional foods offer potential alternatives to address these challenges. Selection of food and nutritive products may also be related to sex. Women attach greater importance to healthy eating when making food choices as compared with men (Wardle et al., 2004). In addition, nutraceuticals are used more frequently by women (Greger, 2001; Marques-Vidal et al., 2009), although sex differences in nutraceutical and functional food use are typically product-specific (de Jong N. et al., 2003).

Traditional whole foods, functional foods and nutraceuticals can, in theory, be used as dietary strategies to increase the EPA and DHA content of a typical Canadian diet to meet

recommendations for healthy individuals (Patterson and Stark, 2008). Providing consumers with functional foods alone, or in combination with fish for ≤1 month can significantly increase intake and blood levels of EPA and DHA (Lovegrove et al., 1997; Mantzioris et al., 2000; Metcalf et al., 2003). An increase in EPA and DHA intake measurements has also been observed when functional foods were provided for six months, but biomarkers of adherence were not assessed in this long term investigation (Patch et al., 2005). Dietary advice to consume oily fish has also been observed to result in long term increases in measures of EPA and DHA intake and blood status in male, post-myocardial infarction patients, but nutraceuticals were also provided to this portion of the study population due to an inability to tolerate fish (Burr et al., 1989). Through diet history interview assessment, adherence up to three months following dietary advice to increase intake of oily fish has been observed as part of a one-year trial (Neale et al., 2012).

Measuring Dietary n-3 HUFA Intake

Dietary survey methods can be used to estimate intakes of EPA and DHA. Quantitative methods, such as dietary records, measure the quantity of all foods consumed over one day and the number of measurement days is increased to better approximate usual intake (Gibson, 2005). Selection of the number of days is typically based on the day-to-day intake variability for the nutrient of interest and the financial and time-related burden of multiple days of recording for both the recorder and analyst. Semi-quantitative methods, such as food frequency questionnaires (FFQ), consist of a list of pre-selected food items and require respondents to indicate the usual portion size and how frequently these items are consumed. Respondents are asked to estimate habitual intake over a given period of time. These questionnaires can either assess total dietary intake by querying the most commonly consumed foods by the population or a specific nutrient

by including only items which supply appreciable amounts of a nutrient of interest. Due to its' brevity, the nutrient-specific FFQ typically provides a more expedient estimate of intake but does not permit examination of the selected nutrient in the context of other nutrients or total energy (Cade et al., 2002). Overall, the food frequency questionnaire provides a faster estimate of habitual intakes than the multiple day dietary record but restricts measurement of nutrient intake to the foods included on the questionnaire. In selecting an appropriate assessment method, considerations of precision and accuracy, recording and analysis time in addition to how well the methodological and statistical limitations have been characterized must be weighed (Cade et al., 2002).

The FFQ requires the user to respond to pre-determined dietary intake questions and therefore, considerable attention is placed on developing and validating the FFQ. A FFQ may be created by modifying an existing questionnaire or by using basic principles to develop a questionnaire *de novo*, with the latter option typically requiring a more extensive investment of resources (Subar, 2004). Considerations include selecting the food list, portion sizes and frequency categories which reflect the intakes of the desired nutrient(s) by the population of interest. Recent dietary surveys describing the population of interest may assist in this process (Cade et al., 2002). A nutrient database must also be chosen or developed to reflect the food list. Before the FFQ can be employed, it should be validated against a reference method which can include another type of dietary survey or a biological measurement of nutrient intake. The validation study typically includes correlation calculations in addition to other statistical procedures, such as the Bland-Altman method which evaluates agreement across the range of nutrient intakes (Bland and Altman, 2010). As no measurement of dietary intake is without error, validation studies provide an indication of whether the FFQ and reference method give

related answers. These types of comparisons can help determine whether the FFQ is sufficiently rigorous to achieve its' goal; usually measurement of habitual intake of a nutrient(s) by a specific population.

FFQ validated for use among Canadian populations are available to assess EPA and DHA intake based on traditional marine (Lucas et al., 2009) or whole food (Lien and Clandinin, 2009) sources alone. A more comprehensive, nutrient-specific questionnaire incorporating the range of Australian conventional and novel dietary sources of n-3 HUFA has been developed. This questionnaire was found to be a valid tool to assess usual n-3 HUFA intakes by Australians when compared with measurements from 3-d dietary records and blood fatty acid composition (Sullivan, Williams, and Meyer, 2006; Sullivan et al., 2008; Swierk et al., 2011). This questionnaire has formed the basis for similar FFQ in other countries, including a Belgian adaptation used to survey EPA and DHA intakes and dietary sources by Flemish women of reproductive age (Sioen et al., 2010).

Misreport of intake is a potential source of error in dietary assessment and can result from both failure to record what was actually consumed or alterations in usual eating patterns in response to the recording process (Gibson, 2005). Both over and under reporting of intake can occur. Energy intake, an important variable as energy and nutrient intakes are mutually dependent, is typically under-reported when compared to estimates of energy expenditure of weight-stable individuals (Livingstone and Black, 2003). Multiple and potentially interacting variables may influence energy misreport including biological characteristics such as sex and body mass index and attitudes towards food such as social desirability (Hill and Davies, 2001).

The rigor of food nutrient composition databases is an important variable in the translation of food sources identified through dietary surveys into nutrient intakes. The Canadian

Nutrient File has been developed to characterize the nutrient composition of whole foods in the Canadian food supply. Such national databases provide good estimates but cannot fully capture the variability inherent to the food supply and additional factors such as diverse cooking methods (Kitson et al., 2008). Functional foods and nutraceuticals are generally not included in national databases and require separate characterization before nutrient intakes from these products can be assessed.

Levels of EPA and DHA in biological tissues offer an alternative and objective strategy to measure EPA and DHA intake which does not rely on self-report. Since endogenous production of EPA and DHA is low, dietary intake has a profound effect on circulating levels of EPA and DHA (Pawlosky et al., 2003b). Indeed, levels of EPA and DHA in blood have been observed to respond to dietary intake (Bjerve et al., 1993; Kobayashi et al., 2001). Consequently, there is potential to relate specific levels of EPA and DHA in easily sampled tissues, like blood, to dietary doses of these fatty acids. In order to relate blood fatty acid composition with dietary intake of EPA and DHA, the specific dose-response relationship must be characterized. For healthy adults, blood levels corresponding to EPA + DHA doses under 1g/d have been examined in plasma (Higgins et al., 2001) but examination of additional blood fractions is limited. Biological determinants of blood fatty acid composition should also be considered. Due to sex differences in endogenous synthesis, blood levels of DHA are higher in women than men when background dietary n-3 HUFA intake is low (Bakewell, Burdge, and Calder, 2006; Metherel et al., 2009) while supplementation at high doses of EPA and DHA (4.8g/d) removes sex differences in blood fatty acid measurements (Metherel et al., 2009).

Both dietary survey and biochemical markers provide complementary information and act as surrogate measures of true dietary intake. Dietary surveys indicate what is actually

consumed in the diet but depend upon the quality of the dietary assessment tool, the recall and reporting of the respondent, and the quality of nutrient composition databases. In contrast, biochemical markers provide objective assessments but their ability to predict nutrient intake must be confirmed through dose-response evaluation.

Table 2.1. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake recommendations for adults[1]

Target	Recommending Body	Recommendation
<1g/d for healthy individuals		
Healthy adults	American Heart Association; 2002[2], 2006[3], 2010[4]	2 fish servings /wk
	USDA Dietary Guidelines for Americans; 2010[5]	0.25 g/d EPA+DHA
	Dietitians of Canada & American Dietetic Association; 2007[6]	0.5 g/d EPA+DHA
	International Society for the Study of Fatty Acids and Lipids; 2004[7]	0.5 g/d EPA+DHA
	United Nations & World Health Organization; 2008[8]	0.25-2[9] g/d EPA+DHA
≥1g/d for 2° prevention		
Patients with coronary heart disease	American Heart Association; 2002[2], 2006[3]	1 g/d EPA+DHA
Triglyceride lowering		2-4 g/d EPA+DHA

[1] EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid
[2] (Kris-Etherton, Harris, and Appel, 2003), [3](Lichtenstein et al., 2006), [4](Lloyd-Jones et al., 2010), [5](United States Department of Agriculture, 2010), [6](Kris-Etherton et al., 2007), [7](International Society for the Study of Fatty Acids and Lipids, 2004), [8](Elmadfa and Kornsteiner, 2009)
[9] Acceptable Macronutrient Distribution Range, upper end for 2° coronary heart disease prevention

CHAPTER 3

RATIONALE AND OBJECTIVES

Rationale

Traditional whole foods, functional foods and nutraceuticals could be used as cost-effective dietary strategies to increase the EPA and DHA content of a typical Canadian diet and bridge the gap between observed and recommended intakes for healthy individuals (Patterson and Stark, 2008). While each of these omega-3 sources has been used to increase intakes and blood levels of these fatty acids, it is unknown whether the same results would be achieved if healthy consumers were required to select and purchase items independently, over the long term based on dietary advice. Furthermore, as selection of nutrient sources can vary between men and women, it is unknown whether the effectiveness of different dietary advice strategies varies by sex. Simultaneous evaluation of long-term adherence to seafood, functional food and nutraceutical strategies in a self-selected manner would assist health care professionals in formulating effective dietary advice strategies.

In order for health care professionals to rapidly evaluate the success of their dietary advice, tools which efficiently and accurately estimate usual dietary intake of EPA and DHA are needed. New dietary surveys should have the scope to assess EPA and DHA intake from their specific, concentrated dietary sources and provide measurements which compare well against estimates from other dietary survey and biomarker-based tools. An FFQ incorporating Canadian whole food, functional food and nutraceutical sources of EPA and DHA and validated for use among Canadians has the potential to address the challenges of estimating usual intake of these fatty acids.

Blood n-3 HUFA status offers a complementary, objective strategy to evaluate dietary intake. From the existing literature, it is difficult to determine specific blood levels of EPA and DHA which correspond to recommended intakes of EPA and DHA for healthy individuals across a variety of markers as doses >1g/d of EPA + DHA are typically examined and methods to measure blood fatty acids are highly variable and not standardized. Comprehensive blood analyses in response to EPA and DHA intakes under 1g/d are needed and should include whole blood due to its increased utility in high throughput finger-tip prick blood fatty acid determinations. Furthermore, examining men and women is necessary to determine if different intake recommendations for EPA and DHA are required for each sex to achieve cardio-protective blood levels of EPA and DHA.

Presently, long term adherence to seafood, functional food and nutraceutical dietary advice strategies to increase intakes of EPA and DHA by male and female Canadian adults will be evaluated. In addition, survey and blood-based assessment tools to efficiently measure EPA and DHA intakes will be characterized; the relative validity of a nutrient-specific, semi-quantitative FFQ, incorporating the range of Canadian EPA and DHA sources, will be assessed and a comprehensive examination of the dose response to EPA and DHA intakes of 0.25, 0.5 and 1.0g/d in men and women will be examined.

Objectives

1. To develop and validate a nutrient-specific FFQ to determine EPA and DHA intakes by Canadian adults from Canadian whole food, functional food and nutraceutical sources.

2. To examine blood levels and dietary intakes of EPA and DHA 4, 8, 12, 24 and 52 weeks after receiving dietary advice to increase EPA and DHA intakes using a seafood, functional food, nutraceutical or combined approach in men and women.

3. To examine the dose-response of 0.25, 0.5 and 1 g/d of supplemental EPA and DHA on corresponding blood fatty acid levels and biomarkers in whole blood, erythrocytes and plasma phospholipids in men and women.

Hypotheses

1. Correlations between FFQ measurements of EPA and DHA intake and whole blood EPA and DHA levels will be stronger than correlations between 3-d dietary record measurements of EPA and DHA intake and whole blood EPA and DHA levels.

2. Dietary intakes and blood levels of EPA and DHA will increase compared with baseline following all of dietary advice strategies with women demonstrating greater adherence as compared with men.

3. The strategy providing combined dietary advice about whole foods, functional foods and nutraceuticals containing EPA and DHA will result in the greatest adherence over one year and the highest increase in dietary intake and blood levels of EPA and DHA.

4. Blood biomarker levels of EPA and DHA will increase in response to dietary supplementation with the highest dose of EPA and DHA, 1g/d, resulting in the highest blood levels, but the blood levels achieved will be below targets associated with protection from cardiovascular disease.

5. Women will have higher blood levels of DHA as compared with males at baseline and the increases in blood levels of DHA in response to supplementation will be higher in females because of differences related to body mass.

CHAPTER 4

GENERAL METHODS AND MATERIALS

Participants

A total of 100 participants took part in the studies described herein. None of the participants had existing cardiovascular disease or diabetes mellitus. The University of Waterloo Human Ethics Committee approved all procedures and protocols associated with these studies and all participants provided informed written consent. All participants completed a 3-d dietary record, FFQ, fasted finger-tip prick blood sample and health screening form and provided anthropometric measurements including weight and height at screening or baseline.

Sample and Data Collection

All sample and data collection took place in the Laboratory of Nutritional and Nutraceutical Research or Manulife Wellness Centre at the University of Waterloo. Participants fasted for 8-12 h overnight before all blood sampling. Fasting finger-tip prick whole blood samples were collected by puncturing the skin with a disposable lancing device (Capiject; Terumo, Somerset, NJ or Unistik 2; Lifescan, Milpitas, CA) and absorbing blood onto a $1cm^2$ area of chromatography paper (Whatman, Maidstone, England) pre-washed in 4:1 chloroform:methanol. Venous blood samples were collected by venipuncture into evacuated 10 mL tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) with whole blood samples aliquoted for storage prior to plasma and erythrocyte separation by centrifugation at 2300 rpm for 15 minutes. Subsequently, aliquots of recovered plasma were collected and stored. Erythrocytes were washed with saline, followed by centrifugation at 2300 rpm for 15 minutes, twice prior to aliquoting and storage. All venous blood fraction samples were stored at -80°C

until analysis as 1-2 mL aliquots. Storage of blood samples at -80°C promotes long-term stability of the HUFA pool (Metherel, 2012).

Written and verbal instructions were provided for dietary surveys including 3-day dietary records (two weekdays and one weekend day) and the FFQ. Completed dietary surveys were checked by a single researcher for completeness and clarification. Dietary surveys were analyzed using the Food Processor SQL Edition dietary analysis software with the latest version available at the time of the study (ESHA Research, Salem, OR; version 10.6.0 and 10.9.0). The dietary analysis software was also updated manually with the Canadian Nutrient File 2007b database (Health Canada, 2007a) and fatty acid compositions determined directly by gas chromatography in the laboratory of select functional food and nutraceutical products identified on the FFQ and 3-d dietary records.

Fatty Acid Composition Determinations

Fatty acid compositions were determined for venous blood fractions, finger-tip prick blood samples and nutraceuticals and functional foods identified on diet intake surveys and food duplicate collections. Venous blood samples were prepared by traditional lipid extraction followed by transesterification methods. A 22:3n-3 ethyl ester (10 ug/sample, NuCheck Prep, Elysian, MN) internal standard was added prior to lipid extraction to quantify fatty acids and to verify complete transesterification to fatty acid methyl esters. A Folch based method (Folch, Lees, and Sloane Stanley, 1957) was used to extract lipids from whole blood and plasma (100 uL aliquots), using 3 mL chloroform:methanol (2:1 v/v) with 50 ug/mL butylated hydroxytoluene (Sigma-Aldrich, Bellefonte, PA) and subsequent addition of 500 uL sodium phosphate. Butylated hydroxytoluene was added to prevent lipid oxidation during analysis. A double lipid

extraction was performed on erythrocytes (200 uL aliquots, weighed) using 2 mL chloroform:methanol (1:1 v/v) with 50 ug/mL butylated hydroxytoluene and subsequent addition of 1.8 mL sodium phosphate buffer (Bligh and Dyer, 1959), with steps to ensure erythrocyte cell membrane lysis (Reed et al., 1960). Fatty acids in the lipid extracts were then transesterified to fatty acid methyl esters using 14% boron trifluoride in methanol (1 mL, Thermo Scientific, Bellefonte, PA) with hexane (300 uL) on a 90°C heat block for 1 h (Morrison and Smith, 1964). Finger-tip prick blood samples were prepared for analysis by direct transesterification. Chromatography paper, pre-washed in 4:1 chloroform:methanol and saturated with 1 cm^2 blood, was placed into the 14% boron trifluoride in methanol with hexane on a 90°C heat block for 1 h without prior lipid extraction (Armstrong, Metherel, and Stark, 2008; Fratesi et al., 2009; Metherel et al., 2009). The fatty acid composition of nutraceutical and functional food products, and food duplicate collections were determined in triplicate analyses in the presence of butylated hydroxytoluene (Sigma-Aldrich) and 22:3n-3 ethyl ester internal standard (NuCheck Prep). Oils isolated from nutraceuticals (10 uL, weighed) were prepared for analysis by extraction followed by transesterification as described for whole blood and plasma above. For functional foods and food duplicate collections, AOAC method 996.06 (Mossoba et al., 2003), with adjustment of solvent volumes for samples containing 6.25 - 12.5 mg of total fat was modified with an initial acid hydrolysis treatment to ensure the extraction of lipids from microencapsulated fish oil powder (Stark, 2012). Fatty acid methyl esters were then prepared from lipid extracts by transesterification with boron trifluoride as the transesterification reagent as described above. The coefficient of variation for fatty acid composition associated with this procedure is 2.5 - 3.5% (unpublished observations).

The fatty acid methyl esters in hexane were collected into sealed vials to prevent evaporation and analyzed on a Varian 3900 gas chromatograph (Varian, Palo Alto, CA) with an autosampler following settings as previously described (Kitson et al., 2008). Briefly, hydrogen was used as a carrier gas at a flow rate of 30 mL/min with a DB-FFAP 15m x 0.10 mm inner diameter x 0.10 μm film thickness capillary column (J & W Scientific, Agilent Technologies, Palo Alto, CA). The flame ionisation detector was set at 300°C with a 50 Hz sampling frequency, a 200:1 split ratio and nitrogen and air make-up gas flow rates of 25 and 300 mL/min respectively. The autosampler injector temperature was set at 250°C with an injection volume of 1μl. The temperature program was as follows: initial, 150°C with a 0.25 min hold; ramp: 35°C/min - 200°C, then by 8°C - 225°C with a 3.2 min hold, then by 80°C/min - 245°C with a 15 min hold. Total run time was 23 min for each sample. An external standard (NuCheck Prep) was run with each batch of samples for fatty acid identification and to verify a linear response of the flame ionisation detector. Both identified and unidentified peaks were included in the total fatty acid summation when individual fatty acids were expressed as a percentage of the total fatty acid pool.

Statistical and Data Analyses

All statistical analyses were performed using SPSS (IBM, Armonk, NY; release 18.0.0). Significance was set at $p < 0.05$. Individual fatty acids were expressed as a percentage of total fatty acids. The % n-3 HUFA in total HUFA was calculated using the following formula: (20:5n-3 + 22:5n-3 + 22:6n-3) / (20:5n-3 + 22:5n-3 + 22:6n-3 + 20:3n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6) x 100 (Stark, 2008a). Variability associated with arithmetic mean values is expressed as a standard deviation.

CHAPTER 5

DIETARY AND BIOMARKER VALIDATION OF A FOOD FREQUENCY QUESTIONNAIRE TO MEASURE EPA AND DHA INTAKES FROM CONVENTIONAL AND NOVEL CANADIAN DIETARY SOURCES[1]

Introduction

The purpose of a nutrient-specific FFQ is to rapidly assess usual intake of a nutrient of interest over the long term. Habitual Canadian EPA and DHA intakes from whole food (Lien and Clandinin, 2009) and seafood sources (Lucas et al., 2009) can be measured using existing FFQ and novel functional food and nutraceutical products have been incorporated into FFQ in other countries (Sioen et al., 2010; Sullivan, Williams, and Meyer, 2006; Sullivan et al., 2008). As neither dietary surveys nor biomarkers assess EPA and DHA intake without measurement error, several recent studies have simultaneously used dietary records and blood samples as reference tools for validation of FFQ to measure n-3 HUFA intake (McNaughton, Hughes, and Marks, 2007; Swierk et al., 2011; Zhang et al., 2009). Both two-way comparisons between FFQ and reference method measurements and three-way comparisons using the method of triads (Kaaks, 1997; Ocke and Kaaks, 1997) were used in these validation studies. The purpose of the present study is to incorporate conventional and novel Canadian sources of EPA and DHA into a nutrient-specific FFQ and assess FFQ validity in measuring EPA and DHA intakes by Canadian adults using dietary record and blood biomarker reference measurements.

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[1] The contents of this chapter has been previously published and copyright has been assigned to the Academy of Nutrition and Dietetics.
Patterson, A. C., Hogg, R. C., Kishi, D. M. et al. (2012) Biomarker and Dietary Validation of a Canadian Food Frequency Questionnaire to Measure Eicosapentaenoic and Docosahexaenoic Acid Intakes from Whole Food, Functional Food, and Nutraceutical Sources. J Acad.Nutr Diet. 112: 1005-1014.

Methods and Materials

Adult men and women were recruited from the Kitchener-Waterloo, Ontario area between January and September 2008 as part of the recruitment strategy described in Chapter 6 and word-of-mouth. A minimum recruitment of 50 participants was targeted based on biomarker and 3-d dietary record validation of an Australian FFQ which served as a template for the present FFQ (Sullivan, Williams, and Meyer, 2006; Sullivan et al., 2008). Fifty participants would be needed to detect an effect size of 1 with 99% power, assuming a correlation between methods of 0.6 ($\alpha = 0.05$). Effect size is the mean difference between assessment methods divided by the standard deviation of either method. To be eligible to participate in the study, individuals had to be $\geq$18 years old and be free of any conditions which could alter blood fatty acid composition including diabetes mellitus, lipid metabolism disorders and pregnancy. At the first visit, participants completed the FFQ (**Appendix A**), a health screening questionnaire (**Appendix B**) and received instructions to complete a 3-d dietary record. A finger-tip prick blood sample and anthropometric measurements were also collected. Completed dietary records were returned to the laboratory within two weeks when they were checked for completeness and clarification. Two researchers performed all recruitment, sample collection and sample and data analysis.

A survey of all Canadian whole food, functional food and nutraceutical sources of EPA and/or DHA was conducted to develop the FFQ. Whole food sources were assessed through a nutrient-based search of the Canadian Nutrient File 2007b database (Health Canada, 2007a) and categorized as follows; marine-based products (including fresh, frozen and canned fish, shellfish, seafood paste and sauce, seaweed and fish broth), eggs (including amounts in baked-goods and cooking) and poultry, meat and related products (including deli meats and sausages). Internet

searches and surveys of local grocery chains and specialty/health stores were conducted to compile available functional food and nutraceutical sources. The functional foods from the following categories were found; eggs and egg products, dairy and dairy alternatives, nut and oil-based spreads, breads and juice. To integrate these Canadian sources into FFQ format, an existing Australian FFQ assessed for validity and reproducibility (Sullivan, Williams, and Meyer, 2006; Sullivan et al., 2008) was used as a template. Serving sizes to reflect Canadian functional food products, such as portions of string cheese, were incorporated. For each category of whole food, functional food or nutraceutical, the questionnaire solicits the following information through identification from a list or user input; the type/brand of product, frequency of intake and serving size usually consumed during the preceding 12 months. To facilitate portion size estimation, visual aids including measuring cups and spoons, product packaging and illustrations of meat/fish portion sizes on plate settings were displayed. The final FFQ included 37 questions (Appendix A), was self-administered and was immediately checked for clarification and completeness upon completion.

Statistical Analyses

Intakes of HUFA determined by the 3-d dietary records and FFQ did not follow a normal distribution and accordingly, non-parametric tests or $\log_e$ transformations followed by parametric tests were conducted. Wilcoxon signed rank test and the Bland-Altman method (Bland and Altman, 2010) were performed on EPA and DHA intakes measured by the FFQ and 3-d dietary record. Pearson correlation coefficients were calculated between fatty acid measurements from the FFQ, 3-d dietary record and blood biomarker. Correlation coefficients were evaluated as low (<0.4), moderate (0.4-0.6) and high (>0.6) (Nelson et al., 2001). Participants were ranked

independently by EPA + DHA measures from the FFQ, 3-d dietary record and blood biomarker as well as the % n-3 HUFA in total HUFA from the blood biomarker and then divided into quartiles. Percent agreement between quartiles of FFQ, 3-d dietary record and blood biomarker measurements was determined.

The Method of Triads, a strategy to compare individual measurements from the FFQ, 3-d dietary record and blood biomarker with an estimated true value, was also performed (Kaaks, 1997; Ocke and Kaaks, 1997). Two-way Pearson correlation coefficients were combined to produce a triangulated estimate of the true value and yield a new correlation coefficient (validity coefficient) for each of the FFQ, 3-d dietary record and blood biomarker measurements with this estimated true value as follows:

$$\text{Validity Coefficient}_{\text{FFQ with True}} = \sqrt{[(r_{\text{FFQ \& Dietary Record}} \times r_{\text{FFQ \& Biomarker}})/r_{\text{Dietary Record \& Biomarker}}]}$$

$$\text{Validity Coefficient}_{\text{Dietary Record with True}} = \sqrt{[(r_{\text{FFQ \& Dietary Record}} \times r_{\text{Dietary Record \& Biomarker}})/r_{\text{FFQ \& Biomarker}}]}$$

$$\text{Validity Coefficient}_{\text{Biomarker with True}} = \sqrt{[(r_{\text{FFQ \& Biomarker}} \times r_{\text{Dietary Record \& Biomarker}})/r_{\text{FFQ \& Dietary Record}}]}$$

For EPA, DHA and EPA + DHA measurements, the validity coefficients 'FFQ with True', 'Dietary Record with True' and 'Biomarker with True' were each calculated. To illustrate, the coefficient of correlation of the FFQ-derived DHA measurement with the estimated true DHA intake is described by the 'Validity Coefficient $_{\text{FFQ with True}}$' for DHA.

The method of triads model assumes a linear relationship of FFQ, dietary record and blood biomarker measurements with the true value and that random errors between the three measurements are uncorrelated. Because self-reported intakes assessed by FFQ and dietary record may share sources of error, positive correlation of random errors will lead to overestimation of the associated validity coefficients. Validity coefficients should therefore be interpreted as upper limits of their real values (Ocke and Kaaks, 1997). In addition, correlation between blood biomarker and intake measurements may be depressed by sources of random

variation, such as metabolic effects. As a result, the correlation coefficients $r_{FFQ\ \&\ Biomarker}$ and $r_{Dietary\ Record\ \&\ Biomarker}$ are interpreted as lower limits of the validity coefficients (Ocke and Kaaks, 1997). Taken together, the true value of the validity coefficient falls within a range; the method of triads provides the upper bound of this range and the correlation coefficients $r_{FFQ\ \&\ Biomarker}$ and $r_{Dietary\ Record\ \&\ Biomarker}$ act as the lower bound.

Results

Participant Flow and Characteristics

A total of 88 participants were recruited and 78 were included in final analyses. Two participants were excluded for implausible 3-d dietary record energy intake (Gibson, 2005) and eight for a missing FFQ, dietary record or blood sample. The characteristics of the study sample are detailed in **Table 5.1**. Participants were largely Caucasian, non-smokers and well-educated. Use of nutraceutical products was reported by 60% of participants and energy intakes measured by 3d food record were 2174 ± 549 kcal/d. The study sample had an average age of 40.3 ± 9.9 y (95% CI: 38.0 - 42.5 y) and body mass index of 26.5 ± 5.5 kg/m^2 (95% CI: 25.2 - 27.7 kg/m^2).

Dietary Intake and Blood EPA and DHA Measurements

The FFQ took approximately 15 minutes to complete and dietary intakes and finger-tip prick whole blood composition for fatty acids of interest are presented in **Table 5.2**. No significant differences were observed between mean EPA + DHA intakes reported by FFQ and 3-d dietary record (p=0.93). Median EPA + DHA intakes were 0.21 g/d by FFQ and 0.13 g/d by 3-d dietary record. The median whole blood % EPA + DHA was 2.44% and % n-3 HUFA in total HUFA was 23.36%.

Comparisons of EPA and DHA Measurements by FFQ and Dietary Records and Whole Blood Fatty Acid Assessment

Results determined by Bland-Altman analyses for EPA, DHA and EPA + DHA intakes are depicted in **Figure 5.1**. The FFQ gives a lower intake measurement at high intakes of EPA + DHA and a higher intake measurement at low intakes of EPA + DHA. For a small proportion of the study population (5%, n=4/78), EPA + DHA intake measurements differed between FFQ and dietary record estimates.

Agreement analysis (**Table 5.3**) was similar across pairs of dietary assessment and blood biomarker measurements. Agreement of quartile ranking based on FFQ measurement of EPA + DHA intake and blood biomarker % n-3 HUFA in total HUFA demonstrates that 42% of participants were classified into the same and 79% into the same or adjacent quartile. By FFQ measurement of EPA + DHA intake, 42% of participants were classified into the same and 77% into the same or adjacent quartile as measurement of EPA + DHA by 3-d dietary record and blood biomarker.

Agreement analysis was also performed to compare 3-d dietary record with biomarker measurements. Overall, a lower percentage of participants were classified into the same quartile as biomarker measurements by 3-d dietary record as compared with FFQ measurement. Agreement of quartile ranking based on 3-d dietary record measurement of EPA + DHA intake and blood biomarker % EPA + DHA demonstrates that 27% of participants were classified into the same quartile, 44% into the adjacent quartile and 29% were misclassified. Agreement of quartile ranking based on 3-d dietary record measurement of EPA + DHA intake and blood biomarker % n-3 HUFA in total HUFA demonstrates that 38% of participants were classified into the same quartile, 36% into the adjacent quartile and 26% were misclassified.

Correlation coefficients were low to moderate (**Table 5.4**). The blood biomarker % n-3 HUFA in total HUFA was moderately correlated with estimates of intake with blood biomarker % n-3 HUFA in total HUFA and FFQ EPA estimates (r= 0.51, $p< 0.001$) being the strongest correlation. The correlation between dietary record DHA intake estimates and blood biomarker %DHA in total fatty acids was the weakest (r= 0.21, $p= 0.07$).

Moderate and high validity coefficients between assessment tools by three-way comparisons were observed (**Table 5.5**). For the FFQ measurements with the true value, the validity coefficient for EPA + DHA was the strongest (Validity Coefficient $_{\text{FFQ with True}} = 0.71$) while the validity coefficient for DHA fell outside the allowable range of 0 to 1 (Validity Coefficient $_{\text{FFQ with True}} = 1.005$). Such an occurrence is usually attributed to small sample sizes and is known as a Heywood case (Ocke and Kaaks, 1997). EPA measurements produced the strongest validity coefficients for dietary record and biomarker estimates with the true value (Validity Coefficient $_{\text{Dietary Record with True}} = 0.61$, Validity Coefficient $_{\text{Biomarker with True}} = 0.61$).

Discussion

The present FFQ is the first, to our knowledge, to specifically evaluate EPA and DHA intake from Canadian whole food, functional food and nutraceutical sources and to be simultaneously validated using dietary survey and blood biomarker reference methods. The strength of the FFQ is that it can be rapidly completed and analyzed and identifies all Canadian dietary sources contributing to usual intake of EPA and DHA. Although FFQ-based measurements showed reasonable agreement with measurements from 3-d dietary records and blood biomarkers, neither of the reference methods can truly be considered a gold standard.

Accordingly, comparisons between the three assessment tools are discussed herein to characterize the information each measurement tool provides.

As concentrated sources of EPA and DHA like oily fish and nutraceuticals are not typically consumed daily, the FFQ may provide a better estimate of usual EPA and DHA intake than a 3-d dietary record (Overby, Serra-Majem, and Andersen, 2009). Although EPA and DHA intake measurements from the FFQ and 3-d dietary record differed for only 5% of the study population, a distinct relationship was observed between measurements from the two dietary surveys; the FFQ gave higher estimates of EPA and DHA intake than the 3-d dietary record at low intakes of EPA and DHA, and lower estimates at high intakes of EPA and DHA by Bland-Altman analysis. Furthermore, the range of EPA + DHA intakes from FFQ estimates (0.01-1.47 g/d) was half as wide as the range from 3-d dietary records (0.01-2.92 g/d). Challenges associated with using 3-d dietary records to measure habitual EPA and DHA intakes have been previously documented and were attributed to the inclusion or exclusion of concentrated sources of EPA and DHA on 3-d dietary records (Fratesi et al., 2009). Use of novel products can be periodic and a 3-d recording period may not fully capture patterns of novel product use over time, which are presently not well characterized (Dwyer, Picciano, and Raiten, 2003). A considerably greater number of recording days may be necessary to approximate usual EPA and DHA intakes using dietary records as it is typical for measures of short-term nutrient intake to show variability above and below long-term, habitual intake (Overby, Serra-Majem, and Andersen, 2009; Willett, 1998). Additional recording days would greatly increase the burden of dietary record assessment for both the recorder and analyst and accordingly, the FFQ could be used to rapidly determine usual intake of EPA and DHA.

Differences between FFQ and 3-d dietary record measurements are further highlighted

through comparisons with the blood biomarker. Discrepancies in the reference time frame may account for the low correlation between DHA measurements from the 3-d dietary record and blood biomarker (r= 0.21, *p*= 0.07). DHA incorporates into and washes out of erythrocytes over the course of months as it is preferentially placed in the *sn*-2 position of phosphatidyl ethanolamine of the inner membrane leaflet of the erythrocyte, while EPA accumulates in phosphatidyl choline on the outer membrane leaflet where it can exchange more rapidly with plasma fatty acids (Metherel et al., 2009). Therefore, measures of blood DHA may not match the short reference time frame of the 3-d dietary record. In contrast, the stronger correlation between FFQ and blood biomarker DHA measurements (r= 0.42, *p*= <0.01) suggest a better alignment of the reference time frames. The FFQ may better approximate usual intakes of DHA. Moderate correlation coefficients were also observed when intake measurements from a comparable Australian FFQ to measure n-3 HUFA intake from conventional and novel sources were compared with erythrocyte and plasma fatty acid composition (Sullivan, Williams, and Meyer, 2006). Moderate correlations between dietary intake and biomarker measures may therefore be appropriate (Overby, Serra-Majem, and Andersen, 2009). Two-way correlation between intake of EPA + DHA measured by the FFQ with whole blood % EPA + DHA, and with % n-3 HUFA in total HUFA biomarkers of n-3 HUFA status were within the generally deemed acceptable range of 0.4 - 0.7 (Subar, 2004). Two-way correlations were within the 0.4 - 0.7 range for EPA and DHA intake measurements from the 3-d dietary record and whole blood biomarkers expressed only as the % n-3 HUFA in total HUFA. Though individual fatty acid levels may vary between blood sub-fractions and tissues, the % n-3 HUFA in total HUFA remains generally consistent between depots as it represents the competition between HUFA for

esterification at the *sn*-2 position of phospholipids (Armstrong, Metherel, and Stark, 2008; Stark, 2008a).

Selection of the 3-d dietary record as a reference method was based on common practice. Intake of EPA and DHA measured by repeat 3-d dietary records may have resulted in better agreement with FFQ measurements in the present study, however selection of a single 3-d dietary record as a reference method presently was based on the Australian study (Sullivan et al., 2008). Dietary validation of the Australian FFQ used as a template for the present FFQ found high correlation and no systematic variation by the Bland-Altman method between EPA and DHA estimates from the FFQ and a 3-d dietary record (Sullivan et al., 2008). Additional methodological considerations may have particularly strengthened comparisons between FFQ and dietary record measurements in the Australian validation study. For instance, weighed food records were used which can diminish inaccuracies in portion size estimation. While some context can be provided for the results of the present FFQ validation through the examination of similar validation studies, direct comparison of validation results has limitations as other FFQ pose different questions, examine different populations and employ different reference measurements, nutrient composition databases and statistical tests.

The dietary survey and biomarker tools used herein provide discrete approaches to estimate the true EPA and DHA intake. Consequently, complete accordance of measurements from each of these three tools was not expected. In addition to incongruence of the reference time frame outlined above, sources of error inherent to dietary surveys, including limitations of the nutrient composition database and misreport may also attenuate agreement between dietary survey and biomarker assessment.

While the fatty acid composition of functional food and nutraceutical products identified by the FFQ was directly determined, fatty acid composition data in the food composition database could limit comparisons between dietary survey and blood biomarker measurements. The Australian national food composition database has been updated for n-3 HUFA content, including measurements of n-3 docosapentaenoic acid (Mann et al., 2003) but similar updates were not available in the Canadian database at the time of survey completion. Among typical Canadian populations with low intakes of n-3 HUFA, n-3 docosapentaenoic acid intakes make an important contribution to total n-3 HUFA intakes and often exceed EPA intakes when fish is not consumed (Fratesi et al., 2009). *In vivo* conversion and retroconversion between EPA, n-3 docosapentaenoic acid and DHA has been observed in individuals with low n-3 HUFA intakes (Brossard et al., 1996; Kitson, Stroud, and Stark, 2010; Pawlosky et al., 2003b). As endogenous synthesis of DHA has been demonstrated to be higher by females than males when consuming low n-3 HUFA intakes (Burdge and Wootton, 2002; Burdge, Jones, and Wootton, 2002; Kitson, Stroud, and Stark, 2010; Pawlosky et al., 2003b), sex differences were also considered. However, sex stratification did not strengthen agreement between dietary intake and biomarker measurements and no sex differences in dietary or blood measurements of EPA or DHA were observed in the present study. Furthermore, it is important to consider the contribution of functional food and nutraceutical sources to dietary intake however, measurement of EPA and DHA intake from novel sources requires frequent database and FFQ updating due to changes in product availability.

Dietary intake assessment may be influenced by misreport. Those who under-report energy intake may simultaneously over-report intake of perceived healthy foods, including fish (Livingstone and Black, 2003). Such selective misreport could impact both FFQ and dietary

record assessments. While this nutrient-specific FFQ provides a relatively quick estimate of EPA and DHA intake, it does not provide a measure of total energy intake. Methods to statistically account for selective misreport are not defined and adjustment of dietary record measurements using the residual method (Willett, 1998) yielded no improvement in correlation coefficients in the present study. Finally, in the present study n-3 HUFA intakes assessed by 3-d dietary record were not anticipated to vary greatly by season over the nine month recruitment period. Greater use of fish liver oils, a source of fat soluble vitamins, in winter has been observed in Polish consumers (Kolanowski, 2008) however, the fish body oils consumed by participants in the present study were not anticipated to be taken seasonally. A prior Canadian study has also found no seasonal variability in intakes of EPA and DHA from whole food sources (Lien and Clandinin, 2009). A one year reference period has previously been used for food frequency questionnaire determination of EPA and DHA intake from both food and nutraceutical sources (Zhang et al., 2009) and was selected in the present study to capture potential variability in novel product use over time (Dwyer, Picciano, and Raiten, 2003) and probe usual intake. Intake over the prior year has been queried in seminal semi-quantitative food frequency questionnaires (Block et al., 1986; Willett et al., 1985) on the basis that respondents will rely on generic memory to recall usual intake in the past and that responses will be independent of fluctuation in usual intake over time (Willett, 1998). In practice, diet recall respondents do not fully restrict their responses to intake during the reference period (Smith, 1993). Although FFQ respondents were asked to consider the past year, diet recall will include both description of the typical diet, regardless of the reference period, and specific memory about intake during the reference period (Smith, 1993).

Conclusion

Comparison of FFQ with food record and blood biomarker estimates suggests that the FFQ is an adequate tool for ranking and estimating habitual EPA and DHA intakes. Comparison of dietary survey with biomarker measurements, particularly blood DHA measurements, suggests that the FFQ has a longer reference time frame than the 3-d dietary record for measurement of EPA + DHA intake. The FFQ appears to have a theoretical advantage over the 3-d dietary record as the FFQ captures EPA and DHA intake from all dietary sources, instead of just those consumed on recording days. This is important in measurement of n-3 HUFA intakes as in the food supply, these fatty acids are found concentrated in a limited number of sources. The FFQ can also have benefits over a 3-day food record in situations where rapid assessment of habitual intake is needed, as the FFQ is more expedient to complete and assess. Finally, as the FFQ provides an overview of all dietary EPA and DHA sources, it could also be used to identify the primary dietary sources and detect underused sources which could be promoted to achieve dietary intake recommendations.

Table 5.1. Characteristics of food frequency questionnaire (FFQ) study participants[1]

Characteristic	*n*	*%*[2]
Sex		
Female	46	59
Male	32	41
Cultural/Racial Background		
Caucasian	66	85
Chinese	6	8
Latin American	3	4
West Asian	2	3
Mixed	1	1
Education		
Some High School	1	1
High School or Current Undergraduate Student	19	24
Trade, Technical/Vocational School or Business College	5	6
Community College, CEGEP or Nursing School	9	12
Bachelor's Undergraduate Degree or Teacher's College	26	33
Master's Degree	13	17
Earned Doctorate	2	3
Professional Degree[3]	3	4
Annual Household Income, *Canadian Dollars*, *n*=77		
<$25,000	8	10
$25,000-$50,000	14	18
$50,000-$75,000	18	23
$75,000-$100,000	12	15
>$100,000	25	32
Smoking, *n*=77		
Non-Smoker	61	78
Ex-Smoker	14	18
Current Smoker	2	3
Regular Nutraceutical Use	47	60
Oral Contraceptive Use	8	10

[1] *n*= 78 unless stated otherwise

[2] Percentage of total study population

[3] Medicine, dentistry, veterinary medicine, optometry or law

Table 5.2. Food frequency questionnaire (FFQ) and dietary record intakes and blood biomarker fatty acid composition of FFQ validation study participants

Fatty Acid	Mean (95% CI)	Min	Max	*p* value[1]
FFQ[2], *g/d*				
EPA	0.06 (0.02 - 0.14)	0.01	0.61	0.84
DHA	0.13 (0.04 - 0.39)	0.01	1.00	0.94
EPA + DHA	0.19 (0.06 - 0.61)	0.01	1.47	0.93
Dietary Record[2], *g/d*				
EPA	0.04 (0.02 - 0.08)	0.01	1.09	
DHA	0.10 (0.04 - 0.29)	0.01	2.01	
EPA + DHA	0.16 (0.05 - 0.48)	0.01	2.92	
Blood Biomarker[2]				
EPA, *wt%*	0.61 ± 0.41	0.08	3.22	
DHA, *wt%*	1.95 ± 0.59	0.81	4.47	
EPA + DHA, *wt%*	2.56 ± 0.90	1.15	7.69	
% n-3 HUFA	24.35 ± 5.47	14.45	45.32	

[1] Wilcoxon signed rank test (2-tailed) of difference between FFQ and dietary record means
[2] Means expressed as geometric mean (95% confidence interval).
[3] Blood biomarker (whole blood) values were expressed as the weight percentage of total fatty acids (*wt%*) or the percentage of n-3 highly unsaturated fatty acids in total highly unsaturated fatty acids (*% n-3 HUFA*). Means expressed as mean ± standard deviation.

Table 5.3. Agreement of quartile assignment by the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intakes by food frequency questionnaire (FFQ) and dietary record and n-3 HUFA blood biomarkers[1]

Quartile	Same Quartile (*n*)	Adjacent Quartile (*n*)	Misclassified[2] (*n*)
FFQ vs. Dietary Record			
1 (n=19)	8	6 (6 below)	5 (5 below)
2 (n=20)	8	10 (8 below, 2 above)	2 (2 below)
3 (n=20)	5	9 (5 below, 4 above)	6 (6 above)
4 (n=19)	12	2 (2 above)	5 (5 above)
Total	33 (42%)	27 (35%)	18 (23%)
FFQ vs. Blood Biomarker EPA+DHA,*wt%*[1]			
1 (n=19)	7	5 (5 below)	7 (7 below)
2 (n=20)	8	10 (3 below, 7 above)	2 (2 below)
3 (n=20)	8	9 (6 below, 3 above)	3 (3 above)
4 (n=19)	10	3 (3 above)	6 (6 above)
Total	33 (42%)	27 (35%)	18 (23%)
FFQ vs. Blood Biomarker *% n-3 HUFA in total HUFA*[1]			
1 (n=19)	8	6 (6 below)	5 (5 below)
2 (n=20)	7	11 (5 below, 6 above)	2 (2 below)
3 (n=20)	8	9 (6 below, 3 above)	3 (3 above)
4 (n=19)	10	3 (3 above)	6 (6 above)
Total	33 (42%)	29 (37%)	16 (21%)

[1] Blood biomarkers were expressed as the sum of the weight percentage of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in total fatty acids (EPA+DHA, *wt%*) or the percentage of n-3 highly unsaturated fatty acids in total highly unsaturated fatty acids

[2] Participants not classified into same or adjacent quartile

Table 5.4. Pearson coefficients of correlation between food frequency questionnaire (FFQ), dietary record and blood biomarker fatty acid measurements[1]

	Dietary Record[2]			Blood biomarker fatty acids[3]			
	EPA	DHA	EPA+DHA	EPA, *wt%*	DHA, *wt%*	EPA+DHA, *wt%*	*% n-3 HUFA*
FFQ[2]							
EPA	0.31**	0.48**	0.37**	0.31**	0.48**	0.45**	0.51**
DHA	0.28*	0.49**	0.37**	0.29*	0.42**	0.41**	0.47**
EPA+DHA	0.30**	0.49**	0.38**	0.30**	0.45**	0.43**	0.49**
Dietary record[2]				-	-	-	-
EPA	-	-	-	0.37**	0.25*	0.33**	0.44**
DHA	-	-	-	0.35**	0.21	0.29**	0.42**
EPA+DHA	-	-	-	0.36**	0.24*	0.32**	0.45**

[1] FFQ: food frequency questionnaire, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid, *p<0.05, **p<0.01

[2] Log_e transformations were applied to intake values (expressed as g/d) prior to statistical analyses

[3] Blood biomarker values were expressed as the weight percentage of total fatty acids (*wt%*) or the percentage of n-3 highly unsaturated fatty acids in total highly unsaturated fatty acids

Table 5.5. Method of triads validity coefficients (VC) and VC ranges of food frequency questionnaire (FFQ), dietary record and blood biomarker fatty acid measurements with the true value[1]

	FFQ with True		Dietary Record with True		Biomarker with True	
Fatty Acid	VC	VC Range	VC	VC Range	VC	VC Range
EPA	0.51	0.31-0.51	0.61	0.37-0.61	0.61	0.31-0.61
DHA	1.00[2]	0.42-1.00[2]	0.49	0.21-0.49	0.42	0.42-0.42
EPA+DHA	0.71	0.43-0.71	0.53	0.32-0.53	0.60	0.43-0.60

[1] Validity coefficients (VC): correlation coefficients of the FFQ and dietary record fatty acid intake and blood biomarker weight % of total fatty acids measurements with the estimated true value. True value: estimated by combining two-way Pearson correlation coefficients between FFQ, dietary record and biomarker measurements. FFQ: food frequency questionnaire, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid

[2] Values >1 (Heywood case) were truncated as validity coefficients must fall between 0 and 1

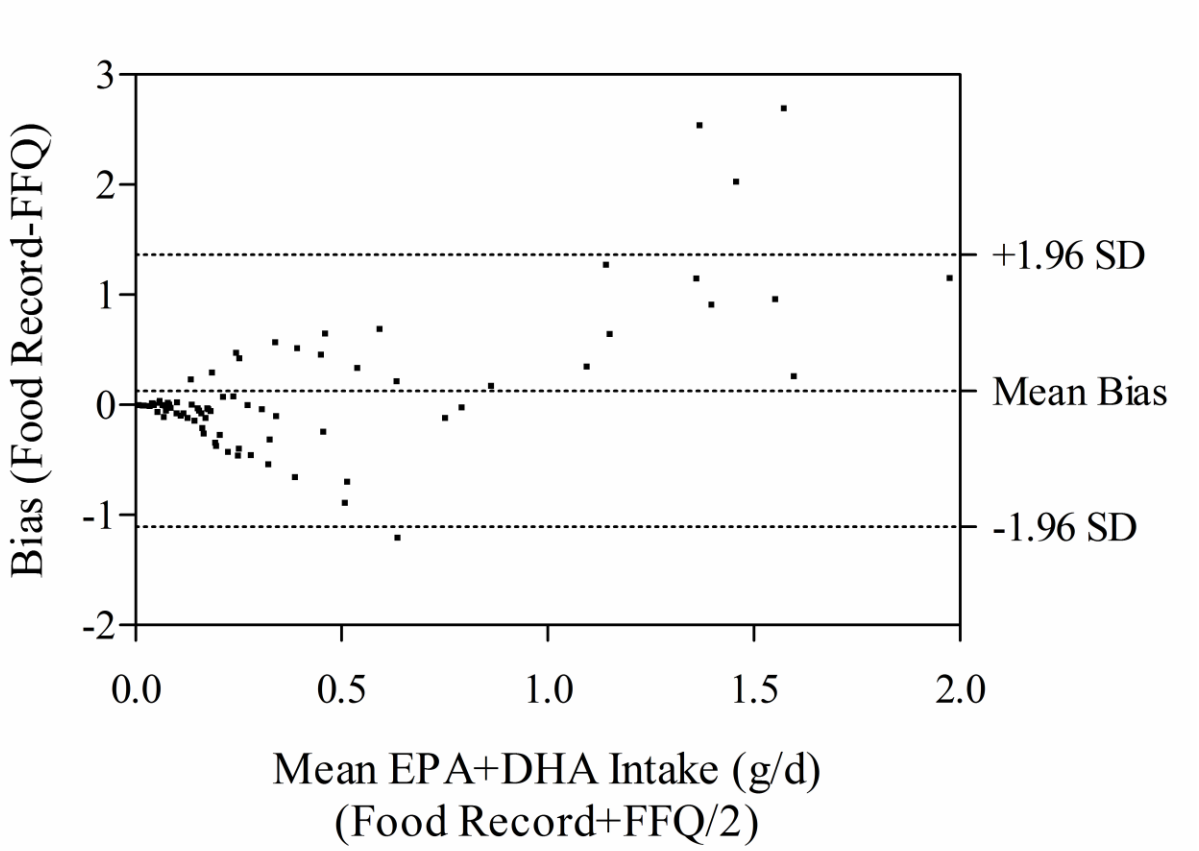

Figure 5.1. Agreement between food frequency questionnaire (FFQ) and 3-d dietary record estimates of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) intake assessed by the Bland-Altman method.

CHAPTER 6

ONE YEAR ADHERENCE TO CONVENTIONAL AND NOVEL DIETARY ADVICE STRATEGIES TO INCREASE INTAKES OF EPA AND DHA

Introduction

The disparity between recommended EPA and DHA intakes and those observed by Canadians could be resolved by increased consumption of seafood, functional food and/or nutraceuticals (Patterson and Stark, 2008). Providing consumers functional foods and/or fish can increase EPA and DHA intake (Lovegrove et al., 1997; Mantzioris et al., 2000; Metcalf et al., 2003; Patch et al., 2005), but greater adherence to dietary recommendations is achieved when food products are provided as compared with a self-selected approach (Metz et al., 1997). Dietary advice to increase intake of oily fish can result in adherence, as assessed by diet history interviews for up to three months (Neale et al., 2012). Long term adherence to seafood, functional food and nutraceutical-based dietary advice in a self-selected manner has not been evaluated through objective dietary assessment tools. The aim of the following study was to assess through dietary survey and blood n-3 HUFA biomarkers, the one-year adherence to Seafood, Functional Food, Nutraceutical and Combined dietary advice strategies to increase EPA and DHA intake to 1g/d by adult men and women.

Methods and Materials

Participants aged 35-50 y were recruited from the Kitchener-Waterloo, Ontario area between January and September 2008. Local newspaper advertisement and flyers posted at local community centres, grocery stores and on the University of Waterloo campus were used to

recruit participants (**Appendix B**). Recruitment of 40 participants was targeted to provide 80% power to detect 1.5 unit differences in erythrocyte % DHA based on 10 subjects per dietary advice group, as determined from variability observed in previous work examining blood fatty acids pre and post supplementation with EPA and DHA ($\alpha = 0.05$) (Metherel, 2007). At a screening visit, individuals provided a finger-tip prick whole blood sample, completed a semi-quantitative FFQ specific for EPA and DHA intake (Patterson et al., 2012) and health screening form (Appendix B), and were given instructions for completing a 3-d dietary record (Appendix B). Individuals consuming nutraceuticals containing EPA and DHA and those with >4% EPA + DHA in total fatty acids from finger-tip prick whole blood samples were excluded from the study. Males and females separately were sequentially assigned to receive Seafood, Functional Food, Nutraceutical and Combined dietary advice in the order in which they were admitted to the study. Neither participants, nor the researchers were blinded to participant allocation and a single researcher recruited, allocated, provided dietary advice, collected samples, completed sample analysis and performed data analysis.

Participants attended a baseline visit approximately two weeks after screening where they were assigned to each of the four dietary advice groups. At this baseline visit, dietary advice was provided as a structured interview and handout (Appendix B) and participants were asked to follow the advice for the subsequent 52 weeks. A 3-d dietary record, anthropometric measurements and a venous blood sample were collected at baseline and at follow-up visits in weeks 4, 8, 12, 24 and 52.

The dietary advice targeted an intake of 1 g/d of EPA + DHA from seafood, functional food, nutraceutical or combined sources. Participants in each of the dietary advice groups received the same information regarding the health benefits associated with EPA and DHA

intake. The Seafood advice group was asked to substitute seafood in place of other meat and meat alternatives in their regular diets. Information on local seafood retailers, safe seafood preparation and cooking techniques, recipes, sample daily menus and recommendations for avoiding frequent intake of fish with high mercury content was provided. Differences in the EPA + DHA content of oily and lean fish were discussed and the amount EPA + DHA of all seafood itemized in the Canadian Nutrient File 2007b (Health Canada, 2007a) was provided to help participants estimate EPA + DHA intake. The Functional Food advice group was asked to substitute EPA and/or DHA-containing functional foods in place of similar foods (e.g., n-3 eggs in place of regular eggs) in their regular diets. Information on how to identify functional foods containing EPA and/or DHA from product labels, recipes, sample daily menus, and the amount of EPA + DHA per serving of available functional foods along with cost comparisons at local retail outlets was provided to the Functional Food advice group. The Nutraceutical advice group was advised to supplement their regular diet with a nutraceutical product. Information on the different types of nutraceutical products available (e.g., capsules, liquid, soft chews, etc), interpretation of product labels, and cost associated with consuming 1 g/d EPA + DHA from various nutraceutical products from local retail outlets was provided. The Combined advice group was given the Seafood, Functional Food and Nutraceutical advice and advised how to simultaneously substitute/supplement their diet with all three sources by providing sample daily menus. Outside of recommendations to increase EPA + DHA intake through substitution/supplementation, all groups were requested to otherwise maintain their usual diet and physical activity regimes. Participants were responsible for purchasing their own food and/or nutraceutical products. At the follow-up visits in weeks 4 through 52, the 3-d dietary records detailing intake of recommended foods/nutraceuticals were reviewed in-person,

challenges associated with following the dietary advice were discussed and continued adherence was encouraged. The week 0 (dietary advice) visit took up to one hour and visits in weeks 4 through 52 took up to 30 minutes, including blood sample and anthropometric measurement collection. Researchers were available to answer any questions from participants between study visits by phone or email.

The anti-coagulant ethylenediamine tetraacetic acid (Sigma-Aldrich, Bellefonte, PA) was added to venous blood samples following collection in preparation for whole blood, plasma and erythrocyte fatty acid determination (Chapter 4). Serum was also collected for blood lipid determinations. For serum collection, whole blood samples were collected into 5 mL serum separating tubes. The whole blood samples clotted for 1 h and were subsequently centrifuged at 2300 rpm for 15 minutes to recover serum. Serum aliquots were stored at -80°C prior to total, HDL and LDL (calculated) cholesterol and triacylglycerol determination by a commercial laboratory (Lifelabs, Toronto, ON, Canada).

Participants were instructed to collect duplicates of all foods, beverages and nutraceuticals consumed on the three days corresponding with the 3-d dietary record returned at the week 4 visit. Returned duplicate collections were then weighed, blended to homogeneity in a 4-Liter blender (Waring Laboratory & Science, Torrington, CT) and stored at –80°C until fatty acid analysis as described in Chapter 4.

Statistical Analyses

Measurements of serum triacylglycerol concentration and HUFA intakes from 3-d dietary records were $\log_e$ transformed to permit parametric analyses, and their values are presented as geometric means with 95% confidence intervals. The linear mixed model procedure was used

for analysis of variance. Following the determination of a significant *F*-value, individual means were examined with Bonferroni's post hoc analyses.

Blood n-3 HUFA status (assessed by the whole blood % n-3 HUFA in total HUFA), adherence to dietary advice (assessed through erythrocyte % DHA) and dietary intake of EPA + DHA from 3-d dietary records, were evaluated as the primary study outcomes. These measurements, along with anthropometric and blood lipid measurements, were compared between advice groups using a three-factor (advice group, sex and time) repeated measures linear mixed model procedure. Measurements of EPA and DHA intake by 3-d dietary records and 3-d food duplicate collections in week 4 were compared by paired t-tests. EPA and DHA intake between diet advice groups at week 4 was examined by the linear mixed models procedure with diet advice as a factor, independently for 3-d dietary records and 3-d food duplicate collections. The association of sex, body mass and dietary fatty acid intake with blood fatty acid measurements were examined by linear regression. Specifically, the association of dietary DHA intake with erythrocyte % DHA, and dietary EPA + DHA intake with whole blood % n-3 HUFA in total HUFA was examined. Regression analyses were performed with dietary DHA and EPA + DHA intake expressed as g/d, % energy, and nutrient residuals (Willett and Stampfer, 1986).

Statistical analyses were performed for participants completing the entire 52-week study, described herein as 'completers' to assess the biological effect of the dietary advice on blood fatty acid levels. The main study outcomes (whole blood % n-3 HUFA in total HUFA, erythrocyte % DHA and dietary intake of EPA + DHA from 3-d dietary records) and anthropometric measurements were also examined according to intention to treat to assess the potential use of the dietary advice in clinical practice. Participants who started, but did not complete the 52-week protocol were included in statistical procedures as described above, using baseline values

for missing data values. The presentation of the following results focuses on the 'completer' analyses, with a summary of the intention to treat analyses afterwards.

Results

Participant Flow and Characteristics

Fifty-nine people responded to advertisements and were screened for study participation. Nine people were not admitted to the study as they did not meet eligibility requirements; eight had blood levels of EPA + DHA above cut-off and/or were consuming a fish oil nutraceutical, and one individual wished to begin a weight-loss diet. Five individuals initially recruited left the study following baseline dietary advice intervention; one became pregnant, one was attempting to become pregnant, one was diagnosed with gallstones, one was unable to attend study visits, and one could no longer be contacted. As blood fatty acid composition would change with pregnancy and gallstone treatment, these two participants were excluded from all analyses herein, including intention to treat. Forty-five individuals participated in the year-long study and were considered 'completers' during analysis and 48 individuals were included in the intention to treat analyses. The four completer dietary advice groups consisted of the following; 11 (6 F, 5 M) were assigned to each of the Seafood, Functional Food and Combined advice groups and 12 (6 F, 6 M) participants were assigned to the Nutraceutical advice group. Individuals not completing the study and included in the intention to treat analysis had been randomized to Seafood advice (1 F, 1 M) and Functional Food advice (1 F). Participants were mainly Caucasian, educated beyond high school, nutraceutical users and not regular smokers (**Table 6.1**).

Anthropometric Measurements and Blood Lipid Measurements

For completers, dietary advice groups were similar in age (baseline: 43.7 ± 4.4 y) and body mass index (baseline: 27.3 ± 5.3 kg/m^2). Overall, men had a higher body mass than women (completers; men: 87.6 ± 17.6 kg, women: 71.7 ± 15.6 kg, effect of sex, $p < 0.01$). The dietary advice groups had similar levels of total cholesterol (baseline: 4.7 ± 0.8 mmol/L) and LDL cholesterol (baseline: 2.8 ± 0.7 mmol/L). Overall, males had higher levels of triacylglycerols [men: 1.4 (1.1 - 1.6) mmol/L, women: 0.8 (0.7 - 0.8) mmol/L] and lower levels of HDL cholesterol (men: 1.2 ± 0.3 mmol/L, women: 1.5 ± 0.3 mmol/L) (effect of sex, $p < 0.01$, for both measurements). Body mass did not change over time. As anticipated for doses of EPA + DHA <2g/d, blood lipid measurements did not change over time (Kris-Etherton, Harris, and Appel, 2003). Study participants reported no adverse events with the dietary advice.

Blood Fatty Acids and Fatty Acid Biomarkers

The % n-3 HUFA in total HUFA in whole blood was 24.0 ± 4.8% at baseline. With the exception of women in the Seafood and Functional Food advice groups, the % n-3 HUFA in total HUFA in whole blood increased at various time points after dietary advice for men and women in each advice group (effect of advice group x time x sex interaction, $p < 0.01$) (**Figure 6.1**, meaningful comparisons shown). Specifically, the % n-3 HUFA biomarker increased from baseline for men in the Seafood advice group in weeks 4 through 52. For men in the Functional Food advice group, the biomarker increased in week 4 from baseline. In the Nutraceutical advice group, the biomarker increased from baseline for men in weeks 8 through 52 and for women in weeks 4 through 52. The % n-3 HUFA biomarker increased in weeks 4, 12, 24 and 52 for the men in the Combined advice group from baseline. Women in the Combined advice group

had increased % n-3 HUFA biomarker at all time points after baseline. The whole blood % n-3 HUFA in total HUFA was also higher for women in the Nutraceutical advice group at weeks 24 and 52 as compared with week 8 for men in the Functional Food advice group. Increases in the whole blood % n-3 HUFA in total HUFA post-intervention were generally accompanied by an increase in the % total HUFA (effect of advice group x time interaction, $p < 0.04$), with no change in the % n-6 HUFA.

Overall, the % DHA in erythrocytes increased from baseline (4.0 ± 1.0 %) in weeks 4 and 8, reached a maximum in week 12 (4.9 ± 0.8 %), and subsequently returned to baseline levels in weeks 24 and 52 (effect of time, $p < 0.01$) (**Figure 6.2**). For all time points combined, the % of DHA in erythrocytes was higher with Seafood advice (4.7 ± 1.0 %) as compared with Nutraceutical (4.0 ± 0.9 %) and Functional Food (4.0 ± 0.8 %) advice (effect of advice group, $p < 0.01$). The Seafood advice group appeared to have a higher % DHA in erythrocytes at baseline however no significant time x advice group interaction was observed ($p = 0.30$).

In contrast with the % of DHA in erythrocytes, the % of DHA in whole blood and plasma and the % of EPA in all three blood fractions was higher in weeks 4 through 52 as compared with the baseline measurement (**Figure 6.2**). This observation, coupled with the relatively slow incorporation and washout of DHA into/from the inner erythrocyte membrane, confirms that erythrocyte DHA is an appropriate marker of sustained adherence to dietary advice over the 52 week study.

Dietary Intake of EPA + DHA

At baseline, EPA + DHA intake was 0.12 (0.07 - 0.21) g/d. Intake of EPA + DHA increased at each post-intervention time point in the Seafood, Nutraceutical and Combined

advice groups from the advice group's own baseline measurement (effect of advice group x time interaction, $p = 0.03$, meaningful comparisons shown) (**Figure 6.3**). For the Functional Food group, EPA + DHA intake did not increase significantly post-intervention. No significant differences in EPA + DHA intake were observed between advice groups at each time point.

Food Duplicate Collections

Measurement of EPA intake in the Seafood advice group was higher when assessed by 3-d dietary record as compared with food duplicates in week 4 ($p = 0.04$) with no other differences between estimates of EPA and/or DHA by 3-d dietary record and food duplicate collections (**Table 6.2**). Unlike dietary records, no differences in food duplicate EPA composition (g/d) were observed between the Seafood and Functional Food advice groups.

Relationship of body mass index, sex and dietary n-3 HUFA intake with blood n-3 HUFA

Body mass index was inversely associated and being female was positively associated with the % of DHA in erythrocytes and the % n-3 HUFA in total HUFA in whole blood (**Table 6.3**). Dietary EPA + DHA intake was also positively associated with the % n-3 HUFA in total HUFA in whole blood (**Table 6.3**). Regression results were similar with DHA and EPA + DHA intakes expressed as % energy and nutrient residuals and absolute body mass was also inversely associated with erythrocyte % DHA (data not shown).

Intention to Treat Analyses

Overall, findings by intention to treat analyses were similar to those by completer analyses. Participant characteristics (**Table 6.1**) and anthropometric measurements were similar

following completer and intention to treat analyses. Also similar to completers, the % n-3 HUFA in total HUFA in whole blood increased from baseline for Seafood advice group men in weeks 4 through 52 while no changes from baseline were observed for Seafood advice group women by intention to treat (effect of advice group x time x sex interaction, $p = 0.01$). For women in the Functional Food advice group, the % n-3 HUFA in total HUFA in whole blood did not increase from baseline, similar to completers. The pattern of a higher % of DHA in erythrocytes in weeks 4 through 12 relative to baseline was also observed by intention to treat (effect of time, $p < 0.01$). Intake of EPA + DHA was unaltered by intention to treat; intake of EPA + DHA increased from baseline in the Seafood, Nutraceutical and Functional Food advice groups, but not in the Functional Food advice group (effect of advice group x time interaction, $p = 0.04$). Results of regression analyses were also unaltered following intention to treat.

Discussion

An examination of changes in the % of DHA in erythrocytes suggest that adherence to dietary advice was maintained during the initial 12 weeks in the study but not sustained in weeks 24 and 52. In contrast, changes in the % of DHA in plasma and whole blood, and the % of EPA in erythrocytes, plasma and whole blood suggest continued adherence after week 12, as did the % n-3 HUFA in total HUFA in whole blood and intake estimates by 3-d dietary records. Based on the % n-3 HUFA in total HUFA in whole blood, n-3 HUFA status increases for men and women following Nutraceutical and Combined advice and for men following Seafood and Functional Food advice. Dietary estimates from 3-d dietary records indicate that intake of EPA + DHA significantly increases following Seafood, Nutraceutical and Combined dietary advice strategies.

A strength of the present investigation is the combination of multiple assessment tools to characterize EPA + DHA intake and blood status over the 52-week period. The return of the % of DHA in erythrocytes to baseline levels after week 12 for the study population overall indicates poor adherence when participants were not in contact with the investigators. In contrast, the % n-3 HUFA in total HUFA in whole blood increased with dietary advice in general and remained increased above baseline throughout the study, indicating good adherence. DHA has been demonstrated to incorporate into and plateau in erythrocytes within 4 weeks (Barcelo-Coblijn et al., 2008; Katan et al., 1997) and washout can take >6 weeks with DHA supplementation of > 1g/d (Brown, Pang, and Roberts, 1991). In contrast, with supplementation of 3.2 g/d EPA + 1.6 g/d DHA for one month, the % n-3 HUFA in total HUFA in whole blood rapidly increases and decreases within 1 week of initiation and cessation, respectively (Metherel et al., 2009). By considering both the changes in the % of DHA in erythrocytes and the % n-3 HUFA in total HUFA in whole blood, it appears that adherence in the present study was inconsistent through weeks 24 and 52. A pattern of poor adherence after the week 12 visit, followed by "compensatory" EPA + DHA intake shortly before the weeks 24 and 52 visits would potentially result in % n-3 HUFA in total HUFA in whole blood values above baseline and relatively low % of DHA in erythrocytes.

DHA is preferentially incorporated into phosphatidyl ethanolamine of the inner leaflet of lipid membrane bilayers while EPA incorporates into phosphatidyl choline of the outer leaflet (Lemaitre-Delaunay et al., 1999). Fatty acid remodelling of inner cell membranes is relatively slow (Seigneuret and Devaux, 1984), therefore EPA can increase and decrease rapidly in erythrocytes, while DHA turnover is relatively slow (Metherel et al., 2009). In contrast, plasma EPA and DHA respond relatively rapidly to dietary intake changes (Metherel et al., 2009).

While plasma phospholipids are predominantly phosphatidyl choline, plasma also contains triacylglycerols and cholesteryl esters that can incorporate fatty acids. In addition, plasma fatty acid composition is largely influenced by the liver, the primary site of lipid and fatty acid metabolism, whereas remodelling of erythrocyte fatty acids would occur in the circulation.

Biomarkers of n-3 HUFA, like the % n-3 HUFA in total HUFA, appear to be appropriate for characterizing short term changes in EPA and DHA intake and describing the n-3 HUFA status of those with stable EPA and DHA intakes. DHA in erythrocytes reflects n-3 HUFA intake over longer periods and appears to characterize adherence to increases in n-3 HUFA intake well. The use of erythrocytes also has limitations as there are additional analytical and storage considerations, such as hemolysis and iron-mediated HUFA oxidation following erythrocyte storage at -20°C but not -80°C (Metherel, 2012).

Adherence to dietary advice up to, but not beyond the initial 12 weeks of the study may be related to follow-up frequency and/or participant fatigue. Study visits occurred monthly from baseline to week 12, and subsequently took place 3 months later at week 24 and then 6 months later at week 52. A similar pattern of adherence up to 12 weeks, but not 52 weeks, was observed in a study examining adherence to dietary advice to consume fatty fish in a self-selected manner (Neale et al., 2012). In this fish advice study, dietary counselling was provided at the same time points as the present study, plus an additional visit at week 36, and adherence was measured by diet history. Taken together, this fish-based advice study and the present show that dietary advice for consumers to increase intake of EPA and DHA sources can be effective and suggest that regular follow-up and motivation may be needed to maintain long-term adherence.

Sex and advice group differences were found by examining the % n-3 HUFA in total HUFA in whole blood and not the % of DHA in erythrocytes. This may be an indication that the

% of DHA in erythrocytes may not be sensitive enough to detect subtle differences that affect EPA and DHA status in other tissues. Tissue differences in n-3 HUFA levels are well documented (Stark et al., 2005). Increases in % n-3 HUFA in total HUFA in whole blood of men but not women after receiving Seafood advice could be interpreted as men achieving higher intakes of EPA + DHA following Seafood advice or that the men engaged in greater "compensation" fish consumption as compared with women. As adult men typically consume larger portions of meat and meat alternatives as compared with women, it is plausible that their intake of EPA + DHA from seafood substitution could be greater (Garriguet, 2007), and compensation intakes of EPA + DHA would be higher. In contrast, men and women could both increase intakes of nutraceuticals prior to study visits without the need for dietary change. This may account for sustained increases in the % n-3 HUFA in total HUFA in whole blood of men and women after Nutraceutical and Combined advice. Dietary assessments performed by both sexes can be subject to response bias as women are influenced by social desirability, a tendency to avoid criticism, and men are influenced by social approval, a tendency to seek praise (Hebert et al., 1997). Knowing blood samples would be measured for n-3 HUFA status at each study visit could have lead to compensatory adherence immediately prior to study visits.

The amount of EPA + DHA per serving of functional food products likely contributes to lower n-3 HUFA intake and blood status measurements following Functional Food advice. As a prelude to the present study, fish, nutraceuticals or functional foods were substituted into theoretical typical Canadian diets to examine the feasibility of these dietary strategies to provide dietary EPA and DHA (Patterson and Stark, 2008). While functional food substitution increased the EPA + DHA content of the theoretical typical Canadian diet, levels attained were below those achieved following nutraceutical or seafood substitution. At the time of the present study,

functional foods available to consumers provided between 0.01 and 0.25 g EPA + DHA per serving and several functional foods needed to be combined and consumed daily to achieve EPA + DHA intake recommendations provided as part of the dietary advice. The amount of EPA + DHA provided by seafood and nutraceuticals also varies from source to source but can supply more than functional foods. Oily fish like farmed Atlantic salmon supplies 2.2 g of EPA + DHA per 100g portion (Health Canada, 2007a) and accordingly, oily fish would not need to be consumed daily to achieve 1 g/d EPA + DHA. Fish oil capsules supplying 0.5 g EPA + DHA are readily available to consumers and a small dose of two capsules per day could be used to achieve study EPA + DHA intake targets for participants following Nutraceutical advice. Functional food advice may be appropriate for those finding fish and nutraceutical intakes unacceptable, or targeting the low end of EPA + DHA intake recommendations. Functional foods could be also included as part of a combined approach.

Unlike nutraceuticals, functional foods supply a variety of nutrients. For example, eggs are a low cost source of protein (Drewnowski, 2010) and lutein and zeaxanthin, which have benefits for macular health, (Vishwanathan et al., 2009), but also supply dietary cholesterol which may increase circulating LDL cholesterol levels for individuals with a specific genotype (Herron et al., 2006). The purpose of the Functional Food advice strategy was to observe, through a substitution approach with existing consumer products, whether EPA and DHA intake could increase following advice based on functional food products. Whether the nutritional advantages of a functional food supplying EPA and DHA outweigh any potential disadvantages may vary at the individual level. In the future, advances in nutrigenomics testing could help individuals identify whether a specific functional food product supplying EPA and DHA is

appropriate for them (Nielsen and El-Sohemy, 2012) however, the focus herein was long-term adherence to EPA and DHA intake from different conventional and novel dietary sources.

Agreement between dietary survey intake measurements and fatty acid analyses of food duplicate collections, with the exception of EPA intakes by the Seafood advice group, indicate that differences in the adherence pattern between 3-d dietary records and the % of DHA in erythrocytes for weeks 24 and 52 are likely attributable to self-report. Dietary intervention can impact diet self-report as a result of a desire to give socially desirable responses and seek approval (Kristal et al., 1998). As a result, dietary intakes of EPA and DHA measured by the 3-d dietary records herein give an indication of EPA + DHA intakes achievable following each of the dietary strategies, but are not an optimal measure of long-term adherence. The estimated intakes of EPA by the Seafood advice group in weeks 4 through 52 may be an overestimation if the discrepancy between 3-d dietary records and duplicate food collections is due to the food composition database rather than participant food collection/recording error.

The n-3 HUFA intakes and adherence to dietary advice over time attained in the present study may be greater than those achievable by the general population. For ethical reasons, study recruitment materials advertised the study as an omega-3 fatty acid intervention and may have attracted individuals with an interest in consuming these fatty acids. However, once recruited for the study, all participants received the same information regarding health benefits of EPA and DHA and advice was consistently provided by the same investigator. Major study conclusions were the same following intention to treat and completer analysis however; loss to follow-up in both Seafood and Functional Food advice groups could indicate difficulty adhering to dietary strategies involving food substitution instead of nutraceutical addition. For interpretation of blood n-3 HUFA biomarkers, the assumption was made that EPA and DHA in the various

dietary sources would be equally bioavailable. Some of the nutraceutical and functional food products selected by participants supplied EPA and DHA in ethyl ester form and may (Harris et al., 2007; Krokan, Bjerve, and Mork, 1993; Nordoy et al., 1991) or may not (Dyerberg et al., 2010; Neubronner et al., 2010) be equally as bioavailable. This study examined adherence to dietary advice in a population with no prior history of cardiovascular disease and therefore cannot be generalized to those with a history of cardiovascular disease following EPA + DHA intake recommendations for secondary coronary heart disease prevention. A larger study population would have increased the power to detect differences in blood and dietary n-3 HUFA measurements between advice groups and sexes.

Conclusion

The % of DHA in erythrocytes captures long term adherence to n-3 HUFA intakes and could be an appropriate marker of adherence in long term trials. Short term blood biomarkers of n-3 HUFA status can increase for men following dietary advice to consume EPA + DHA from seafood, functional food or nutraceutical sources alone or in combination while n-3 HUFA biomarkers can increase for women following Nutraceutical or Combined advice. Any of these strategies could be used to increase intakes of EPA + DHA to those currently recommended for healthy individuals (0.25 - 0.5 g/d) while functional foods should be consumed in combination with other sources to achieve higher intakes of EPA + DHA (≥0.5 g/d). Strategies to improve adherence to such dietary advice beyond 12 weeks are needed and the effect of increased follow-up frequency over the long term warrants investigation. Sex differences in blood n-3 HUFA biomarker responses also indicates that advice tailored to the traits of individuals should be explored.

Table 6.1. Characteristics of dietary advice study participants.

	All Diets, n=45 (48)		Seafood, n=11 (13)		Functional, n=11 (12)		Nutraceutical, n=12		Combined, n=11	
	n	*%*[1]	*n*	*%*[1]	*n*	*%*[1]	*n*	*%*[1]	*n*	*%*[1]
Sex										
Female	24 (26)	53 (54)	6 (7)	55 (54)	6 (7)	55 (58)	6	50	6	55
Male	21 (22)	47 (46)	5 (6)	46 (46)	5 (5)	45 (42)	6	50	5	45
Cultural/Racial Background										
Caucasian	39 (40)	87 (83)	10 (11)	91 (85)	10 (10)	91 (83)	10	83	9	82
Chinese	3 (3)	7 (6)	0 (0)	0 (0)	1 (1)	2 (2)	1	8	1	9
Latin American	2 (3)	4 (6)	1 (1)	9 (8)	0 (1)	0 (2)	1	8	0	0
West Asian	0 (1)	0 (2)	0 (1)	0 (8)	0 (0)	0 (0)	0	0	0	0
Mixed	1 (1)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0	0	1	9
Education										
Some High School	1 (1)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0	0	1	9
High School or Current Student	3 (4)	7 (8)	1 (2)	9 (15)	0 (0)	0 (0)	2	17	0	0
Trade, Technical/Vocational School or Business College	4 (4)	9 (8)	0 (0)	0 (0)	2 (2)	18 (17)	1	8	1	9
Community College, CEGEP or Nursing School	7 (7)	16 (15)	3 (3)	27 (23)	1 (1)	9 (8)	2	17	1	9
Bachelor's Undergraduate Degree or Teacher's College	20 (20)	44 (42)	4 (4)	36 (31)	7 (7)	64 (58)	4	33	5	45
Master's Degree	8 (9)	18 (19)	3 (4)	27 (31)	1 (1)	9 (8)	1	8	3	27
Earned Doctorate	1 (1)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1	8	0	0
Professional Degree[2]	1 (2)	2 (4)	0 (0)	0 (0)	0 (1)	0 (8)	1	8	0	0
Annual Household Income, *Canadian Dollars*										
<$25,000	3 (3)	7 (6)	0 (0)	0 (0)	1 (1)	9 (8)	0	0	2	18
$25,000-$50,000	6 (7)	13 (15)	1 (2)	9 (15)	3 (3)	27 (25)	1	8	1	9
$50,000-$75,000	15 (16)	33 (33)	6 (6)	55 (46)	1 (2)	9 (17)	3	25	5	45
$75,000-$100,000	4 (5)	9 (10)	0 (1)	0 (8)	2 (2)	18 (17)	2	17	0	0
>$100,000	16 (16)	36 (33)	4 (4)	36 (31)	4 (4)	36 (33)	5	42	3	27
undisclosed	1 (1)	2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	1	8	0	0
Smoking										
Non-Smoker	30 (33)	67 (69)	6 (8)	55 (62)	8 (9)	73 (75)	8	67	8	73
Ex-Smoker	13 (13)	29 (27)	4 (4)	36 (31)	2 (2)	18 (17)	4	33	3	27
Current Smoker	2 (2)	4 (4)	1 (1)	9 (8)	1 (1)	9 (8)	0	0	0	0
Regular Nutraceutical Use	31 (33)	69 (69)	7 (8)	64 (62)	8 (9)	73 (75)	8	67	8	73
Oral Contraceptive Use	2 (2)	4 (4)	1 (1)	9 (8)	1 (1)	9 (8)	0	0	0	0

Values outside brackets represent completers and (values inside brackets represent intention to treat participants).
[1] Percentage of dietary advice group population. [2] Medicine, dentistry, veterinary medicine, optometry or law.

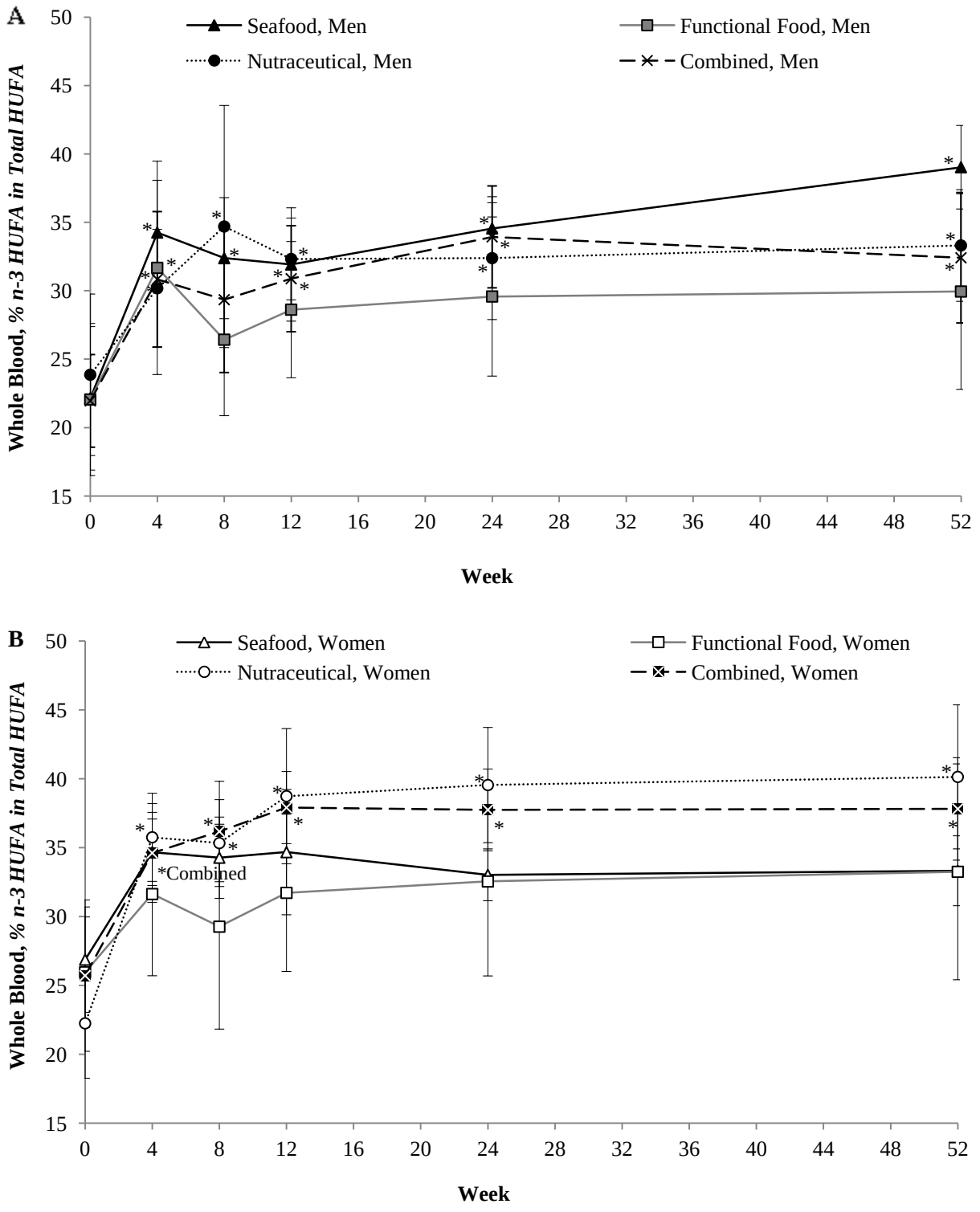

Figure 6.1. Whole blood % n-3 HUFA in total HUFA by men (A) and women (B) completers in each advice group over time. (*) represents a significant difference from baseline within each advice group (by sex) by Bonferroni's post-hoc test following a significant F-value by the linear mixed model procedure. Values are means ± standard deviation. ($p < 0.05$, n = 45)

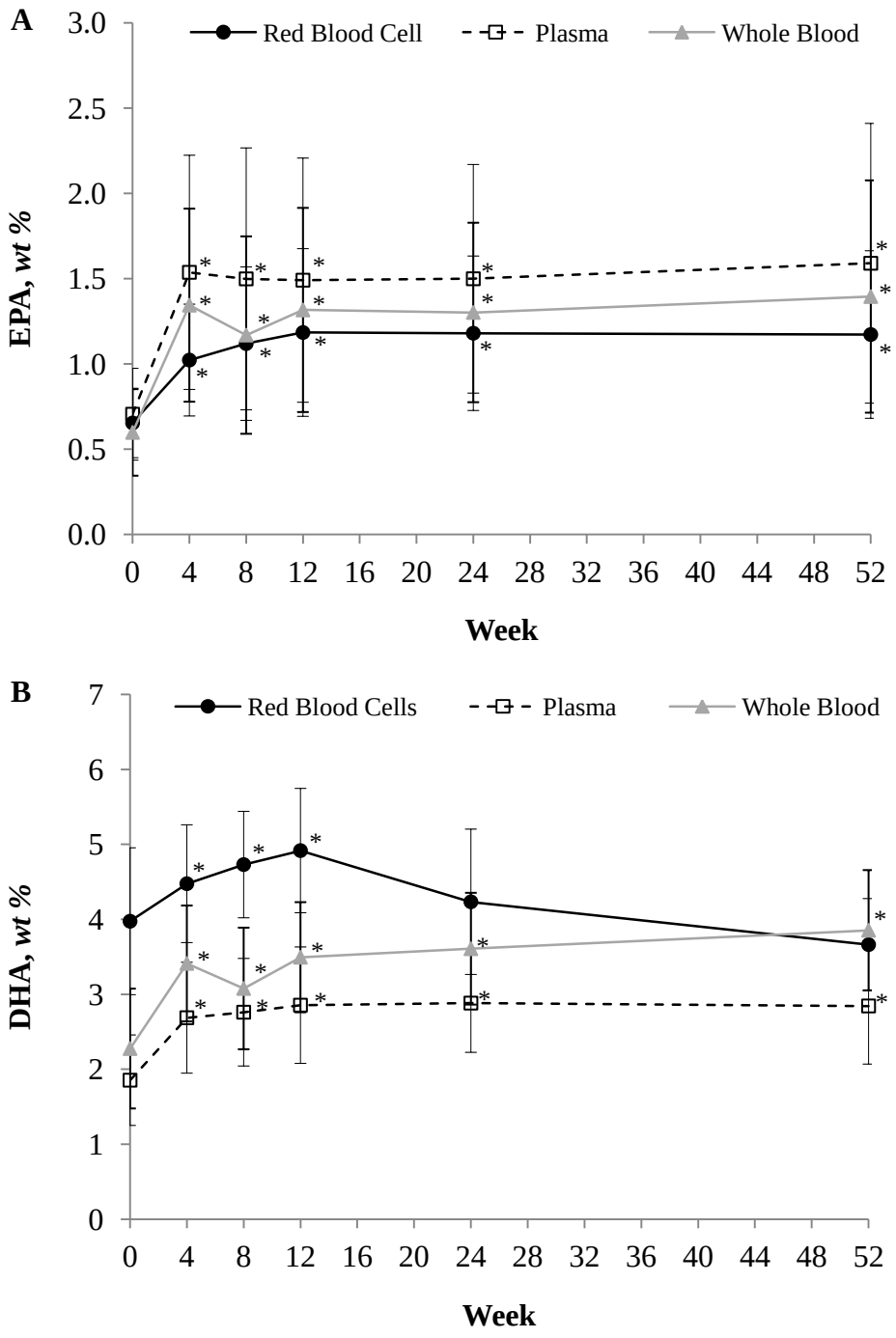

Figure 6.2. Percent DHA (A) and EPA (B) over time in erythrocytes, plasma and whole blood by completers. (*) represents a significant difference from the week 0 measurement within each blood fraction over time by Bonferroni's post-hoc test following a significant F-value by the linear mixed model procedure. ($p < 0.05$, n=45)

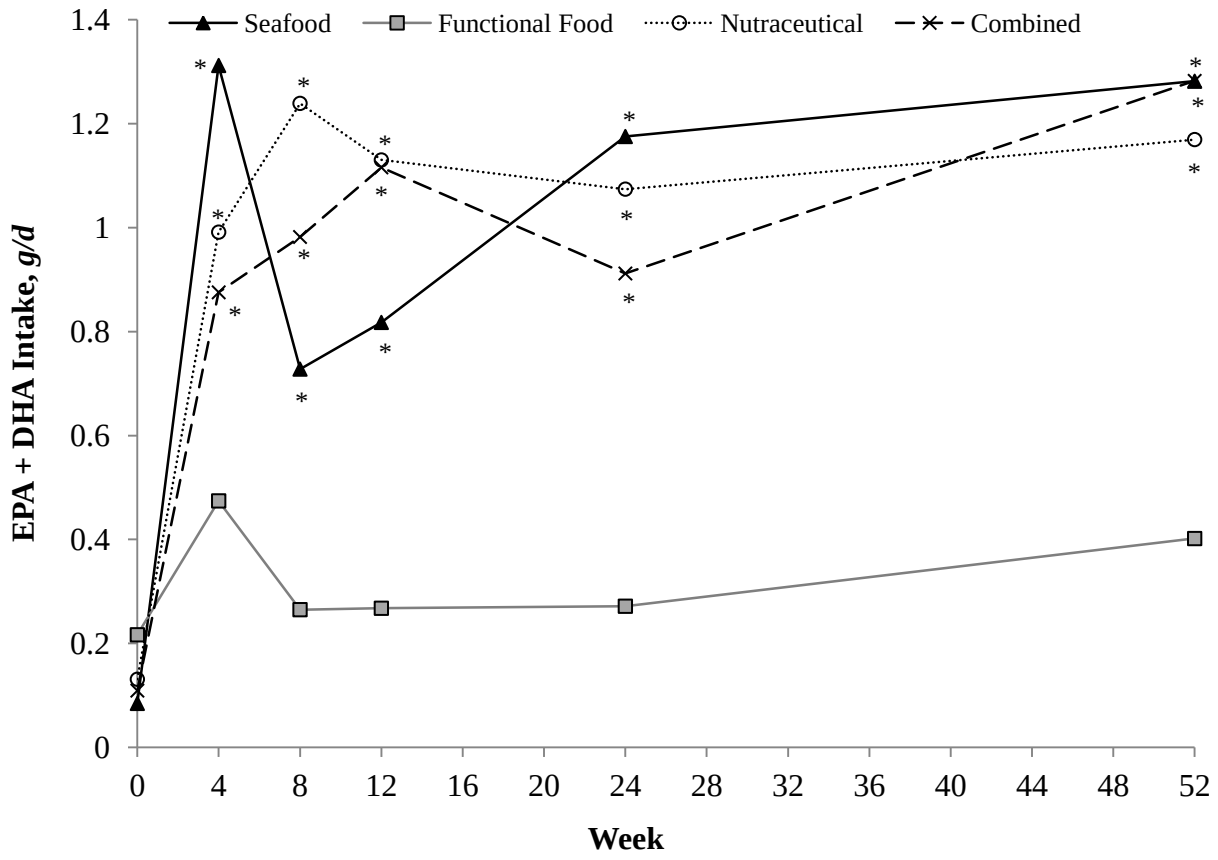

Figure 6.3. Intake, by completers, of the sum of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from 3-d dietary records in Seafood, Functional Food, Nutraceutical and Combined advice groups. Values are geometric means. (*) represents a significant difference from the week 0 measurement in each dietary advice group by Bonferroni's post-hoc test following a significant F-value by repeated measures linear mixed model procedure. ($p < 0.05$, n=45).

Table 6.2. EPA and DHA estimates by 3-d dietary records and 3-d food duplicate collections by completers.

	3-d Dietary Records		3-d Food Duplicates	
	Mean	95% CI	Mean	95% CI
EPA[1], *g/d*				
All, *n=45*	0.37	(0.27 - 0.51)	0.30	(0.21 - 0.41)
Seafood [2]	0.48 [a]	(0.30 - 0.75)	0.31 [a,b]	(0.22 - 0.44)
Functional Food	0.15 [b]	(0.05 - 0.48)	0.12 [b]	(0.05 - 0.29)
Nutraceutical	0.60 [a]	(0.51 - 0.71)	0.62 [a]	(0.32 - 1.19)
Combined	0.40 [a,b]	(0.29 - 0.55)	0.32 [a,b]	(0.20 - 0.53)
DHA[1], *g/d*				
All, *n=45*	0.46	(0.35 - 0.59)	0.38	(0.30 - 0.49)
Seafood	0.82 [a]	(0.53 - 1.27)	0.57 [a]	(0.39 - 0.82)
Functional Food	0.30 [b]	(0.12 - 0.79)	0.22 [b]	(0.11 - 0.44)
Nutraceutical	0.39 [a,b]	(0.32 - 0.47)	0.46 [a,b]	(0.28 - 0.76)
Combined	0.44 [a,b]	(0.29 - 0.65)	0.37 [a,b]	(0.25 - 0.55)

[1] Values are geometric means and were $\log_e$ transformed for statistical analyses.
[2] Significant difference between dietary record and food duplicate measurements within row by paired t-test ($p < 0.05$).
Different letters represent differences between advice groups for a fatty acid measurement within a column by the linear mixed models procedure followed by Bonferroni's post-hoc test ($p < 0.05$).

Table 6.3. Results of linear regression determining the association of sex, body mass index and n-3 HUFA intake on blood biomarkers for completers (n=45)

	Erythrocyte DHA wt% of total fatty acids			Whole Blood % n-3 HUFA in total HUFA		
	B	*p*	95% CI	B	*p*	95% CI
Sex	**0.26**	**0.02**	**0.04 - 0.48**	**3.51**	**<0.01**	**2.10 - 4.92**
Body Mass Index, *kg/m*^{2}	**-0.05**	**<0.01**	**-0.07 - -0.03**	**-0.19**	**<0.01**	**-0.33 - -0.05**
DHA / EPA + DHA Intake[1], *g/d*	0.06	0.19	-0.03 - 0.14	**1.90**	**<0.01**	**1.42 - 2.38**

[1]DHA intake evaluated for association with erythrocyte DHA; EPA + DHA intake evaluated for association with whole blood % n-3 HUFA in total HUFA. DHA and EPA + DHA intakes were $\log_e$ transformed prior to regression analyses.

CHAPTER 7

BLOOD BIOMARKER RESPONSES TO LOW DOSES OF EPA AND DHA IN MEN AND WOMEN

Introduction

Data linking diet and blood n-3 HUFA levels is mainly limited to interventions with EPA + DHA doses of 1g/d or higher (Blonk et al., 1990; DiStasi D. et al., 2004; Katan et al., 1997) and background dietary intake of EPA and DHA is not estimated. In addition, at low levels of EPA + DHA intake typical of Western diets, women have higher blood levels of DHA as compared with men (Bakewell, Burdge, and Calder, 2006; Stark, 2008a), but this sex difference disappears with high dose fish oil supplementation (Metherel et al., 2009). Characterization of the blood dose-response relationship following low intakes of EPA + DHA in men and women is necessary to enable a mechanistic link between dietary EPA and DHA intake and proposed EPA + DHA blood level targets for coronary heart disease protection (Armstrong, Metherel, and Stark, 2008; Harris and Von Schacky, 2004; Stark, 2008a). Clearly defining the diet-blood relationship will also be informative for establishing dietary recommendations for EPA + DHA. The aim of the present study is to examine whole blood, plasma phospholipid and red blood cell n-3 HUFA levels in men and women in response to intakes of 0.25 and 0.5 g/d EPA + DHA. The dose-response to 1.0 g/d EPA + DHA will also be examined in a subset of participants.

Methods and Materials

Participants aged 18-35 y were recruited from October 7-21, 2011 from the University of Waterloo community through flyers and emails (**Appendix C**). Recruitment of 20 participants

(50/50% males/females) was initially targeted. This provides 80% power to detect a 0.8 unit difference in % DHA in erythrocytes measurements between men and women, as determined from variability of observed in previous work in a similar population ($\alpha = 0.05$) (Metherel et al., 2009). At a screening visit, individuals provided a finger-tip prick whole blood sample and completed a semi-quantitative FFQ specific for EPA and DHA intake (Patterson et al., 2012) and health screening form (Appendix C). Individuals consuming nutraceuticals containing EPA and DHA and those with >3% EPA + DHA in total fatty acids as determined by finger-tip prick fatty acid profiling were excluded from the study.

At baseline, anthropometric measurements and a fasting venous blood sample were collected. Participants were instructed to not consume any seafood, EPA/DHA-enriched functional foods or EPA/DHA-containing nutraceuticals other than those provided, and to otherwise maintain their usual diet and physical activity regimes during the study period. Verbal and written instructions were provided to complete a 3-d dietary record to measure background caloric and macronutrient intake during the study (Appendix C). Dietary records were returned within two weeks. Fish body oil, supplying EPA and DHA largely in triacylglycerol form, was provided in 1g capsules (Webber Naturals Pharmaceuticals, Coquitlam, BC; NPN# 80002435). The ratio of EPA to DHA in the fish oil was 1.8:1 and the fatty acid composition of the fish oil capsules, assessed by gas chromatography, is presented in Appendix C. Although industrial deodorization fish oil can generate trans isomers of fatty acid, their concentrations are typically low (Sciotto and Mjos, 2012). The present gas chromatography protocol was not able to resolve all trans isomers. Participants were instructed to consume one capsule per day (providing 0.24 g/d EPA + DHA) in weeks 1 through 4. More fish oil capsules were provided at week 4 and participants were instructed to take two capsules per day (providing 0.48 g/d EPA + DHA) in

weeks 5 through 8. A convenience subset of participants (n=5; 1 F, 4 M) continued the study in weeks 9 through 12 and consumed four capsules per day (providing 0.95 g/d EPA + DHA). Adherence to the supplementation protocol was verified with finger-tip prick blood samples analyzed for fatty acid composition weekly from baseline through week 8 and again in week 12. Additional strategies were discussed to ensure adherence with the study protocol such as routinely taking capsules at the same time every day and setting a daily electronic reminder. Capsules were provided in excess and counts were performed weekly from baseline through week 12 as an additional measure of adherence.

Venous blood samples were collected in tubes spray-coated in lithium heparin (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) at baseline, weeks 4 and 8 (n=20), and at week 12 (n=5) for whole blood, plasma phospholipid and erythrocyte fatty acid determinations. A 22:3n-3 ethyl ester internal standard (NuCheck Prep, Elysian, MN) was used for all venous blood fatty acid analyses. Individual fatty acids were expressed as a percentage of total fatty acids and as a concentration (fatty acid mass per mass of sample). The baseline FFQ and anthropometric measurements were repeated at week 8. Participants were instructed to consider their intake during the study period only for the week 8 FFQ.

Plasma phospholipid lipid classes were isolated using thin layer chromatography. Plasma total lipids were extracted in the presence of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), triheptadecanoin (Nu-Check Prep, Elysian, MN) and cholesteryl heptadecanoate (Nu-Check Prep, Elysian, MN) as internal standards. Total lipids were applied to silica gel plates (250 µm thickness, 60 Å pore size, Whatman, Maidstone, England), which had been pre-washed in 4:1 chloroform:methanol and oven dried, and developed in heptane/diethyl ether/acetic acid (60:40:2, v/v/v; Fisher Scientific,

Ottawa, ON). The plates were then treated with a 0.1% solution of dichlorofluorescein (Sigma, St. Louis, MO) and examined under ultraviolet light to visualize and the isolated phospholipid fraction was collected, in the presence of the anti-oxidant butylated hydroxytoluene, for transesterification and fatty acid analysis.

Participants were not blinded to the intervention. A single researcher who was not blinded performed recruitment, sample collection, partial sample analyses and all data analysis. Additional researchers performed fatty acid analyses of the blood samples that were identified by a numeric code.

Statistical Analyses

Entry characteristics, including baseline anthropometric and dietary intakes were compared between males and females using independent *t* tests. Sex differences were not examined at week 12 due to the small sample (n=5). Baseline and week 8 anthropometric and n-3 HUFA intake measurements were compared by paired *t* tests. The linear mixed models procedure for repeated measures was used to examine the effect of EPA + DHA dose, sex and their interaction as fixed factors on blood fatty acid measurements in plasma phospholipids, erythrocytes and whole blood in weeks 0 through 8 (n=20). This procedure was also conducted on the subgroup (n=5) for weeks 0 through 12 with EPA + DHA dose alone included as a fixed factor in the model. Blood n-3 HUFA biomarker measurements were compared between blood fractions at baseline and following the 0.25 and 0.5 g/d EPA + DHA doses by the linear mixed models procedure. Following the determination of a significant *F*-value by the linear mixed models procedure, individual means were examined with Bonferroni's post hoc analyses. Pearson correlation coefficients were calculated between EPA + DHA intake measurements and

blood n-3 HUFA biomarkers. The association of sex, body mass index and dietary fatty acid intake with blood fatty acid measurements were examined by linear regression. Specifically, the association of dietary DHA intake with the % DHA in erythrocytes and dietary EPA + DHA intake with % n-3 HUFA in total HUFA in whole blood was examined (n=20).

Results

Participant Flow, Characteristics and Anthropometric Measurements

A total of 35 (17 M, 18 M) individuals were screened and 21 (11 M, 10 F) individuals met the eligibility criteria and participated in the 8-week study. Of those who were screened but not admitted to the study; 11 had blood levels of EPA + DHA above the cut-off and/or were consuming fish oil nutraceuticals, one had anemia, one would not be able to attend weekly study visits, and one could not consume the gelatin fish oil capsules for religious reasons. During the study period, one female participant increased fish consumption, as determined by high blood EPA + DHA and self report, despite explicit instructions to avoid seafood. Given the primary purpose of the present study on the metabolic relationship between diet intake and blood levels of EPA + DHA, this individual was excluded from data analyses. A total of 20 (11 M, 9 F) participants were included in the final results.

The final study population consisted of adult men and women, 23.3 ± 4.0 years of age. At baseline, men had a higher body mass (men: 83.4 ± 15.4 kg, women: 64.2 ± 4.4 kg) than women ($p < 0.01$). By paired *t* test, body mass did not change between baseline and week 8 for men ($p = 0.23$) or women ($p = 0.20$). Body mass index was similar for men and women (baseline; men: 25.9 ± 3.8 kg/m^2, women: 23.4 ± 2.6 kg/m^2) ($p = 0.11$). One female participant reported loose stools periodically in weeks 1 through 8 of supplementation. No adverse events

were otherwise reported.

Dietary Intake

Background energy and macronutrient intakes were determined by 3-d dietary record at baseline. Energy intake was higher by men (men: 2699 ± 754 kcal/d, women: 1862 ± 412 kcal/d, $p = 0.01$) but the percentage of energy from protein (men: 17.3 ± 4.2 % kcal/d, women: 16.4 ± 3.6 % kcal/d, $p = 0.62$), carbohydrate (men: 49.2 ± 4.6 % kcal/d, women: 55.6 ± 9.2 % kcal/d, $p = 0.06$) and total fat (men: 32.3 ± 4.0 % kcal/d, women: 28.8 ± 5.3 % kcal/d, $p = 0.12$) was similar between men and women. Background EPA + DHA intake was 0.07 ± 0.05 g/d at baseline and 0.03 ± 0.03 g/d at week 8 as determined by FFQ (**Table 7.1**). Background intake of EPA + DHA did not differ by sex at baseline or week 8 (baseline: $p = 0.93$, week 8: $p = 0.31$). For women, background EPA intake decreased in week 8 from the baseline measurement ($p = 0.04$). By summing background intake estimated by the FFQ and EPA + DHA supplied by the fish oil capsules, EPA + DHA intake was 0.27 ± 0.03 g/d (men) and 0.26 ± 0.02 g/d (women) in weeks 1-4, 0.51 ± 0.03 g/d (men) and 0.50 ± 0.02 g/d (women) in weeks 5-8 and 0.98 ± 0.02 g/d (men & women, n=5) in weeks 9-12.

EPA and DHA in Finger-Tip Prick Whole Blood

The % EPA and % DHA in finger-tip prick whole blood rose following one week of supplementation and reached a plateau after two weeks at each of the 0.25g/d and 0.5g/d EPA + DHA doses (effect of dose, $p < 0.01$, for both % EPA and % DHA) **(Figure 7.1**). This pattern indicates good compliance with the fish oil supplementation protocol and that four weeks were sufficiently long for EPA and DHA to fully incorporate in whole blood following each of the

0.25 and 0.5 g/d EPA + DHA doses. The % EPA in finger-tip prick whole blood was higher in males at baseline (males: 0.46 ± 0.13, females: 0.31 ± 0.05) and higher in females at week 8 (males: 1.07 ± 0.18, females: 1.24 ± 0.15, effect of dose x sex interaction, $p < 0.01$).

Omega-3 Blood Biomarkers

Intake of EPA + DHA was highly correlated with both the % n-3 HUFA in total HUFA in whole blood (n=20: r =0.88, n=5: r =0.86, $p < 0.01$ for both) and the % EPA + DHA in erythrocytes (n=20: r =0.66, n=5: r =0.78, $p < 0.01$ for both) (**Figure 7.2**). The % n-3 HUFA in total HUFA in whole blood was also highly correlated with the % EPA + DHA in erythrocytes (n=20: r =0.77 and n=5: r =0.82, $p < 0.01$ for both) **Figure 7.3**. The 95% confidence intervals for the % n-3 HUFA in total HUFA biomarker were distinct at each level of EPA + DHA intake in each of the venous blood fractions and in finger-tip prick blood (**Table 7.2)**. For the % EPA + DHA in erythrocytes, the 95% confidence intervals measured at baseline and following 0.25 g/d EPA + DHA overlapped in each of the venous blood fractions. Following 1 g/d EPA + DHA, the % n-3 HUFA in total HUFA in whole blood by finger-tip prick was 34.7 ± 2.1 % (95% CI: 32.04 - 37.3 %) and the % EPA + DHA in erythrocytes was 6.1 ± 0.3 % (95% CI: 5.7 - 6.4 %).

As in finger-tip prick blood, the % n-3 HUFA in total HUFA increased following the 0.25g/d and the 0.5 g/d EPA + DHA doses in venous blood fractions (effect of dose, $p < 0.01$, for each blood fraction) (**Figure 7.4**). Differences between blood fractions for the % EPA + DHA measurement varied following baseline and post-supplementation measurements but generally, values were lowest in the finger-tip prick whole blood, intermediate in venous whole blood and highest in erythrocytes and plasma phospholipids (Figure 7.4).

EPA and DHA in Specific Blood Fractions

In general, the EPA and DHA responses in whole blood reflected observations in finger-tip prick blood. The % EPA in whole blood increased with increasing EPA + DHA intakes however the increase in the % DHA was only significant after the 0.5 g/d EPA + DHA dose (effect of dose, $p < 0.01$, for both fatty acids) with evidence of higher DHA levels in women (effect of sex, $p = 0.01$). The consumption of 1g/d EPA + DHA resulted in further increases in the % EPA and % DHA in whole blood (effect of dose, $p < 0.01$, for both fatty acids). In addition to these relative percentage changes in EPA and DHA, the total concentration of fatty acids in whole blood increased during the intervention as concentrations of total saturated, monounsaturated, and polyunsaturated fatty acids all increased ($p < 0.01$, for each). The changes in the % EPA and DHA in erythrocytes were similar to those in whole blood, but total fatty acid concentrations did not change ($p = 0.15$). In plasma phospholipids, the % EPA responded to increasing intakes similar to whole blood and erythrocytes. The % DHA increased in the plasma phospholipid of women only at 0.5g/d EPA+DHA (effect of dose x sex interaction, $p = 0.01$). The subgroup (n=5) taking 1.0 g/d EPA+DHA was predominantly male (n=4) and significant increases in the % DHA in phospholipids was observed relative to baseline and 0.5 g/d of EPA + DHA (effect of dose, $p < 0.01$). The total fatty acid concentration in plasma phospholipids increased at 0.5 g/d EPA + DHA in men and at 0.25 g/d EPA + DHA in women (effect of dose x sex interaction, $p < 0.01$) with men having higher total fatty acid concentrations in plasma phospholipids after 0.5 g/d EPA + DHA. These changes in total fatty acid concentration were similar in each of the major fatty acid classes (effect of dose x sex interaction, saturates: $p = 0.01$, monounsaturates: $p < 0.01$, polyunsaturates: $p < 0.01$).

Sex Differences in Omega-3 HUFA Ratios

The ratio of DHA:EPA was higher in women as compared with men at baseline in each of the venous blood fractions and in finger-tip prick blood (**Figure 7.5**, meaningful comparisons shown). Following one week of supplementation, the DHA:EPA ratio decreased in both men and women and sex differences disappeared (effect of dose x sex interaction in finger-tip prick blood, $p < 0.01$). The ratio of DHA:DPAn-3 was higher in women than men in each blood fraction and increased in week 8 in whole blood (effect of time, $p < 0.01$).

Relationship of body mass index, sex and dietary n-3 HUFA intake with blood n-3 HUFA

Overall, the % DHA in erythrocytes was positively associated with DHA intake and female sex, and the % n-3 HUFA in total HUFA in whole blood was positively associated with EPA + DHA intake and female sex (**Table 7.3**). There was no association of body mass index or body mass (data not shown) with blood n-3 HUFA biomarkers.

Discussion

The aim of this study was to examine blood biomarker response following intakes of 0.25 and 0.5 g/d EPA + DHA in men and women. The response to 1 g/d EPA + DHA was also examined in a small subset of participants. Strengths of this study are the combination of detailed dietary intake information, through controlled dietary supplementation and measurement of background EPA + DHA intake, with comprehensive blood fatty acid assessment, and weekly visitations that included blood fatty acid profiling to encourage adherence. A dose-response relationship was observed for blood n-3 HUFA biomarkers with low doses of EPA + DHA. The dose-response equations presented in Figure 7.2 are useful for predicting intake of EPA + DHA

from blood n-3 HUFA biomarkers or conversely, predicting the blood biomarker response to dietary EPA + DHA intake. The linear relationship presented in Figure 7.3 has application in conversion between commonly reported blood n-3 HUFA biomarkers. The ratio of blood DHA:EPA was greater in women than men at baseline and this sex difference disappeared with supplementation.

Quartiles of the % EPA + DHA in erythrocytes associated with risk of primary cardiac arrest have been examined (Siscovick et al., 1995). Specifically, 2-4% EPA + DHA in erythrocytes was associated with the greatest risk of primary cardiac arrest. Relative to this quartile, quartiles defined by 4.1-4.6%, 4.7-5.4% and 5.5-10.9% EPA + DHA in erythrocytes were associated, respectfully, with 50%, 70% and 90% primary cardiac arrest risk reduction. In the present study, the baseline 3.80 ± 0.55% EPA + DHA in erythrocytes falls within the quartile associated with the greatest risk of primary cardiac arrest. Supplementation at 0.25 g/d EPA + DHA resulted in 4.15 ± 0.56 % EPA + DHA in erythrocytes, which would correspond with 50% risk reduction, and supplementation at 0.5 g/d EPA + DHA resulted in 4.94 ± 0.57 % EPA + DHA in erythrocytes, which would correspond with 70% risk reduction. Supplementation at 1 g/d EPA + DHA resulted in 6.1 ± 0.3 % EPA + DHA in erythrocytes, which would correspond with 90% risk reduction. Relating results of the present dose-response study with quartiles of primary cardiac arrest risk suggests that current intake guidelines of <1 g/d EPA + DHA for healthy individuals may result in partial, but not maximal coronary heart disease protection.

Current evidence suggests that EPA and DHA have shared and complementary benefits for cardiovascular health (Mozaffarian and Wu, 2012). Expressing n-3 HUFA status as a biomarker, such as the % n-3 HUFA in total HUFA which models competition between HUFA for incorporation into the *sn*-2 position of phospholipids (Stark, 2008a), reflects the fact that EPA

and DHA are typically consumed together but in varying ratios. The distinct 95% confidence intervals for the % n-3 HUFA in total HUFA measured following baseline and the 0.25 and 0.5 g/d EPA + DHA doses provides support for application of n-3 HUFA biomarkers towards characterizing intakes of EPA and DHA (Table 7.2). The % n-3 HUFA in total HUFA is a ratio, therefore its value is consistent when determined from blood fatty acid composition expressed as a percentage of the total fatty acid pool or as a concentration and thereby overcomes any effects of total blood fatty acid concentration changes. Using the equation in Figure 7.3, levels of the % EPA + DHA in erythrocytes associated with primary cardiac arrest (Siscovick et al., 1995) can be converted to the whole blood % n-3 HUFA in total HUFA. Specifically, the % EPA + DHA in erythrocytes quartiles of 2.0 - 4.0%, 4.1 - 4.6%, 4.7 - 5.4% and 5.5 - 10.9% is equivalent to 15.5 - 25.1%, 25.5 - 27.9%, 28.4 - 31.8% and 32.3 - 58.1% n-3 HUFA in total HUFA in whole blood. Supplementation with 0.25 g/d, 0.5 g/d and 1.0 g/d in the present study was associated with 27%, 31% and 35% n-3 HUFA in total HUFA.

A prior dose-response study equated intake of 0.5 and 1 g/d EPA + DHA with 8% and 10% EPA + DHA in erythrocytes, respectfully (Harris and Von Schacky, 2004). Background dietary EPA + DHA intake was not considered in calculation of the total EPA + DHA dose and high baseline % EPA + DHA in erythrocytes of 4.7 ± 0.9% suggests 0.4 - 0.5 g/d of EPA + DHA were regularly consumed based on the present data. The 8% and 10% values were based on fatty acid determinations using a 10-minute heating protocol that fails to transesterify fatty acids in amide linkages of sphingolipids to fatty acid methyl esters (Armstrong, Metherel, and Stark, 2008). Although sphingomyelin is a minor component of erythrocyte lipids (8% by wt of total lipids (Christie, 1985)), the EPA and DHA content of sphingolipids is lower than glycerophospholipids. Therefore incomplete transesterification of fatty acids in sphingolipids

results in a lower summation of the total fatty acids that in turn overestimates the % of EPA + DHA in total fatty acids. Unfortunately, the number and identity of fatty acids included in the total fatty acids is typically undefined. The number of fatty acids included can vary due to chromatography techniques, but also the practice of only including fatty acids that have been identified. The ability to identify fatty acids is also dependent on analytical methods and includes differences in peak identification practices such as external references of mathematical determinations in addition to chromatography protocols. Any omission of a fatty acid from the total fatty acids summation leads to a smaller denominator and a higher estimated % EPA + DHA. Total fatty acids included in calculations herein include all those presented in a prior blood fatty acid characterization study (Armstrong, Metherel, and Stark, 2008) and unidentified peaks determined to be fatty acids are included in the calculation of total fatty acids. In contrast, the % n-3 HUFA in total HUFA has a clearly defined numerator and denominator (Stark, 2008b). Methodological consistency and transparency is critical to establish a clear link between EPA + DHA intake and blood biomarker targets and method standardization is needed to facilitate between-study comparisons.

Blood levels of DHA are generally higher in women as compared with men in societies consuming low n-3 HUFA intakes (Bakewell, Burdge, and Calder, 2006; Crowe et al., 2008; Giltay et al., 2004; Metherel et al., 2009). Although baseline sex differences in % DHA in blood did not reach statistical significance in the present study, the baseline sex differences in the ratio of DHA:EPA in erythrocytes, plasma phospholipid and whole blood were significant and disappeared with EPA + DHA supplementation. Sex differences in endogenous synthesis of DHA along the n-3 elongation/desaturation pathway may contribute to baseline sex differences in the DHA:EPA ratio and intake of pre-formed n-3 HUFA is known to remove sex differences

in endogenous DHA synthesis, likely through feedback inhibition. The conversion of stable-isotope labelled 22:5n-3 to DHA is greater in women than men consuming very low amounts of EPA + DHA (0.06 g/d) and sex differences in 22:5n-3 to DHA conversion disappear following higher intakes of EPA + DHA (0.56 g/d) (Pawlosky et al., 2003a). Although tracer studies with fatty acid stable isotopes is the preferred measure of endogenous DHA synthesis, substrate to product ratios for n-3 HUFA are often interpreted as crude indicators of endogenous synthesis. The ~2:1 ratio of EPA:DHA in the fish oil capsules may also contribute to the convergence of the DHA:EPA ratio between men and women, and the decrease of the ratio with fish oil supplementation.

Levels of n-3 HUFA biomarkers achieved following supplementation by the present study population reflect those of adults under the age of 35, consuming <0.25 g/d EPA + DHA at baseline as determined by FFQ. The n-3 HUFA levels achieved reflect 4-week supplementation periods at each dose. Weekly finger-tip prick analysis suggests four weeks were sufficient for whole blood n-3 HUFA levels to plateau following 0.25 and 0.5 g/d EPA + DHA doses. The potential for plasma phospholipid and erythrocyte n-3 HUFA levels to continue to rise beyond four weeks cannot be discounted but is not anticipated for these low EPA + DHA doses based on prior work (Barcelo-Coblijn et al., 2008; Katan et al., 1997). The stepwise supplementation protocol was selected to allow further time for full fatty acid incorporation into blood pools at the higher doses while keeping the overall study duration sufficiently short to promote adherence with the protocol. This design has been previously used to examine the blood dose-response relationship to n-3 HUFA supplementation (Von Schacky C., Fischer, and Weber, 1985). Although no control group was examined, background intakes of EPA + DHA were measured by FFQ and successfully identified a participant who increased background EPA and DHA intake.

A larger study population would add to the predictive quality of dose-response curves and a wider range of ages would assist in generalization of predictive equations to additional populations including infants, children and the elderly.

Sex-specific changes in plasma phospholipid total fatty acid concentrations following the 0.5 g/d EPA + DHA dose may contribute to the sex differences in the % DHA in plasma phospholipid response. Sex and dose-related differences in triacylglycerol-lowering following EPA + DHA supplementation, accompanied by lower VLDL cholesterol and higher LDL and HDL cholesterol, have been observed (Caslake et al., 2008). Sex-related differences in the % DHA in plasma phospholipid response may be related to lipoprotein metabolism. This observation provides support that the plasma phospholipid is not an ideal blood fraction for routine n-3 HUFA status assessment. The requirement of additional time and resources for plasma phospholipid isolation prior to fatty acid analysis make it unfeasible for routine fatty acid profiling to determine n-3 HUFA status.

The observation of measureable increases in n-3 HUFA biomarkers over baseline levels following an EPA + DHA dose as low as 0.25 g/d is potentially of great interest to food, beverage, and nutraceutical industries and related ingredient suppliers. The ability to enhance the n-3 HUFA composition of a functional food product is limited by potential changes to sensory properties and regulations specific to countries (Health Canada, 2007b; Kishi et al., 2011). Nutraceutical products supplying doses of EPA + DHA ≤0.5 per serving are widely available to consumers and additional low-dose products have become available recently such as smaller, easy-to-swallow capsules, and products for children. The ability of a low dose of EPA + DHA to increase blood n-3 HUFA biomarkers above levels associated with the lowest coronary heart disease protection (2-4 % EPA + DHA in erythrocytes) (Harris and Von Schacky, 2004;

Siscovick et al., 1995) may allow industry to better communicate the effectiveness of their products.

Conclusions

Levels of the % n-3 HUFA in total HUFA blood biomarker determined in this dose-response study could be applied towards determining whether EPA and DHA intake is below, at, or above currently recommended levels for adults. The % n-3 HUFA in total HUFA is a particularly useful biomarker of n-3 HUFA status due to ease of sample preparation, equivalent magnitude in whole blood and erythrocytes at each level of EPA and DHA intake, responsiveness to increases in EPA + DHA intake as low as 0.18 g/d, a specifically defined numerator and denominator, resistance to changes in total blood fatty acid concentration, and applicability to high-throughput fatty acid analyses through minimally-invasive finger-tip prick assessment. Supplementation with 0.25 g/d EPA + DHA at a ratio of 2:1 removed baseline sex differences in the blood DHA:EPA ratio. This finding could be applied towards EPA + DHA dose targeting in costly fatty acid stable isotope studies to determine the n-3 HUFA dose at which sex differences in endogenous DHA synthesis disappear. Understanding sex differences in endogenous DHA synthesis has application in establishing dietary intake recommendations and investigating the relationship between n-3 HUFA status and sex differences in chronic diseases.

Table 7.1. Dietary intake of EPA and DHA at baseline and week 8 by FFQ.

	Men (n=11)			Women (n=9)		
	mean ± sd	95% CI	CV	mean ± sd	95% CI	CV
	g/d	*g/d*	*%*	*g/d*	*g/d*	*%*
Week 0						
EPA	0.02 ± 0.02	(0.01 - 0.03)	90	0.02 ± 0.01	(0.01 - 0.03)	60
DHA	0.05 ± 0.04	(0.02 - 0.08)	83	0.05 ± 0.03	(0.03 - 0.08)	58
EPA + DHA	0.07 ± 0.06	(0.03 - 0.11)	85	0.07 ± 0.04	(0.04 - 0.11)	56
Week 8						
EPA	0.01 ± 0.01	(0.00 - 0.02)	96	0.01 ± 0.01	(0.00 - 0.01)	113
DHA	0.03 ± 0.02	(0.01 - 0.04)	67	0.02 ± 0.02[1]	(0.01 - 0.03)	89
EPA + DHA	0.04 ± 0.03	(0.02 - 0.05)	71	0.02 ± 0.02	(0.01 - 0.04)	100

[1]Significant difference from week 0 measurement by paired *t* test ($p < 0.05$).

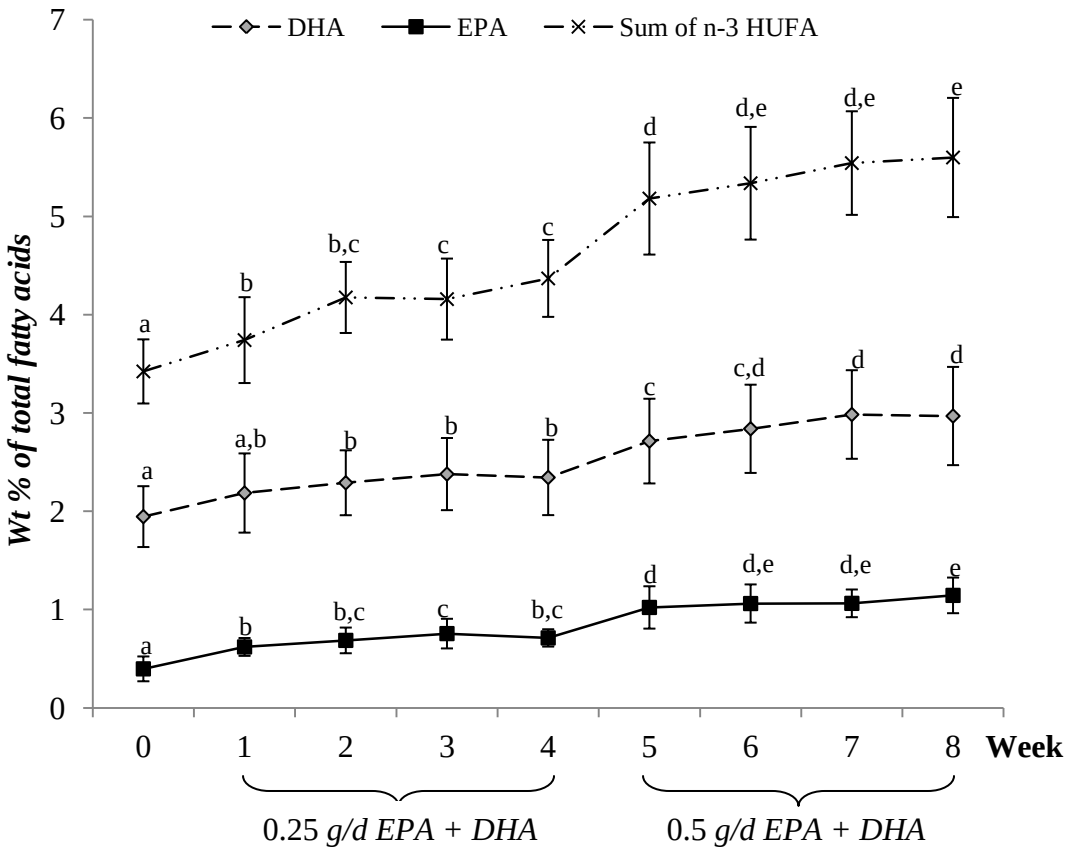

Figure 7.1. EPA, DHA and sum of n-3 HUFA in finger-tip prick whole blood at week 0 and following EPA + DHA intakes of 0.25g/d (weeks 1-4) and 0.5g/d (weeks 5-8). Different letters represent differences in individual means were determined by Bonferroni's post-hoc test following a significant F-value by repeated measures linear mixed model procedure ($p < 0.05$).

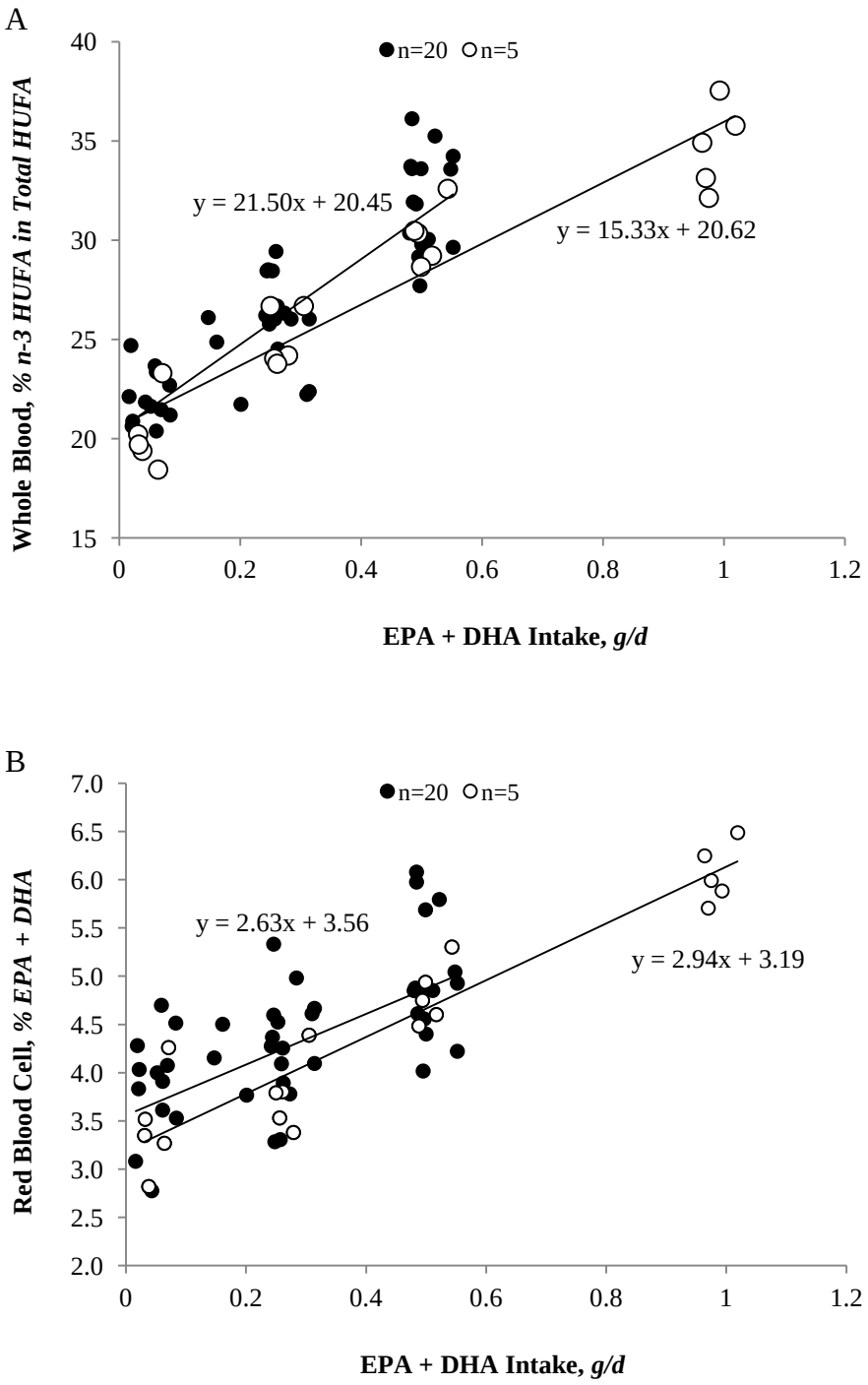

Figure 7.2. Response of the % n-3 HUFA in total HUFA in whole blood (A) and the % EPA + DHA in erythrocytes (B) to intake of EPA + DHA. Intake is the sum of background EPA + DHA measured by FFQ and EPA + DHA supplied by fish oil supplementation. Whole blood measurements are from finger-tip prick samples. Linear dose-response equations are presented for participants consuming 0.25 g/d EPA + DHA in weeks 1-4 and 0.5 g/d EPA + DHA in weeks 5-8 (n=20) and the sub group also consuming 1.0 g/d EPA + DHA in weeks 9-12 (n=5) from fish oil supplements.

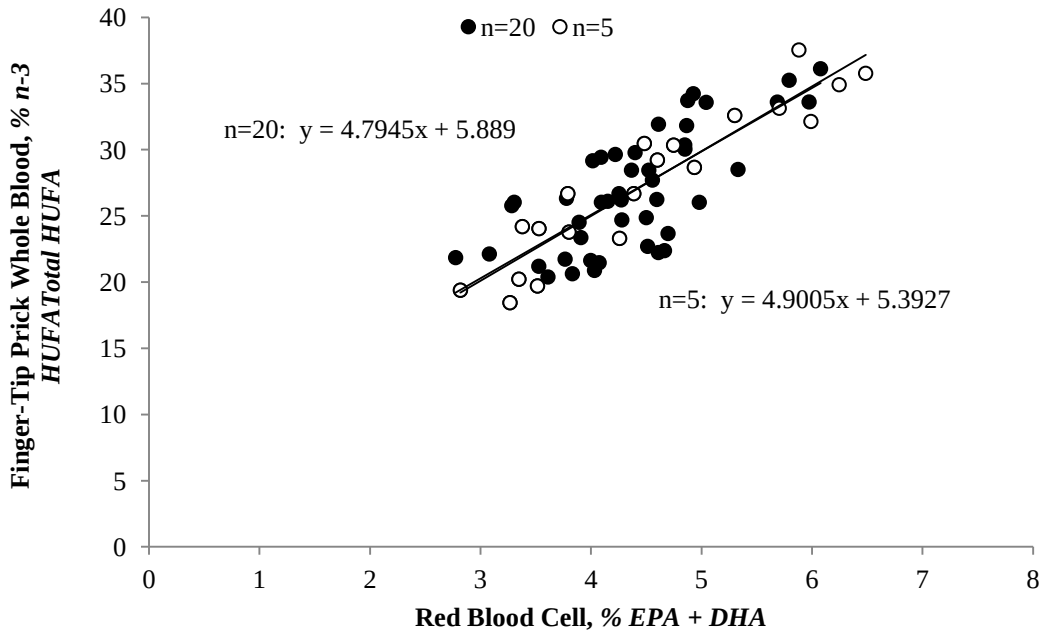

Figure 7.3. Relationship between the % n-3 HUFA in total HUFA in whole blood and the % EPA + DHA in erythrocytes. Whole blood measurements are from finger-tip prick samples. Linear dose-response equations are presented for participants consuming 0.25 g/d EPA + DHA in weeks 1-4 and 0.5 g/d EPA + DHA in weeks 5-8 (n=20) and the sub group also consuming 1.0 g/d EPA + DHA in weeks 9-12 (n=5) from fish oil supplements.

Table 7.2. n-3 HUFA biomarkers in erythrocytes, plasma phospholipid and whole blood fractions with supplementation.

	Baseline			0.25 g/d EPA + DHA			0.5 g/d EPA + DHA		
	mean ± sd	%CV	95% CI	mean ± sd	%CV	95% CI	mean ± sd	%CV	95% CI
				All Participants (n=20)					
Erythrocytes									
EPA + DHA, *wt%*	3.80 ± 0.55	14.5	(3.60 - 4.05)	4.15 ± 0.56	13.5	(3.96 - 4.40)	4.94 ± 0.57	11.5	(4.74 - 5.19)
HUFA Score	23.97 ± 2.03	8.5	(23.05 - 24.98)	26.25 ± 2.11	8.0	(25.32 - 27.26)	30.03 ± 2.08	6.9	(29.12 - 31.06)
Plasma Phospholipid									
EPA + DHA, *wt%*	3.75 ± 0.57	15.2	(3.49 - 4.03)	4.03 ± 0.65	16.3	(3.74 - 4.28)	4.93 ± 0.73	14.8	(4.71 - 5.25)
HUFA Score	24.07 ± 2.66	11.0	(22.99 - 23.32)	29.56 ± 2.04	6.9	(28.48 - 30.81)	35.04 ± 3.71	10.6	(34.10 - 36.42)
Venous Whole Blood									
EPA + DHA, *wt%*	3.01 ± 0.36	11.9	(2.85 - 3.60)	3.42 ± 0.37	10.9	(3.25 - 3.60)	4.49 ± 0.55	12.3	(4.36 - 4.70)
HUFA Score	23.28 ± 2.40	10.3	(22.35 - 24.31)	26.60 ± 1.94	7.3	(25.68 - 27.64)	31.47 ± 2.50	7.9	(30.64 - 32.60)
Finger-Tip Prick Whole Blood									
EPA + DHA, *wt%*	2.34 ± 0.33	14.1	(2.19 - 2.50)	3.06 ± 0.39	12.7	(2.87 - 3.24)	4.11 ± 0.61	14.8	(3.83 - 4.30)
HUFA Score	21.92 ± 1.97	9.0	(20.99 - 22.84)	25.93 ± 1.96	7.6	(25.01 - 26.85)	31.59 ± 2.38	7.5	(30.48 - 32.70)

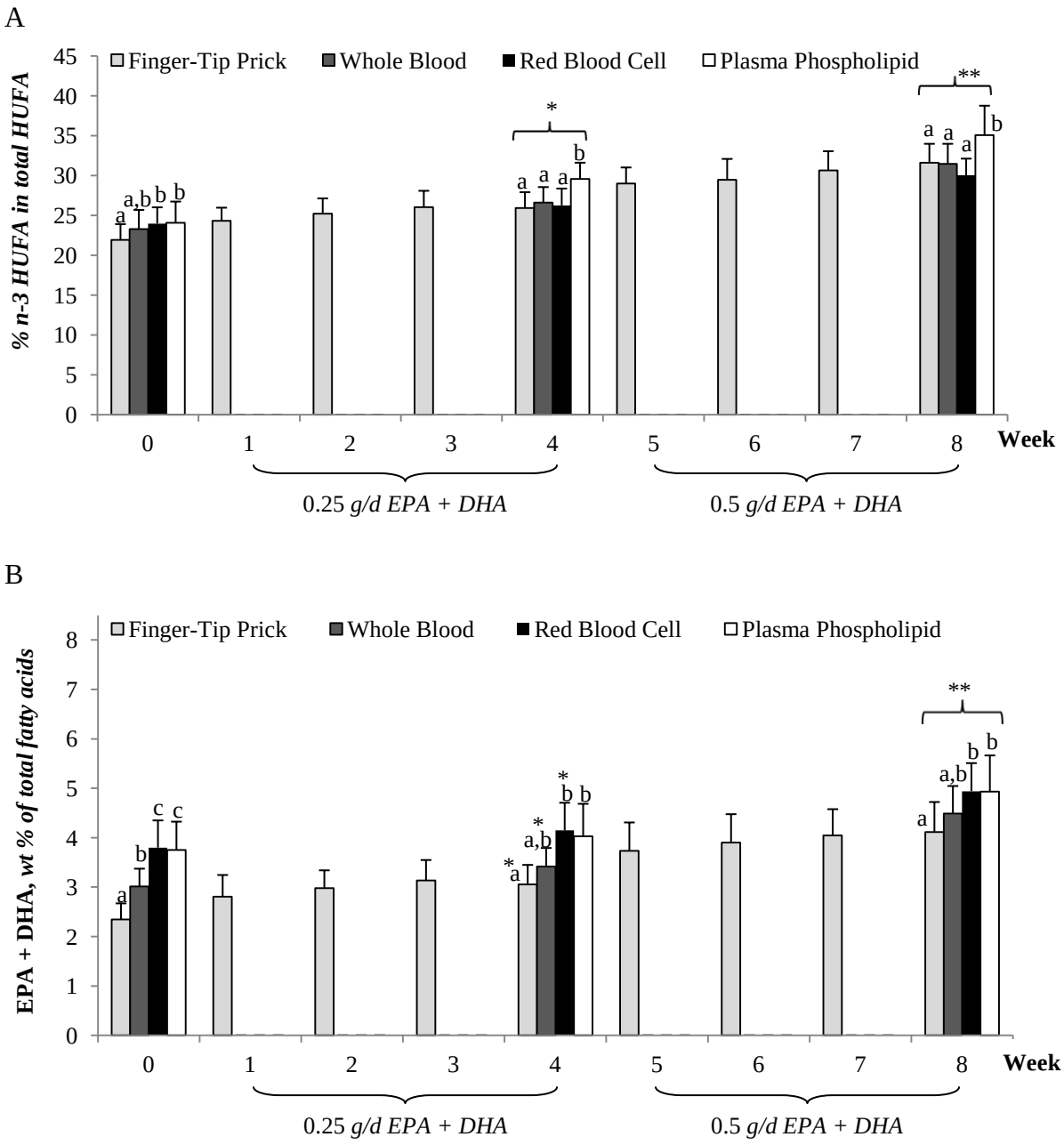

Figure 7.4. The % n-3 HUFA in total HUFA (**A**) and % EPA+ DHA (**B**) throughout fish oil supplementation (weeks 1-4: 0.25 g/d EPA + DHA, weeks 5-8: 0.5 g/d EPA + DHA). Columns with different letters within a time point are significantly different by Bonferroni's post-hoc test following a significant F-value by the linear mixed model procedure ($p < 0.05$). (*) Represents a significant difference from the baseline measurement and (**) represents a significant difference from the week 4 measurement within each blood fraction. Finger-tip prick determinations at weeks 1-3 and 5-7 are shown by not included in the statistical analyses.

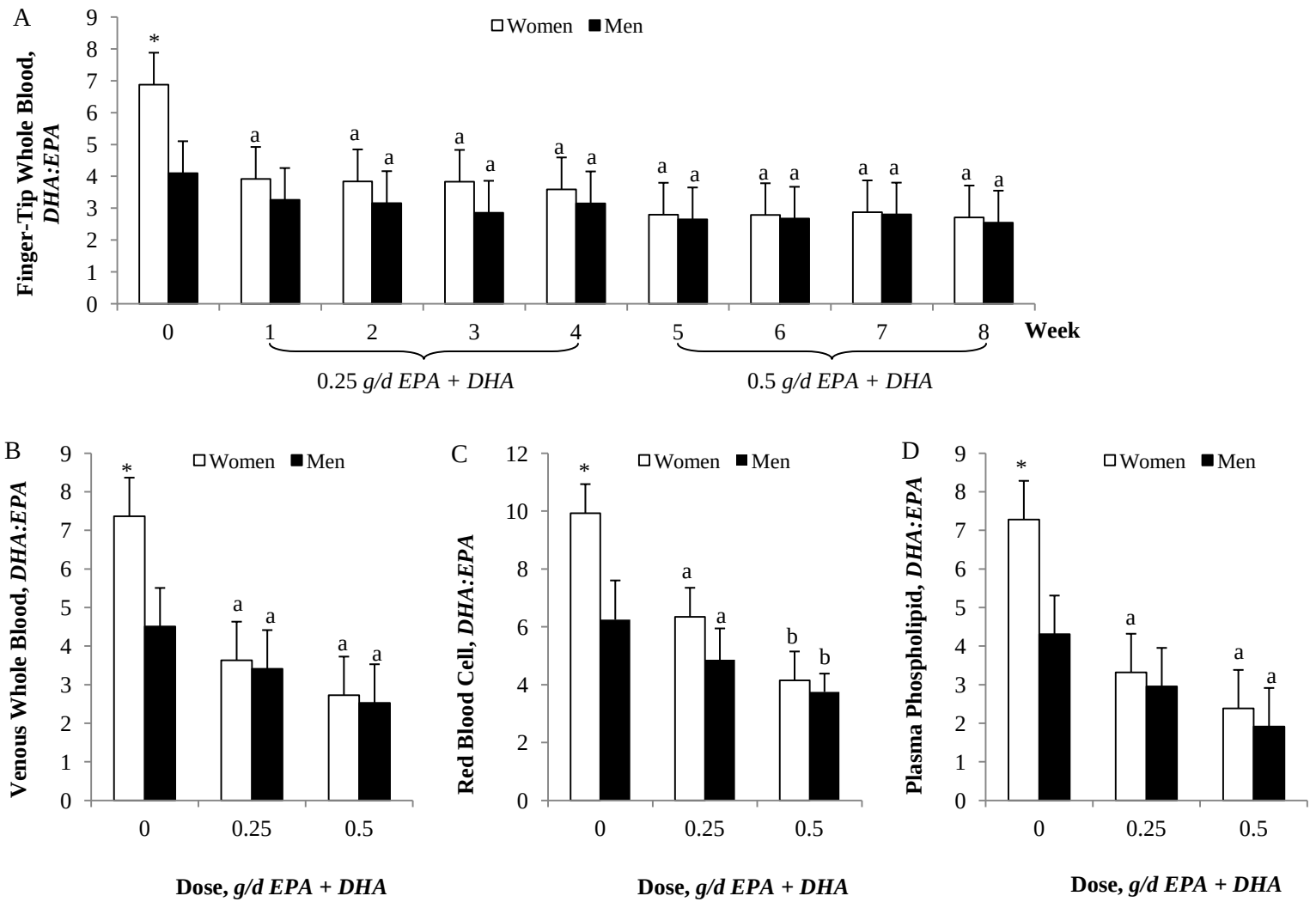

Figure 7.5. DHA:EPA ratio for men and women in (A) finger-tip prick blood, (B) whole blood, (C) erythrocytes and (D) plasma phospholipids with supplementation. (*) represents a significant difference from men at a time/dose, (a) and (b) respectively represent a significant difference from the week 0 (0 g/d EPA+DHA) and week 4 (0.25 g/d EPA+DHA) measurements within each sex by Bonferroni's post-hoc test following a significant F-value by the linear mixed model procedure with a significant dose x sex interaction ($p < 0.05$).

Table 7.3. Results of linear regression models determining the association of sex, body mass or body mass index and n-3 HUFA intake on n-3 HUFA blood biomarkers

	Erythrocyte DHA, wt% of total fatty acids			Whole Blood % n-3 HUFA in Total HUFA		
	B	*p*	95% CI	B	*p*	95% CI
Model 1						
Sex	**0.71**	**<0.01**	**0.35 - 1.06**	**3.73**	**<0.01**	**1.34 - 4.12**
Body Mass, *kg*	<0.01	1.00	-0.01 - 0.01	0.04	0.10	-0.01 - 0.08
DHA or EPA + DHA Intake[1], *g/d*	**3.52**	**0.04**	**0.11 - 6.92**	**14.87**	**<0.01**	**12.75 - 17.00**
Model 2						
Sex	**0.64**	**<0.01**	**0.39 - 0.88**	**2.14**	**<0.01**	**0.95 - 3.33**
Body Mass Index, kg/m^2	-0.01	0.54	0.39 - 0.88	0.05	0.56	-0.12 - 0.23
DHA or EPA + DHA Intake[1], *g/d*	**3.98**	**<0.01**	**-0.05 - 0.03**	**14.71**	**<0.01**	**12.55 - 16.87**

[1]DHA intake evaluated for association with red blood cell DHA; EPA + DHA intake evaluated for association with whole blood % n-3 HUFA in total HUFA.

CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

The purpose of this thesis was to address the gap between observed and recommended intakes of EPA and DHA (Institute of Medicine of the National Academies, 2005; Kris-Etherton, Harris, and Appel, 2003; Kris-Etherton et al., 2007; United States Department of Agriculture, 2010) through an examination of long-term adherence to conventional and novel dietary advice strategies. Furthermore, the blood biomarker response to low intakes of EPA + DHA that are currently recommended was characterized to facilitate objective assessment of EPA + DHA intake and to inform the relationship between EPA + DHA intake and disease risk. Due to infrequent consumption of concentrated n-3 HUFA sources, dietary survey assessment of EPA + DHA intake is challenging (Fratesi et al., 2009; Overby, Serra-Majem, and Andersen, 2009). To complement biomarker and 3-d dietary record assessments, validation of an FFQ to measure EPA + DHA intakes from Canadian whole food, functional food and nutraceutical dietary sources was performed. Overall, this thesis research is distinct in that it simultaneous characterizes EPA and DHA intake through comprehensive blood fatty acid and dietary survey assessment, examines sex effects on study outcomes, and considers conventional and novel dietary sources. This information is useful for both basic research and clinical practice.

Findings herein facilitate interpretation of null conclusions in recent trials examining reduction in cardiovascular endpoints with EPA and DHA supplementation <1g/d. In the Alpha-Omega trial, an intervention targeting an intake of 0.38g/d EPA + DHA from a functional food margarine over 40 months did not reduce the rate of secondary cardiovascular events (Kromhout, Giltay, and Geleijnse, 2010). Although functional foods were provided in the Alpha-Omega

trial, present findings indicate that adherence over 40 months to functional food intake is unlikely. Accordingly, findings herein do not support intention to treat analyses used to examine the effect of EPA + DHA intake on Alpha-Omega trial endpoints. The % of EPA and DHA in plasma cholesteryl esters increased relative to the placebo group at 3, 20 and 40 months post-intervention in a random subset of Alpha-Omega trial participants and was interpreted as a marker of adherence (Kromhout, Giltay, and Geleijnse, 2010). EPA + DHA levels in plasma cholesteryl esters can change rapidly in response to recent dietary intake (Marsen et al., 1992). While the fatty acid composition of cholesteryl esters can reflect dietary fatty acids, it also reflects the specificity of lecithin:cholesterol acyl transferase for 18:1n-9 and 18:2n-6 and may be a poor marker of long term adherence relative to other fatty acid determinations of plasma fractions and erythrocytes (Stark, 2008a). Presently, examining the % of DHA in erythrocytes was required to properly characterize long term adherence as plasma-based and EPA + DHA composite biomarkers appear to be influenced by recent diet intakes. Accordingly, adherence was likely overestimated in the Alpha-Omega trial. Examination of % DHA in erythrocytes as a marker of adherence and blood n-3 HUFA biomarkers in relation to the rate of cardiovascular events could be applied in future long-term trials. Furthermore, the predictive equations developed herein have potential utility for interpreting the intervention literature when intake estimates are neglected.

The present observation of cessation of adherence following 12 weeks indicates that strategies to promote long-term adherence to intake of dietary n-3 HUFA sources are needed. Fatty acid profiling of finger-tip prick whole blood (Armstrong, Metherel, and Stark, 2008; Stark, 2008a) and the EPA + DHA specific FFQ can be rapidly evaluated. To further promote rapid intake assessment, examining the contribution of individual n-3 HUFA sources identified

by the FFQ to variability in EPA + DHA intake could be used to create a short screener tool from the present FFQ (Briefel, 2007). The FFQ could also be made electronically available (Swierk et al., 2011) to facilitate provision of feedback, which could be made available instantly using automated software analysis. Self monitoring with feedback can result in long-term weight loss following weight loss intervention (Burke et al., 2012) and application of feedback to sustaining EPA + DHA intake is worthy of investigation. The FFQ and finger-tip prick fatty acid profiling are also useful research tools to screen candidates for study participation. Alternatively, routine clinical screening could be applied towards identifying individuals with low n-3 HUFA intakes/blood status so they can receive targeted advice to increase EPA + DHA intake.

Relating blood n-3 biomarkers to EPA + DHA intake and to coronary heart disease risk categories could be informative for deriving nutrient intake recommendations, especially given proposals that dietary reference intakes for EPA + DHA be made (Harris et al., 2009; Kris-Etherton, Grieger, and Etherton, 2009). Dietary guidelines, which inform consumers how to achieve nutrient intake recommendations through dietary sources, have conventionally promoted whole food sources. Canada's food guide recommends two servings of fish per week (Health Canada, 2011). Advice to use nutraceuticals are now appearing in official dietary guidelines, such a daily vitamin D supplement is recommended to Canadian adults over 50 years (Health Canada, 2011). Based on the increase in blood n-3 HUFA biomarkers and dietary EPA + DHA intakes following dietary advice to consume nutraceuticals and full retention of participants in Nutraceutical and Combined advice groups presently, dietary recommendations for EPA + DHA nutraceuticals should be considered for inclusion in dietary guidelines. Sex-specific advice may also be warranted as blood n-3 HUFA biomarkers increased for men but not women following seafood advice.

Risk of primary cardiac arrest has been evaluated in relation to quartiles of % EPA + DHA in erythrocytes (Siscovick et al., 1995). This type of categorical classification helps account for inter-individual variability in the relationship between blood biomarkers and disease risk. Presently, ranking individuals based on FFQ intake estimates resulted in reasonable agreement with ranks based on blood biomarker and dietary records. FFQ intake measurements could also be expressed categorically to reduce measurement error associated with dietary assessment. Categorization as meeting or not meeting intake guidelines would facilitate translating FFQ intake measurements to consumers, although may artificially relay the impression that risk is an all-or-nothing phenomenon.

Body mass negatively associated with n-3 HUFA blood biomarkers in the dietary advice study, but not in the controlled dose-blood response study. The tight dietary controls in the latter study combined with the lack of a body mass association indicates that this may not be related to metabolic processing, but rather may be an indication of differences in adherence associated with body mass. Higher baseline body mass index was an independent predictor of attrition in a trial examining dietary advice strategies for weight loss (Greenberg et al., 2009), a lower body mass index has been associated with following a Mediterranean dietary pattern (Schroder et al., 2004), and osteoporotic women with a lower body mass index had greater adherence to calcium and vitamin D supplementation as compared with women with a higher body mass index (Sanfelix-Genoves et al., 2009). It is also possible that the small study population in the dose-blood response study diminished the ability to detect an association of body mass with blood n-3 HUFA biomarkers.

The overarching aims of this work were to examine practical strategies to meet EPA and DHA intake recommendations and improve characterization of dietary EPA + DHA intake and

its relationship to n-3 HUFA blood biomarker status. Examination of the dose-response to low intakes of EPA + DHA facilitates an important connection between EPA + DHA intake, blood levels and disease risk. Application of n-3 HUFA blood biomarkers to monitor adherence and evaluate disease risk has significant implications for interpreting long term trials examining n-3 HUFA intake and chronic disease relationships. Furthermore, equating n-3 HUFA blood biomarkers achieved following low doses with quartiles of primary cardiac risk reduction highlights need for defined and consistent EPA + DHA intake recommendations. In practice, however, long term adherence to sustained intake of EPA + DHA presents a challenge for consumers and strategies, such as trait-based preferences, follow-up frequency and self-monitoring, to improve adherence should be explored. Finally, accurate dietary assessment is facilitated through combining multiple assessment tools. Self-reported intakes by FFQ can be verified against predictive dose-response equations when combined with biomarker assessment and this comprehensive approach should be considered in study designs involving EPA and DHA intake.

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APPENDIX A

University of Waterloo — Laboratory of Nutritional and Nutraceutical Research

Food Frequency Questionnaire

INSTRUCTIONS: Please answer the following background questions and subsequent 40 questions about your **usual** eating habits over the **past 12 months**.

Date you answered this questionnaire: _____

Age:

( ) Under 19 years
( ) 19-24 years
( ) 25-29 years
( ) 30-34 years
( ) 35-39 years
( ) 40-44 years
( ) 45-49 years
( ) 50-54 years
( ) 55-59 years
( ) 60-64 years
( ) 65 years and over

Gender: ( ) Male ( ) Female

Sociodemographic

1. What is your cultural/racial background?

( ) Caucasian
( ) Chinese
( ) South Asian (ex: East Indian, Pakistani, Sri Lankan)
( ) African
( ) Filipino
( ) Latin American
( ) Southeast Asian (ex: Cambodian, Indonesian, Laotian, Vietnamese)
( ) Arab
( ) West Asian (ex: Afghan, Iranian)
( ) Japanese
( ) Korean
( ) Aboriginal
( ) Other: _____

2. What is the highest level of education you have attained?

( ) Some High School
( ) High School Diploma
( ) Diploma/Certificate from trade, technical or vocational school or business college
( ) Diploma/Certificate from community college, CEGEP or nursing school
( ) Bachelor's undergraduate degree or teacher's college
( ) Master's degree
( ) Degree in Medicine, Dentistry, Veterinary Medicine, Optometry or Law
( ) Earned doctorate (ex: Ph.D.)
( ) Other: _____

3. What is your annual household income?

( ) less than $25,000
( ) $25,000 - $50,000
( ) $50,000 - $75,000
( ) $75,000 - $100,000
( ) more than $100,000

Diet

4. What type of milk do you usually use?

( ) I do not drink milk
( ) Regular cow's milk (non-omega-3)
( ) Regular soy milk (non-omega-3)
( ) *Nielson, Dairy Oh!* 1% or 2% milk
( ) *Nielson, Dairy Oh!* Homogenized milk
( ) Silk Plus, Omega-3 DHA soy milk
( ) So Good, Omega-3 DHA soy milk
( ) Other Omega-3 enriched milk
Please specify Brand:
_____

5. How much **omega-3** milk do you usually use **per day** (including milk added to tea, coffee, cereal, etc.)?

( ) None
( ) 1 tablespoon
( ) 2 tablespoons
( ) ¼ cup (62.5 mL)
( ) ½ cup (125 mL)
( ) 1-2 cups (250-500 mL)
( ) 2-3 cups (500-750 mL)
( ) 3 cups (750 mL) or more

6. What kinds of margarine/spread do you usually use? (ex: On bread, vegetables, in cooking and baking, etc.)

( ) I don't use any margarine/spread
( ) Regular margarine (non-omega-3)
( ) *Becel*, *Omega-3 Plus* Margarine
( ) Other Omega-3 enriched margarine
Please specify brand:
_____

7. How much **omega-3** margarine/spread do you usually use **per day**?

( ) None
( ) 1-2 teaspoons
( ) 1 tablespoon
( ) 2 tablespoons
( ) 3 tablespoons
( ) 4 tablespoons
( ) 5-7 tablespoons
( ) 8 or more tablespoons

8. What kinds of eggs do you usually eat (include eggs in baked goods/cooking)?

( ) *Naturegg Breakfree* omega-3 liquid eggs
( ) *Naturegg* Omega-3 eggs (in shell)
( ) *Naturegg* Omega-3 Pro eggs (in shell)
( ) *Complements* Omega-3 eggs
( ) *President's Choice* Omega-3 eggs
( ) *Gray Ridge* Omega-3 large eggs
( ) *Gray Ridge* Omega-3 extra-large eggs
( ) Regular Chicken eggs
( ) Duck eggs
( ) Other Omega-3 enriched eggs
Please specify brand:
_____

9. On average, how many eggs do you usually eat **per week (**including eggs in baked goods/cooking)?

( ) I don't eat eggs
( ) Less than 1 egg
( ) 1 to 2 eggs
( ) 3 to 5 eggs
( ) 6 or more eggs

10. What kind of juice do you usually drink?

( ) I don't drink juice
( ) Regular juice (non-omega-3)
( ) *Tropicana Essentials*, Omega-3 orange juice
( ) *Minute Maid Fruit Solutions*, Omega-3 mango, orange & passion juice
( ) *President's Choice, Oh Mega J* orange juice
( ) Other Omega-3 enriched juice
Please specify brand:
_____

11. How much **omega-3** juice do you usually drink **per day**?

( ) None
( ) less than one cup (<250 mL)
( ) 1 cup (250 mL)
( ) 1-2 cups (250-500 mL)
( ) 2-3 cups (500-750 mL)
( ) 3 cups (750 mL) or more

12. What kind of yogurt do you usually eat?

( ) I don't eat yogurt
( ) Regular yogurt (non-omega-3)
( ) *Danone Danino* yogurt
( ) *Danone Danino Go* yogurt drink
( ) Other Omega-3 enriched yogurt
Please specify brand:
_____

13. How much **omega-3** yogurt do you usually eat **per week**?

( ) none
( ) less than 1, 100 g pot
( ) 1, 100 g pot
( ) 2 to 3, 100 g pots
( ) 4 to 5, 100 g pots
( ) 6 to 7, 100 g pots
( ) 8 or more 100 g pots

14. What kind of bread do you usually eat?

( ) I don't eat bread
( ) Regular bread (non-omega-3)
( ) *Wonder*+ Omega-3 bread with DHA
( ) Other Omega-3 enriched bread
Please specify brand/type:

_____

15. How much omega-3 bread do you usually eat per day (fresh and toasted)?

( ) none
( ) less than 1 piece
( ) 1 piece
( ) 2 pieces
( ) 3 pieces
( ) 4 pieces
( ) 5-7 pieces
( ) 8 or more pieces

16. What kind of cheese do you usually eat?

( ) I don't eat cheese
( ) Regular cheese (non-omega-3)
( ) Black Diamond, Smart Growth Cheese Strings with DHA
( ) Other omega-3 enriched cheese
Please specify brand:

_____

17. How much omega-3 cheese do you usually eat **per week**?

( ) None
( ) less than 1oz (~1 cheese string)
( ) 1 oz (~1 cheese string)
( ) 2-3 oz (2-3 cheese strings)
( ) 4-5 oz (4-5 cheese strings)
( ) 6-7 oz (6-7 cheese strings)
( ) 8 or more oz (8+ cheese strings)

18. What kind of nut butter (ex: peanut) do you usually eat?

( ) I don't eat nut butter
( ) Regular nut butter (non-omega-3)
( ) Life Brand, Omega-3 Peanut Butter
( ) Other omega-3 enriched nut butter
Please specify brand:

_____

19. How much omega-3 nut butter do you eat per week?

( ) None
( ) less than 1 tablespoon (tbsp)
( ) 1 tbsp
( ) 2-3 tbsp
( ) 4-5 tbsp
( ) 6-7 tbsp
( ) 8 or more tbsp

20. On average, how often do you eat fish paste? (ex: Surimi, often in Asian dishes)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Specify the **type** of fish paste, **brand** and **amount** usually eaten (number of teaspoons)
Type of fish paste *Brand* *#tsp*
_____

21. On average, how often do you eat seaweed?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of seaweed (or **dish** containing seaweed) and **amount** eaten on a typical occasion
Type of seaweed/Dish *Amount*

_____

22. On average, how often do you eat seafood-containing sauce? (ex: fish sauce oyster sauce, etc (often in Asian dishes))?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of seafood sauce, **brand** and **amount** usually eaten (# tsp)
Type of sauce *Brand* *#tsp*
_____

23. On average, how often do you eat fish-based broth (ex: fish broth-based soup)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

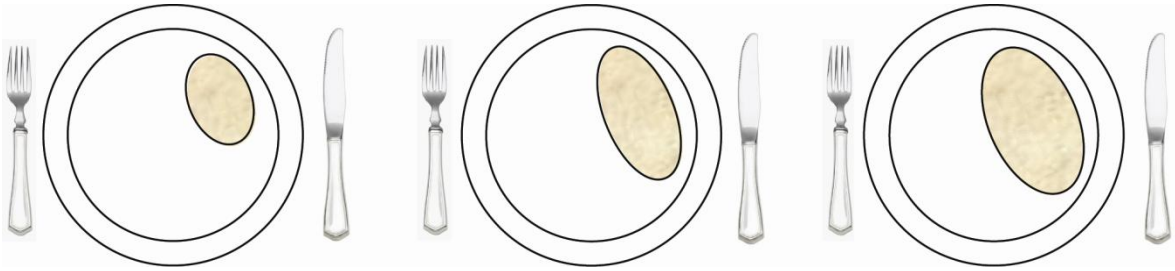
Please specify the **brand** (or 'homemade') and **amount** of fish broth usually eaten
Brand *Amount*
_____

24. How many fish oil supplements (ex:capsules) do you usually take **per day**

( ) None
( ) Number: _____

Please Specify **brand** and **name** of fish oil capsules: (ex: Jamieson, omega-3 Complete)
Brand *Product Name*
_____
_____

25. When you eat meat or poultry, what size serving do you usually eat?



***A**: Deck of cards sized portion = 3oz / 90g*

( ) Less than **A**
( ) **A**
( ) Between **A** and **B**

***B**: 5oz / 150g*

( ) **B**
( ) Between **B** and **C**

***C**: 7oz / 200g*

( ) **C**
( ) More than **C**

26. On average, how often do you eat chicken or turkey?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **types** of chicken/turkey eaten most frequently and whether the **skin** is eaten (ex: Turkey thigh, with skin)
_____

27. On average, how often do you eat duck or goose?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of duck/goose eaten most frequently and whether the **skin** in eaten. (ex: Duck breast, without skin)

---

28. How often do you usually eat beef?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of beef most often eaten (ex: Rump steak):

---

29. On average, how often do you eat lamb?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of lamb most often eaten (ex: Loin chop):

---

30. On average, how often do you eat veal?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of veal most often eaten (ex: Veal steak):

---

31. On average, how often do you eat pork? (do not include ham or bacon)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of pork most often eaten (ex: Pork chop):

---

32. On average, how often do you eat bacon?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **number of slices** of bacon usually eaten on each occasion:

---

33. On average, how often do you eat ham (not deli/sandwich meat)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **number of slices** of ham usually eaten on each occasion:

---

34. On average, how often do you usually eat sliced deli meat (ex: luncheon meats or salami)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** (ex: lean turkey breast), **brand** and the **number of slices** of sliced deli meat most often eaten:
Type *Brand* *# slices*

---

35. On average, how often do you eat sausages?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** (ex: Beef, pork, turkey) and **number** of sausages usually eaten on each occasion:
Type *Number*

---

36. On average, how often do you eat canned fish? (ex: canned tuna, salmon, sardines, etc.)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify up to 4 **types** of canned fish that you regularly eat, including the **brand** and the **amount** typically eaten. (ex: White tuna in water, *Goldseal*, ½ 120g tin)

Type of Fish *Brand* *Amount*

_____
_____
_____

37. On average, how often do you eat fresh or frozen fish? (Include fish meals at home, at a restaurant and take out).

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify up to 4 **types** of fresh or frozen fish that you regularly eat and the **amount** typically eaten

(Include the **brand** for frozen fish. If type of fish is unknown for restaurant fish please write 'restaurant fish').
(ex: Wild Atlantic Salmon, *Highliner*, 150g)

Type of Fish *Brand* *Amount*

_____
_____
_____

38. How often do you eat fresh, frozen or canned shellfish? (ex: shrimp, oysters, scallops, crab, calamari, lobster, etc).

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** and **amount** of shellfish eaten on a typical occasion:

Type of Shellfish *Amount*

_____
_____

39. Are there any other meat, poultry or fish products that you eat regularly?

Please specify the **type, amount** and **how often** other meat or fish products are eaten

Type *Amount* *How often*

_____
_____
_____

40. Have you changed your diet in any significant way over the past year?

( ) Yes
( ) No

If yes, please specify the changes:

_____
_____
_____

Thank you for completing this survey

APPENDIX B

University of Waterloo

Laboratory of Nutritional and Nutraceutical Research

Dietary Advice Package

GENERAL INFORMATION

What are Omega-3 Fatty Acids?

Omega-3 polyunsaturated fats are a type of dietary fat that have been associated with preventing disease and promoting optimal health. In order to maximize heath benefits, it is important to understand that there are various types of omega-3 fatty acids.

One type of omega-3 fatty acid is called alpha-linolenic acid (ALA). ALA can be found in various plant-based foods such as flax seeds (linseed), soybeans, canola, walnuts, pumpkin seeds and their oils. However, specific cardiovascular benefits such as reduction in heart attacks and stroke have been associated with intakes of two other omega-3 fatty acids called Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). EPA and DHA are most commonly found in fish oils. ALA can be converted into EPA and DHA in the body however, this process occurs at very low rates in humans. For this reason, this study will focus on increasing your dietary intake of EPA and DHA.

Why Should I Increase my EPA and DHA Intake?

Populations with high intakes of EPA and DHA, such as Greenland Inuit and Japanese populations, have a low incidence of deaths due to heart disease. EPA + DHA intake in Japanese populations approaches 2 g per day and intake is even greater in Greenland Inuit populations. In comparison, Western populations consume very low amounts of EPA and DHA and Canadian mortality rates due to heart disease are much higher compared to those of Greenland. Various studies have estimated that typical Canadian adult consumption of EPA + DHA is around 0.1 to 0.25 g per day. The American Heart Association has recommended intakes of 1 g/day of EPA + DHA for heart disease prevention. Therefore, there is evidence to indicate that increasing EPA+DHA intakes may have cardiovascular benefits for Canadians.

How can I Increase my EPA and DHA Intake?

There are various means to increase your EPA + DHA intake to meet a potential target of 1 g per day of EPA+ DHA. The traditional source of EPA + DHA is fatty fish. EPA + DHA can also be consumed in the form of fish oil capsules, often referred to as ‘nutraceuticals’. Finally, the food industry has developed various omega-3 enriched foods, referred to as ‘functional foods’, which may include dairy products, eggs and egg products, oil-based spreads, peanut butter and juices, etc.

At present it is unknown which source of EPA + DHA is the most effective and sustainable method to increase EPA+DHA intakes in Canadians at risk for heart disease. Accordingly, this study will ask you to use a combination of the fish, nutraceutical and functional food strategies of increasing EPA + DHA intake, while other participants are being asked to follow one of the other strategies, in order to determine the most practical means for Canadians to consume more EPA + DHA.

COMBINED STRATEGY

How Can I used the Combined Strategy to Increase my EPA+DHA Intake?

The ultimate goal is to attempt to consume American Heart Association recommendations of 1 g/day of EPA+DHA (or EPA+DHA consumption that averages to 1 g/day over the course of a week). To accomplish this goal you may incorporate fish, nutraceutical and functional food strategies into your regular diet. More details about each of these strategies are detailed on the following pages.

As an example, on one day you may choose to eat fish with a high EPA + DHA content for dinner as an alternative to meat, poultry or meat alternatives to meet your 1 g of EPA+DHA goal. The next day you may consume omega-3 liquid eggs instead of regular eggs for a breakfast (0.5 g of EPA+DHA) and a fish oil capsule containing 0.5 g of EPA+DHA for a total of 1 g of EPA+DHA. We recommend that you try many fish and functional food sources to determine which sources best fit your taste preferences, food preparation habits and budget so that you can sustain your EPA+DHA intake over the course of the year. We recommend that you supplement your diet with a fish oil nutraceutical on days when your EPA+DHA intake from fish or functional foods is less than 1 g.

Other than substituting fish and EPA+DHA-enriched functional foods into your regular diet and consuming fish oil nutraceuticals, we ask that you do not make drastic changes to the rest of your diet.

Sample Daily Diet (1)

Meal	Food	Amount	mg EPA+DHA
Breakfast	Naturegg, Breakfree omega-3 liquid eggs	100g	500
	Becel, omega-3 margarine with EPA+DHA	1 tsp	25
	President's Choice, Oh Mega J orange juice	1 cup	50
	Wonder+, Headstart bread with DHA	2 pieces	15
	Becel, omega-3 margarine with EPA+DHA	2 tsp	50
	Banana	1 medium	
Snack	Apple	1 medium	
	Black Diamond, Smart Growth Cheese String	1	20
Lunch	Wonder+, Headstart bread with DHA	2 pieces	15
	Becel, omega-3 margarine with EPA+DHA	2 tsp	50
	Ham lunchmeat with Low fat Swiss cheese	1 oz each	
	Romaine lettuce and tomato		
	President's Choice, Oh Mega J orange juice	1 cup	50
Snack	Danone, Danino omega-3 yogurt	100g pot	40
	Baby carrots	10	
Dinner	Fish sticks (Haddock)	100g	238
	Mixed green salad with ranch dressing	1.5 cups	
	Brown rice	1 cup	
	Becel, omega-3 margarine with EPA+DHA	1 tsp	25
	Silk Plus, Omega-3 DHA soy milk	1 cup	30
TOTAL			**1108mg (~1.1g)**

Sample Daily Diet (2)

Meal	Food	Amount	mg EPA+DHA
Breakfast	Oatmeal with syrup	¾ cup	
	Coffee	1 cup	
	Banana	1	
Lunch	Whole wheat tortilla	1	
	Tuna salad (made with canned white tuna)	60 g tuna (1/2 can)	~515
	Mixed green salad with French dressing	1 cup	
	Water	500 mL	
Snack	Granola bar	1	
Dinner	Grilled chicken breast	125 g	
	Cooked mixed vegetables	1 cup	
	Baked Potato with light sour cream	1	
	1 fish oil nutraceutical	1	500
	Water	500mL	
	Apple	1	
	Chocolate chip cookies	2	
TOTAL			**1015mg (~1g)**

Sample Daily Diet (3)

Meal	Food	Amount	mg EPA+DHA
Breakfast	Waffles with syrup	2	
	Applesauce	½ cup	
	Tea	1 cup	
Snack	Cheddar Cheese	1.5 oz	
	Crackers	6	
Lunch	Beef and barley soup	1.5 cups	
	Raw veggies with dip		
	Fruit Salad	¾ cup	
	Fruit Juice	1 cup	
Snack	Digestive cookies	4	
Dinner	Baked rainbow trout (farmed)	120 g (4 oz)	~1400
	Grilled mixed vegetables	1 cup	
	Whole wheat roll	1	
	Melon	1 cup	
	Vanilla Pudding	¾ cup	
TOTAL			**1400mg (1.4g)**

*Sample Diet #3 contains more than 1g of EPA+DHA so less EPA+DHA could be consumed on another day of the week

Sample Daily Diet (4)

Meal	Food	Amount	mg EPA+DHA
Breakfast	Smoothie with:	1	
	Danone Danino yogurt	100 g	40
	Nielson Dairy Oh! 2% milk	1/2 cup	5
	Banana and Berries		
	Wonder+, Headstart bread with DHA	1 piece	7.5
	Life Brand peanut butter with EPA+DHA	1 tbsp	25
	Coffee	1 cup	
Snack	Walnuts and Dried Cranberries	handful	
Lunch	President's Choice Blue Menu, Ginger Glazed Salmon (frozen personal entrée)	1 (~2.5 oz salmon)	~750
	Salad with oil and vinegar dressing	1 cup	
	President's Choice, Oh Mega J orange juice	1 cup	50
Snack	Low fat muffin	1	
Dinner	Omelet with:	1	
	Naturegg Omega-3 shelled eggs	2	150
	Ham and Spinach	¼ cup each	
	Grilled Mixed Vegetables	1 cup	
	Fruit Pie	1 slice	
	Nielson Dairy Oh! 2% milk	1 cup	10
TOTAL			**1038 mg (~1g)**

FISH (Traditional Whole Foods) STRATEGY

COMMON CONCERNS REGARDING FISH CONSUMPTION

Mercury and Fish

Mercury occurs naturally in the environment and as a result of human activities such as mining. Mercury in fish has been given much media attention in recent years because at very high intakes it has the potential to affect the nervous system. However, the majority of fish sold in Canada contains levels of mercury considered safe for consumption and Health Canada recommends that fish be included in any healthy, Canadian diet. In addition, many carefully controlled studies have concluded that mercury does not increase overall cardiovascular disease risk and the net effect of fish consumption is beneficial for the heart.

Different fish contain different amounts of mercury. Canada has a two-tier system for acceptable levels of mercury in fish for sale to consumers. Most fish and shellfish sold in Canada are classified as Tier One and contain low levels of mercury. The Canadian Food Inspection Agency tests fish and shellfish regularly to ensure that Tier One fish and shellfish sold in Canada have a low mercury content; less than 0.5 parts per million(ppm). 0.5ppm is a very small amount of mercury representing 0.0000005 grams of mercury per gram of fish. Health Canada has not put a limit on how much of these Tier One fish may be consumed as it is believed that adults consuming tier one fish regularly will not be exposed to mercury levels that pose a threat to human health.

Tier Two fish contain slightly higher levels of mercury (less than 1 part per million or 0.000001g mercury/g fish) but are generally consumed in lower amounts. Tier Two fish include fresh/frozen tuna (not canned), shark, swordfish, escolar, marlin, and orange roughy. Health Canada states that you may consume up to 5 oz/150 g **per week** of these Tier Two fish species combined (Note that a 3oz serving of fish is approximately the size of a deck of cards or a cheque book). Women who are or may become pregnant and breastfeeding mothers may consume up to 150 g of Tier Two fish **per month**. Young children between 5 and 11 years of age can eat up to 125 g **per month** and very young children between 1 and 4 years of age should eat no more than 75 g **per month** of these Tier Two fish.

Separate advice has been issued by Health Canada only for canned albacore (white) tuna. This advice does NOT include canned light tuna which has a lower mercury content. Women who are or may become pregnant and breastfeeding mothers may consume up to 300 g (~10 oz) per week of canned albacore tuna (about two 170-g cans of albacore tuna per week). Children between the ages of 5 and 11 years of age may consume 150 g (~5 oz) per week (two servings or about 1, 170-g can) and children between the ages of 1 and 4 may eat 75 g (~2.5 oz) per week (one serving or about ½ of a 170-g can). *There are no consumption limits on albacore (white) tuna for individuals who do not fall into these categories.*

Summary of Health Canada Fish Consumption Guidelines with Reference to Mercury

	Source	**Max Limit Total Mercury in fish**	**Health Canada Consumption Limits**
Tier 1	Most retail fish	0.5 ppm (0.0000005g mercury/ g fish)	No consumption limits
Tier 2	Fresh/frozen tuna (not canned), shark, swordfish, escolar, marlin, and orange roughy	1.0 ppm (0.000001g mercury/ g fish)	·150g/**week** for adults ·150g/**month**: pregnant women, women of child-bearing age & breastfeeding women ·125g/**month**: children between 5 and 11 yrs

			·75g/**month**: children between 1 and 4 yrs
Other	Canned albacore (white) tuna NOT light tuna	0.5 ppm (0.0000005g mercury/ g fish)	·300g/**week** (2, 170g cans): pregnant women, women of child-bearing age & breastfeeding women ·150 g/**week**: children between 5 & 11 yrs ·75 g/**week**: children between 1 & 4 yrs

PCB and Dioxin contamination associated with fish

Polychlorinated biphenyls (PCBs) were used in commercial and industrial processes before they were banned in 1977. Dioxins are a by-product of processes such as pesticide production and paper bleaching. Regulations have worked to reduce dioxin emissions by more than 90% since the late 1980's. While levels of PCBs and dioxins in the environment are declining, these chemicals persist for long periods of time and are present in many foods at low concentrations. Fish have been given much media attention for their PCB and dioxin content, however, beef, chicken, pork, dairy products and vegetables provide greater exposure to these two compounds than fish. The coronary heart disease benefits associated with EPA+DHA in fish greatly outweigh any risks associated with the PCB and dioxin content of fish.

Selecting Fish: Wild vs. Farmed Fish

You may have heard media reports contrasting wild and farmed fish. Reports have found that some farmed fish may contain higher levels of contamination than wild fish. However, Health Canada maintains that levels of contamination in both farmed and wild fish sold in Canada do not represent a health risk. Reports have also been released by Health Canada stating that farmed fish industries are carefully monitored by the government of Canada for environmental impact and human health and that both wild and farmed fish industries provide safe and healthy foods. Some farmed fish may have a slightly higher EPA + DHA content versus their wild counterparts because they are fed a consistent diet of fishmeal and fish oils. It is your choice whether you choose wild or farmed fish sources.

INCORPORATING FISH INTO YOUR DIET

The ultimate goal is to attempt to reach American Heart Association recommendations of 1 g/day (1000 mg/day) of EPA+DHA through fish, functional food and nutraceutical consumption. The tables shown on pages 28-31 illustrate the amount of EPA+DHA per 100g of various species of fish to assist you in reaching this goal. Your EPA + DHA consumption may be less than 1 g on some days and greater than 1 g on others but average to 1 g/day over the course of a week. These tables on pages 28-31 also list approximate mercury content of various fish species. *We recommend you use these tables to select fish with the highest EPA + DHA content and the lowest mercury content (Tier One fish) most frequently.*

Fish can be incorporated into your diet when you would normally consume meat, poultry or a meat alternative. The cost associated with fish can vary widely from being very economical (canned fish or some varieties of frozen whole fish) to more expensive (fresh fillets of some varieties). We recommend that you select sources of fish that fit your budget so that you can maintain high fish intakes over the course of a year.

Purchasing/Preparing Seafood

Fish can be purchased fresh or frozen. Fresh fish should be used within two days of purchase. Otherwise, fresh fish should be stored in the freezer. Frozen seafood can be thawed by placing it in the refrigerator overnight. For faster thawing, immerse fish sealed in a plastic bag in cold water or defrost it in the microwave until the fish is bendable but still icy.

Fish can be purchased from grocery and specialty stores, the farmer's market, etc. You may wish to try a local fish store such as *T & J Seafood* [26 Elm Street , Kitchener (519-578-3080)] or *Caudle's Catch Seafood Ltd*. [St. Jacob's farmer's market and 60 Otonabee Dr., Kitchener (519-894-0442) http://www.caudlescatchseafood.com/index.html]

Additional ingredients such as fish and oyster sauce, fish broth and seaweed also contain some EPA and DHA. These ingredients can be purchased in the Asian section of your grocery store or at an Asian grocery such as *Korean and Japanese Grocery* [510 King St. W., Kitchener (519-569-7199)].

Preparing Whole Fish (adapted from http://www.gortons.com/cookbook/cleanfreshfish.php)

Whole fish can be an economical way to purchase fish.

1. Wash the fish in cold water.
2. If your fish requires scaling (fish with delicate scales such as salmon do not require scaling), hold the fish by the tail and scrape towards the head with a knife.
3. Remove the tail and fins with kitchen scissors (optional).
4. With the fish on its side, press down on the top side with one hand while slicing from the belly (2/3 of the way from the head to the tail) to the base of the head. Be sure to use a sharp knife and not to cut too deeply.
5. Intestines can be removed by pulling them towards the tail. Scrape out any other organs with a knife (such as the kidney - a while sack beside the spine).
6. Wash the inside and outside of the fish in cold water.
7. To remove the head, cut straight through the spine, just below the gills (optional).

Whole fish can now be cooked. Be careful of bones when eating.
Fish can also be cooked whole and filleted later. Have a look at the following web site if you require some step-by-step pictures: http://beyondsalmon.blogspot.com/2006/03/how-to-cook-whole-fish.html.

How to Know When Fish is Cooked

A meat thermometer will tell you that fish is cooked when the middle of the thickest part of the fish reaches an internal temperature of 145 degrees F. A general rule is to cook fish for about 10 minutes per inch (measure fish at thickest part).
If you do not have a meat thermometer:

- **Fish:** When cutting into the thickest part of the flesh, it should appear opaque and should separate easily. Check the flesh of microwaved fish in more than one spot.
- **Shrimp and Lobster:** The flesh should appear pearly-opaque.
- **Scallops:** The flesh should turn milky white or opaque and firm.
- **Clams, Mussels, and Oysters:** When their shells open they are ready to eat. Throw out those with shells that don't open.

FISH COOKING METHODS AND RECIPES

The taste of fish can vary between species and the method of preparation can also affect the taste and texture. As a result, we recommend you try a variety of species and preparation methods detailed on the following pages to determine the fish and fish preparation methods that best suit your tastes. Try searching on the internet or in your favourite cookbooks for other fish recipes. Prepared fish meals can also be purchased.

Poaching Fish: (http://allrecipes.com/HowTo/Healthful-Ways-to-Cook-Fish/Detail.aspx)

-Select a pan large enough to lay fish in a single layer.
-Select a liquid in which to poach fish. Chicken or vegetable stock add a nice flavour to the fish. Peppercorns, lemon juice, herbs, etc can be added to stock for extra flavour.
-Pour in enough liquid to just barely cover fish.
-Bring liquid to a simmer (not boiling). Ideal temperatures for poaching are 165-180 degrees F.

Poached Fish with Dill Mustard Sauce
(from: http://allrecipes.com/Recipe/Quick-Poached-Salmon-with-Dill-Mustard-Sauce/Detail.aspx)
INGREDIENTS

- 1/2 cup plain yogurt
- 1/4 cup Dijon mustard
- 1 tablespoon honey
- 1/4 cup fresh lemon juice
- 3 tablespoons chopped fresh dill
- 1 pound (455 g) fish
- 1 cup white wine
- 1/2 cup water
- 1/4 cup chopped shallots

DIRECTIONS (Poached Fish with Dill Mustard Sauce)

1. In a small bowl, blend the plain yogurt, Dijon mustard, honey, lemon juice, and dill. Cover, and refrigerate until serving.
2. In a medium saucepan over medium heat, place the fish in the white wine and water. Adjust the amount of water as necessary to just cover the fish. Sprinkle with shallots. Cover the saucepan, and cook 10 to 12 minutes, until fish is easily flaked with a fork. Drain, and serve with the yogurt sauce.

Makes 4 servings

Grilling Fish: (http://allrecipes.com/HowTo/Healthful-Ways-to-Cook-Fish/Detail.aspx)

-Dense fish may be placed directly on a grill (you may have to first spray/brush grill with a non-stick cooking spray or cooking oil).
-Delicate fish can be cooked on a grilling basket or on aluminum foil.
-Place the fish around the edges of the grill, away from the hottest part of the fire.
-Grill fish on a medium to medium-high heat.
-Fish should require 4-6 minutes per side (per inch of thickness).

Glazed Fish
(from: **http://allrecipes.com/Recipe/Glazed-Salmon-Fillet-2/Detail.aspx**)

INGREDIENTS
- 2 tablespoons and 2 teaspoons reduced-sodium soy sauce
- 1 tablespoon + 1 teaspoon brown sugar
- 1/8 teaspoon crushed red pepper flakes
- 1/8 teaspoon ground ginger
- 1/8 teaspoon sesame oil
- 1 pound (455 g) fish (ex: salmon)

DIRECTIONS (Glazed Fish)
1. In a bowl, combine the first five ingredients.
2. Grill fish, covered, over medium heat or broil 4-6 in. from the heat for 5-6 minutes on each side or until fish flakes easily with a fork
3. Baste frequently with glaze as fish cooks.

Makes 4 servings

Lemon Salmon Burgers (from http://allrecipes.com/Recipe/Yummy-Lemon-Salmon-Burgers/Detail.aspx)

INGREDIENTS
- 1 (16 ounce) can salmon, drained and flaked
- 2 eggs
- 1/4 cup chopped fresh parsley
- 2 tablespoons finely chopped onion
- 1/4 cup Italian seasoned dry bread crumbs
- 2 tablespoons lemon juice
- 1/2 teaspoon dried basil
- 1 pinch red pepper flakes
- 1 tablespoon vegetable oil
- 2 tablespoons light mayonnaise
- 1 tablespoon lemon juice
- 1 pinch dried basil

DIRECTIONS
1. In a medium bowl, mix together the salmon, eggs, parsley, onion, breadcrumbs, 2 tablespoons of lemon juice, 1/2 teaspoon of basil, and red pepper flakes. Form into 6 firmly packed patties, about 1/2 inch thick.
2. Grill, flipping when light grill marks appear.
3. In a small bowl, mix together the mayonnaise, 1 tablespoon of lemon juice and a pinch of basil. Use as a sauce for your patties.

Makes 6 servings

Steaming Fish: (http://allrecipes.com/HowTo/Healthful-Ways-to-Cook-Fish/Detail.aspx)

-To flavour fish, rub with ex: herbs, ginger, spices, chili peppers, etc
-Place fish on a metal or bamboo steamer in a single layer



-Pour about 1 ½ inches of water into a pan or pot and place the steamer above the water (steamer should not touch water)
-Cover the pot and bring water to a boil
-After about 10 minutes check fish for doneness (fish should be flaky but moist which may take 15-20 minutes)

Chinese-Style Steamed Fish (http://allrecipes.com/Recipe/Chinese-Style-Steamed-Fish/Detail.aspx)

INGREDIENTS

- 1 1/2 pounds (680 g) halibut or any white-fleshed fish, cut into 4 pieces
- 3 green onions, cut into 3 inch lengths
- 2 fresh mushrooms, sliced
- 6 leaves napa cabbage, sliced into 4 inch pieces
- 2 slices fresh ginger root, finely chopped
- 2 cloves garlic, chopped
- 1/4 cup low-sodium soy sauce
- 1/8 cup water
- crushed red pepper flakes to taste
- fresh cilantro sprigs, for garnish

DIRECTIONS

1. Arrange 1/2 of the green onions on the bottom of the steamer (it is important to steam in a container such as a pot in order to retain the steam and juices around the fish). Place 1/2 of the mushrooms and Napa cabbage sections on top of the onions. Place fish on top of the vegetables. Sprinkle ginger, garlic, and red pepper flakes over fish. Top with the remaining green onions, mushrooms, and napa cabbage. Drizzle soy sauce and water over everything.
2. Place steamer in pot or another container containing 1 inch of boiling water, and cover. Steam for 15 to 20 minutes, or until fish flakes easily. Garnish with cilantro, if desired.

Makes 4 servings

Baking/Broiling Fish: (http://www.heb.com/mealtime/CT-easyFish.jsp)

-Preheat oven to 450 degrees F.
-Spray/brush a baking sheet or shallow baking dish with non-stick cooking spray or a cooking oil.
-Place fish on baking sheet in a single layer.
-Season fish as desired.
-For each inch of thickness, bake fish for 10 minutes.

-If using a meat thermometer, fish is cooked when the internal temperature of the thickest part reaches 145 degrees F.

Mediterranean Baked Fish (http://allrecipes.com/Recipe/Mediterranean-Baked-Fish/Detail.aspx)

INGREDIENTS

- 1 cup thinly sliced leeks, white parts only
- 2 garlic cloves, minced
- 2 teaspoons olive oil
- 12 large fresh basil leaves
- 1 1/2 (680 g) pounds fish fillets
- 1 teaspoon salt
- 2 plum tomatoes, sliced
- 1 (60 g) can sliced ripe olives, drained
- 1 medium lemon
- 1/8 teaspoon pepper
- 4 sprigs fresh rosemary

DIRECTIONS

1. In a non-stick skillet, sauté leeks and garlic in oil until tender; set aside.
2. Coat a 13" x 9" x 2" baking dish with non-stick cooking spray. Arrange basil in a single layer in dish and top with fish fillets. Sprinkle with salt. Top with leek mixture.
3. Arrange tomatoes and olives over fish. Thinly slice half of the lemon and place over the top. Squeeze juice from remaining lemon over all. Sprinkle with pepper.
4. Cover and bake at 425 degrees F for 15-20 minutes or until fish flakes easily with a fork. Garnish with rosemary.

Makes 4 servings

Fish Parmesan (http://allrecipes.com/Recipe/Baked-Trout-Fillets/Detail.aspx)

INGREDIENTS

- 1 pound fish fillets
- 1 cup sour cream
- 1/4 cup grated Parmesan cheese
- 1 tablespoon lemon juice
- 1 tablespoon finely chopped onion
- 1/2 teaspoon salt
- Paprika

DIRECTIONS

1. Place fish in a greased shallow 3-Litre. baking dish. In a small bowl, combine the sour cream, Parmesan cheese, lemon juice, onion and salt; spread over fish. Sprinkle with paprika. Bake, uncovered, at 350 degrees F for about 20 minutes or until fish flakes easily with a fork.

Makes 4 servings

Microwaving Fish: (http://www.heb.com/mealtime/CT-easyFish.jsp)

-Spray/brush a microwave-safe dish with non-stick cooking spray or cooking oil.
-Place fish in dish in a single layer.

-Microwave on High for 5-10 minutes depending on the strength of your microwave and the thickness of the fish.

Fish with Summer Tomato Salsa (adapted from http://allrecipes.com/Recipe/Chilled-Salmon-With-Summer-Tomato-Salsa/Detail.aspx)

INGREDIENTS

- 450 g fish fillets
- 1 cup chopped fresh tomato
- 1/2 avocado, chopped
- 1 garlic clove, crushed
- 1 tablespoon balsamic vinegar
- 1 teaspoon olive oil
- 1/2 cup cooked corn kernels
- 1/4 cup minced red onion
- 1/4 cup chopped fresh cilantro
- Salt and pepper, to taste
- 1 lime, cut in wedges

DIRECTIONS (Fish with Summer Tomato Salsa)

1. In a small bowl, combine tomato, avocado, garlic, balsamic vinegar, olive oil, corn, onion, cilantro, salt and pepper. Refrigerate 30 minutes.
2. Place fish in a shallow, lightly oiled microwave bowl. Cover and microwave on high 5-10 minutes or until fish is cooked as desired.
3. Serve fish surrounded by the salsa and lime wedges.

Makes 4 servings

Other Fish Recipes

Pasta de Sardine (http://allrecipes.com/Recipe/Pasta-De-Sardine/Detail.aspx)

INGREDIENTS

- 8 ounces (225 g) dry fettuccine pasta
- 2 tablespoons olive oil
- 1 medium yellow onion, chopped
- 3 cloves garlic, crushed
- 1 lemon, juiced
- 1 (106 g) can sardines in tomato sauce (could use fresh sardines)
- 1 pinch red pepper flakes, or to taste
- 1/4 cup freshly grated Parmesan cheese

DIRECTIONS (Pasta de Sardine)

1. Bring a large pot of lightly salted water to a boil. Add pasta, and cook for about 8 minutes, or until almost tender.
2. While the pasta is cooking, heat olive oil in a skillet over medium heat. Add the onion, and cook for a few minutes until soft, then add the garlic, and cook until fragrant. Stir in the sardines with their sauce. When the sardines heat through, reduce heat to low, and simmer until the pasta is ready.

3. When the pasta is almost done, drain, and add it to the sardine sauce. Stir, cover, and turn the heat off. Let stand for a few minutes to absorb the flavors of the sauce. Squeeze juice from the lemon over the pasta. Divide onto serving plates, and top with red pepper flakes and grated Parmesan cheese.

Makes 4 servings

Salmon Salad Sandwich (adapted from http://www.cooks.com/rec/search/0,1-0,salmon_sandwich,FF.html)

INGREDIENTS

- 1, 213g can of salmon (or any other canned fish)
- Enough low-fat mayonnaise or cream cheese to hold salmon together (try 2-4 tbsp)
- ¼ cup diced celery
- ½ tsp lemon juice
- ¼ tsp dried dill
- ¼ cup (4 tbsp) chopped walnuts (optional)
- Bread, tortilla wraps, bagels, English muffins, etc
- Lettuce
- Pepper to taste

DIRECTIONS

- Drain salmon and flake with a fork.
- Mix in mayonnaise/cream cheese, celery, lemon juice, dill, walnuts and pepper.
- Spread on your favourite bread, top with lettuce and refrigerate until serving.
- Try adding other ingredients to change the flavour of your sandwich such as relish, chopped onions, olives, tomatoes, cucumbers, pickles and apples.

Fish Stock (adapted from http://allrecipes.com/Recipe/Fish-Stock/Detail.aspx)

INGREDIENTS

- 5 cups water
- 1/2 pound of firm fillets, cubed (ex: cod, halibut)
- 1 stalk celery, cut into 2 inch pieces
- 1 small onion, quartered and sliced thickly
- 1 bay leaf
- 1/2 teaspoon salt
- 1/2 teaspoon ground black pepper

DIRECTIONS

1. Combine water, fish, celery, onion, bay leaf, and salt and pepper in a large pot. Bring to rolling boil over high heat. Reduce heat to low, cover, and cook for approximately 1/2 hour or until fish falls apart.
2. Strain broth thoroughly. Be especially careful to remove all bones. Use as directed in any recipe that calls for stock or fish stock.
3. To make into a soup try adding the following to the fish stock and let simmer:

- Asian noodles (ex: rice or egg noodles, follow directions on package)
- Seaweed (purchase dried, break a few sheets into pieces and add to soup)

- Low sodium soy sauce and sesame oil to taste (start with small amounts ~1/2 tsp)
- Asian vegetables ex: bok choy, mushrooms
- Garlic, miso paste (an Asian soy paste) and other flavouring agents

SELECTING FISH BASED ON EPA, DHA and MERCURY CONTENT

Select fish with the highest EPA+DHA and the lowest Mercury content (Tier 1 fish) most often

Fish/Seafood with VERY HIGH EPA+DHA Content (more than 1.2 g (1200mg) of EPA + DHA / 100g fish)

Fish Species and Description	**g DHA / 100 g fish**	**g EPA / 100 g fish**	**g DHA + EPA/ 100 g fish**	**Tier**	**Mercury Concentration (ppm)**
Fish, anchovy, raw	0.911	0.538	1.449	1	0.043[a]
Fish, anchovy, canned in oil, drained	1.292	0.763	2.055	1	0.043[a]
Fish, herring, Atlantic, kippered	1.179	0.970	2.149	1	0.140[b]
Fish, herring, Atlantic, cooked	1.105	0.909	2.014	1	0.140[b]
Fish, herring, Pacific, cooked	0.883	1.242	2.125	1	0.040[b]
Fish, mackerel, Atlantic, cooked	0.699	0.504	1.203	1	0.220[b]
Fish, mackerel, Pacific and jack, mixed species, cooked	1.195	0.653	1.848	1	0.088[b]
Fish, sablefish, cooked	0.920	0.867	1.787	1	0.220[a]
Fish, sablefish, smoked	0.945	0.891	1.836	1	0.220[a]
Fish, salmon, Atlantic, farmed, cooked	1.457	0.690	2.147	1	0.140[b]
Fish, salmon, Atlantic, wild, cooked	1.429	0.411	1.840	1	0.140[b]
Fish, salmon, Chinook, cooked	0.727	1.010	1.737	1	0.040[b]
Fish, salmon, Coho, farmed, cooked	0.871	0.408	1.279	1	0.040[b]
Fish, salmon, pink (humpback), fillet, cooked	0.751	0.537	1.288	1	0.040[b]
Fish, salmon, pink (humpback), canned	1.083	0.604	1.687	1	0.040[b]
Fish, salmon, sockeye (red), fillet, cooked	0.700	0.530	1.230	1	0.040[b]
Fish, salmon, sockeye (red), canned	1.113	0.739	1.852	1	0.040[b]
Fish, tuna, fresh, bluefin, cooked	1.141	0.363	1.504	2	0.380[b]
Fish, whitefish, mixed species (ex: lake whitefish), cooked	1.206	0.406	1.612	1	0.069[a]
Mollusks, oyster, Pacific, steamed	0.500	0.876	1.376	1	0.060[b]

Fish with HIGH EPA+DHA Content (0.8-1.2 g (800-1200mg) of EPA + DHA /100g fish)

Fish Species and Description	g DHA / 100 g fish	g EPA / 100 g fish	g DHA + EPA/ 100 g fish	Tier	Mercury Concentration (ppm)
Fish, bass, striped, cooked	0.750	0.217	0.967	1	0.219 [a]
Fish, bluefish, cooked	0.665	0.323	0.988	1	0.337 [a]
Fish, salmon, chum, canned	0.702	0.473	1.175	1	0.040 [b]
Fish, salmon, Coho, wild, cooked	0.658	0.401	1.059	1	0.040 [b]
Fish, sardine, Atlantic, canned in oil	0.509	0.473	0.982	1	0.016 [a]
Fish, smelt, rainbow, cooked	0.536	0.353	0.889	1	Not Determined
Fish, swordfish, cooked	0.681	0.138	0.819	2	0.976 [a]
Fish, trout, mixed species, cooked	0.677	0.259	0.936	1	0.072 (freshwater) [a]
Fish, trout, rainbow, farmed, cooked	0.820	0.334	1.154	1	0.072 [a]
Fish, trout, rainbow, wild, cooked	0.520	0.468	0.988	1	0.072 [a]
Fish, tuna, albacore (white), canned in water	0.629	0.233	0.862	1*	0.353 [a]

*Albacore tuna has specific consumption guidelines for pregnant, breastfeeding or women of child-bearing age and children.

Fish/Seafood with MODERATE TO LOW EPA+DHA CONTENT (less than 0.8g (800 mg) of EPA+DHA/100g fish)

Fish Species and Description	g DHA / 100 g fish	g EPA / 100 g fish	g DHA + EPA/ 100 g fish	Tier	Mercury Concentration (ppm)
Crustaceans, crab, Alaska king, cooked	0.118	0.295	0.413	1	0.060 [a]
Crustaceans, crab, blue, cooked	0.231	0.243	0.474	1	0.060 [a]
Crustaceans, crab, Dungeness, cooked	0.113	0.281	0.394	1	0.150 [b]
Crustaceans, crab, queen (snow), cooked	0.145	0.332	0.477	1	0.060 [a]
Crustaceans, lobster, northern, cooked	0.031	0.053	0.084	1	0.310 [a]
Crustaceans, shrimp, mixed species, cooked	0.144	0.171	0.315	1	Not Detectable [a]
Crustaceans, spiny lobster, mixed species, cooked	0.139	0.341	0.480	1	0.169 [a]
Fish, catfish, farmed, cooked	0.128	0.049	0.177	1	0.049 [a]
Fish, catfish, wild, cooked	0.137	0.100	0.237	1	0.049 [a]
Fish, cod, Atlantic, cooked	0.154	0.004	0.158	1	0.060 [b]
Fish, cod, Pacific, cooked	0.173	0.103	0.276	1	0.110 [b]

Fish, flatfish (flounder and sole), cooked	0.258	0.243	0.501	1	0.045 [a]
Fish, grouper, cooked	0.213	0.035	0.248	1	0.465 [a]
Fish, haddock, cooked	0.162	0.076	0.238	1	0.031 [a]
Fish, hake (Whiting), mixed species, cooked	0.235	0.283	0.518	1	0.014 [a]
Fish, halibut, Atlantic and Pacific, cooked	0.374	0.091	0.465	1	0.252 [a]
Fish, ocean perch (rockfish), Pacific, cooked	0.262	0.181	0.443	1	0.220 [a]
Fish, ocean perch, Atlantic, cooked	0.271	0.103	0.374	1	0.080 [b]
Fish, perch, mixed species, cooked	0.223	0.101	0.324	1	0.140 [a]
Fish, pike, northern, cooked	0.095	0.042	0.137	1	Not Determined
Fish, pike, walleye, cooked	0.288	0.110	0.398	1	Not Determined
Fish, pollock, Atlantic, cooked	0.451	0.091	0.542	1	0.020 [b]
Fish, orange roughy, cooked	0	0.002	0.002	2	0.554 [a]
Fish, salmon, chinook, smoked/lox	0.267	0.183	0.450	1	Not Determined
Fish, sea bass, mixed species, cooked	0.556	0.206	0.762	1	0.219 [a]
Fish, shark (dog fish), mixed species, fried	0.431	0.258	0.689	2	0.990 [a]
Fish, snapper, mixed species, cooked	0.273	0.048	0.321	1	0.190 [c]
Fish, tilapia, cooked	0.130	0.005	0.135	1	0.010 [a]
Fish, tuna, light, canned in oil, drained solids	0.101	0.027	0.128	1	0.118 [a]
Fish, tuna, light, canned in water, drained solids	0.223	0.047	0.270	1	0.118 [a]
Fish, tuna, skipjack, fresh, cooked	0.237	0.091	0.328	1	0.205 [a]
Fish, tuna, yellowfin, fresh, cooked	0.232	0.047	0.279	1	0.325 [a]
Mollusks, clam, cooked	0.146	0.138	0.284	1	0.010 [b]
Mollusks, mussel, blue, cooked	0.506	0.276	0.782	1	<0.150 [c]
Mollusks, oyster, farmed, cooked	0.211	0.229	0.440	1	<0.050 [c]
Mollusks, oyster, wild, cooked	0.291	0.260	0.551	1	0.013 [a]
Mollusks, scallop, mixed species, fried	0.103	0.086	0.180	1	0.050 [a]
Mollusks, squid (calamari), fried	0.380	0.162	0.542	1	0.070 [a]

Other Traditional Sources of EPA and DHA

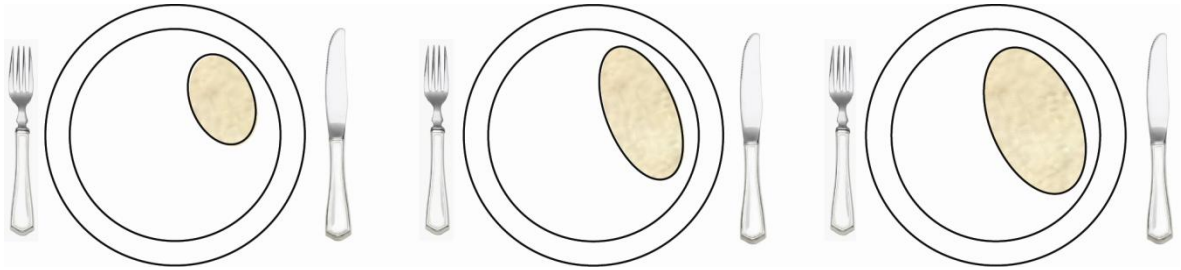
Source	Serving Size	(g) DHA	(g) EPA	(g) DHA + EPA
Fish broth/Stock	250 mL (1 cup)	0.121	0.086	0.207
Seaweed, agar, dried	125ml (1/2 cup) = 7.9 g	0	0.007	0.007
Seaweed, agar, raw	125ml (1/2 cup) = 42.3 g	0	0.003	0.003
Seaweed, dulse (laver, nori), dried	125ml (1/2 cup) = 7.9 g	0	0.040	0.040
Seaweed, dulse (laver, nori), raw	125ml (1/2 cup) = 42.3 g	0	0.034	0.034
Seaweed, irishmoss, raw	125ml (1/2 cup) = 42.3 g	0	0.019	0.019
Seaweed, kelp (kombu, tangle), raw	125ml (1/2 cup) = 42.3 g		0.002	0.002
Seaweed, wakame, raw	125ml (1/2 cup) = 42.3 g	0	0.079	0.079

Sources:

EPA and DHA content:
Canadian Nutrient File, version 2007b. http://www.hc-sc.gc.ca/fn-an/nutrition/fiche-nutri-data/index_e.html. 2007
United States Department of Agriculture. http://www.health.gov/dietaryguidelines/dga2005/report/HTML/table_g2_adda2.htm. 2004.

Mercury:

a) Canadian Nutrient File, version 2007b. http://www.hc-sc.gc.ca/fn-an/nutrition/fiche-nutri-data/index_e.html. 2007.
b) Sunderland EM. (2007). Mercury exposure from domestic and imported estuarine and marine fish in the U.S. seafood market. *Environ Health Perspect* 115:235-242.
c) Mozaffarian D, Rimm EB. Fish intake, contaminants, and human health: evaluating the risks and the benefits. JAMA 2006;296:1885-99.



***A**: Deck of cards/cheque book sized portion = 3oz / 90g*

***B**: 5oz / 150g*

***C**: 7oz / 200g*

Traditional Whole Foods (Fish): Summary Sheet

Cardiovascular benefits associated with two omega-3 fatty acids:

- Eicosapentaenoic Acid (*EPA*) and Docosahexaenoic Acid (*DHA*)

Goal: **1 gram/day of EPA + DHA** from fish and shellfish

How to accomplish goal:

- Substitute fish and shellfish into your regular diet in place of meat, poultry or meat alternatives
- Do not make other significant changes to your diet for 12 months
- Select fish with high EPA+DHA and low mercury content (Tier One Fish) most frequently
- Select types of fish that fit your food budget
- EPA+DHA intake should *average* to 1gram/day (intake can be more than 1g on some days and less on others)
- See Tables p. 28-31 for the EPA+DHA/mercury content of various fish

Summary of Health Canada Fish Consumption Guidelines with Reference to Mercury

	Source	**Max Limit Total Mercury in fish**	**Health Canada Consumption Limits**
Tier 1	Most retail fish	0.5 ppm (0.0000005g mercury/ g fish)	No consumption limits
Tier 2	Fresh/frozen tuna (not canned), shark, swordfish, escolar, marlin, and orange roughy	2.0 ppm (0.000001g mercury/ g fish)	·150g/week for adults ·150g/month: pregnant women, women of child-bearing age & breastfeeding women ·125g/month: children between 5 and 11 yrs ·75g/month: children between 1 and 4 yrs
Other	Canned albacore (white) tuna NOT light tuna	0.5 ppm (0.0000005g mercury/ g fish)	·300g/week (2, 170g cans): pregnant women, women of child-bearing age & breastfeeding women ·150 g/week: children between 5 & 11 yrs ·75 g/week: children between 1 & 4 yrs

NUTRACEUTICAL STRATEGY

Nutraceutical Selection

There are many nutraceutical products on the market that advertise their omega-3 fatty acid content. However, it is important for you to know that not all of these products contain the same amount of EPA and DHA.

Flax Seed Oil

As previously described, flax seed oil contains the omega-3 fatty acid alpha linolenic acid (ALA) but no EPA or DHA. Flax seed oil nutraceutical products highlight the importance of reading nutritional information labeling as not all products advertised as containing omega-3 fatty acids contain EPA and DHA. You are **not** being asked to consume flax seed oil nutraceuticals in this study.

Essential Oil Blends

These products contain fatty acids which are considered essential because they are not synthesized by your body and therefore must be obtained through diet or supplements.

A common essential oil blend is "Omega 3-6-9" which contains fatty acids omega-3, 6 and 9 (even though omega 9 is not considered essential). Accordingly, this product contains many fatty acids in addition to EPA and DHA and therefore the percent of EPA and DHA is fairly low. Typically, these essential oil blends contain about 72mg EPA and 48 mg DHA per 1200 mg of oil. These nutraceuticals are **not** the most cost effective means to increase your EPA + DHA intake.

Fish Oil Blends

Fish oil blends, such as salmon oil (blended with other fish oils like sardine and anchovy) are also available. These products supply approximately 180 mg of EPA and 120 mg of DHA per 1000 mg of oil and therefore 3-4 capsules would be needed per day to supply approximately 1000 mg of EPA + DHA. More concentrated EPA + DHA formulations are available and therefore, these less concentrated products are not the most effective means to increase your EPA + DHA intake.

More concentrated fish oil blends are typically derived from Anchovy, Mackerel and Sardine oils and contain approximately 300 mg of EPA and 200 mg of DHA per 1000 mg of oil. Two of these capsules per day will allow you to meet the 1000 mg/day target of EPA + DHA and therefore may be the most cost effective means to supply EPA + DHA in nutraceutical form.

There will be a few varieties of fish oil nutraceuticals that may have higher concentrations of DHA relative to EPA by the inclusion of tuna oil. It is important to examine the cost per gram of EPA + DHA of various nutraceutical to determine the most cost effective source.

See chart on page 32 for the most cost effective products to obtain EPA+DHA and where to purchase these products. Continue to check your local grocery and drug stores for new nutraceutical products which may provide EPA+DHA more cost effectively.

Taking Nutraceuticals

Some individuals experience a fishy taste after consuming fish oil capsules. It is recommended that you consume the fish oil capsules with food to prevent the fishy taste. Many people find that the longer they consume these fish oil capsules, the less noticeable the fishy taste becomes. Also, when consuming more than one fish oil capsule a day, it may help to spread your intake throughout the day as opposed to consuming all capsules at once.

For those who have difficulty swallowing large (1000 mg) capsules, soft chews containing EPA+DHA and liquid fish oil formulations are also available. Unfortunately the soft chews have a much lower concentration of EPA+DHA and as a result, fish oil in capsule form is more cost effective. Some liquid fish oil formulations contain a high concentration of EPA+DHA and are a more cost effective option. Taking capsules with sufficient liquids may also help swallowing issues.

Your goal is to ingest approximately 1000 mg of EPA+DHA per day. Read the label of the nutraceutical product you select in order to determine how much of the product you need to take to meet this daily goal. Your EPA + DHA consumption may be less than 1 g on some days and greater than 1 g on others but average to 1 g/day over the course of a week.

Costs Associated with Nutraceutical Source of EPA + DHA

Product Category	Brand/Product	Serving Size	EPA+DHA (mg) /serving	Cost ($) / 1000mg EPA+DHA			
				Sobeys	Zehrs	Shoppers Drug Mart	Pharma Plus
400 EPA:200 DHA Capsules	Webber Naturals Omega-3 Super Concentrate	1065 mg	600	0.41		0.38	0.33
	Jamieson Omega-3 Complete	1065 mg	600	0.31	0.44	0.35	0.35
	Genuine Health Omega-3 extra strength	1000 mg	600		0.75		
	Life Brand Omega-3 Super Concentrate	1000 mg	600			0.31	
	Inno-Vite Fish Oil Trio Concentrate	1000 mg	600		0.43		
300 EPA:200 DHA Capsules	Swiss Natural Sources omega-3 triple fish blend	1000 mg	500	0.60	0.56	0.29	0.27
	Jamieson Omega-3 Select	1000 mg	500	0.27		0.30	0.31
	Compliments Omega-3	1000 mg	500	0.13			
	Rexall Naturals Omega-3	1000 mg	500				0.31
	Life Brand Omega-3	1000 mg	500			0.42	
Other Formulation Capsules	Presidents Choice Mind	1100 mg	620 (90 EPA, 530 DHA)		0.27		
	Presidents Choice Body	1100 mg	700 (400 EPA, 300 DHA)		0.24		
Soft Chews	Webber Naturals Omega-3 soft chews	5 g	100			3.20	3.00
	Life Brand Omega-3 soft chews	5 g	100			2.80	
Liquid	Webber liquid omega-3 super concentrate	5mL (1tsp)	1525				0.47

Costs presented above were collected August 2007/January 2008 and may change over time. These costs do not include any applicable taxes. Keep checking your local grocery store for new products containing EPA and DHA

Nutraceutical Dietary Advice Package
Summary Sheet

Cardiovascular benefits associated with two omega-3 fatty acids:

- Eicosapentaenoic Acid (*EPA*) and Docosahexaenoic Acid (*DHA*)

Goal:

- **1 gram/day of EPA + DHA** from Fish oil capsule (Nutraceutical) Supplementation

How to accomplish goal:

- Consume enough fish oil capsules to supplement your diet with 1 gram/day of EPA+DHA
- Do not make other significant changes to your diet for 12 months
- See Tables p. 32 to help you select fish oil capsules with the highest EPA+DHA content for the lowest cost

Fish oil capsule consumption:

- Take fish oil capsules with meals
- Spread fish oil capsule consumption throughout the day

FUNCTIONAL FOOD STRATEGY

What are Functional Foods?

A*ccording to Health Canada a functional food:*

- resembles to or may be a conventional food
- is consumed as part of a usual diet
- has physiological benefits and/or reduces the risk of chronic disease beyond basic nutritional functions.

An example of a functional food is omega-3 yogurt. In this case, EPA and DHA have been purified from fish oil and added to regular yogurt. This process of adding EPA + DHA from fish oil (or from algae) is also used in omega-3 juices, bread, milk and soy milk, oil-based spreads, peanut butter and cheese. The fish oil added to these functional foods has been purified to remove any mercury or other contaminants. Another example of omega-3 functional foods are omega-3 eggs. In this case, chickens or cows are fed a special diet to increase the EPA+DHA in their eggs.

How Can I Incorporate Omega-3 Functional Foods into my Diet?

Functional foods are intended to be substituted for their normal counterpart in your diet. The best way to learn about the EPA + DHA content of the foods you purchase is to read the nutritional information on the packaging. Highlighted below are some products available at grocery stores in the KW area that contain added EPA + DHA. Our goal is not to convince you to purchase specific products and brand-names but to educate you to make your own informed food choices.

Omega-3 Eggs

Omega-3 eggs can be an excellent source of EPA + DHA however, it is still important to read nutritional labels to get the most EPA + DHA for the lowest cost.

Liquid egg products tend to contain the highest amount of EPA + DHA out of all of the omega-3 functional foods (up to 0.5 g EPA + DHA per two egg serving). Another benefit is that some liquid egg products contain less dietary cholesterol than regular-shelled eggs. The Heart and Stroke Foundation of Canada recommends that your daily dietary cholesterol intake does not exceed 300mg. A two egg serving of liquid egg products typically contains 80 mg of dietary cholesterol, well within these daily cholesterol maximums. These products taste the same and have the same baking properties as regular shelled eggs. For use, follow instructions on the package.

Shelled omega-3 eggs typically contain less EPA + DHA per serving than the liquid egg products but are preferable for making foods like hard boiled eggs. If a typical egg contains roughly 200 mg of cholesterol it may not be advisable to consume two or more shelled eggs every day. However, a two egg serving a couple times a week will allow you to keep your dietary cholesterol within recommendations.

Other Omega-3 Functional Foods.

There are various omega-3 *yogurt, milk, soy milk, bread, cheese, peanut butter, bread, juice and oil-based spread (margarine)* products on the market. Be sure to read nutritional labels to select the brand with the most EPA + DHA. Many products advertise their omega-3 content on the packaging. However, if the packaging does not list the amount of EPA + DHA or the ingredient list does not include "fish oil" or "DHA from algae" then the omega-3 fatty acids likely consist solely of alpha linolenic acid

(ALA), usually from flax seed or canola oil. You should focus on consuming functional foods with added EPA and DHA.

Product Containing EPA+DHA:

Ingredients

Pasteurized orange juice, encapsulated fish oil (anchovy and sardine oil, fish gelatin, sodium polyphosphate, sodium ascorbate, soybean oil, canola oil, natural flavour, tocopherols, citric acid, transglutaminase).

MEG-3™ trust the source™

Product with NO EPA+DHA

Ingredients

Milk, cream, modified milk ingredients (milk and whey proteins), blackberries, sugar, modified cornstarch, carrageenan, pectin, flax oil, natural flavour, rosemary extract, lemon juice concentrate, colour and active bacterial cultures (S. Thermophilus, L. Bulgaricus, L. Acidophilusand Bifidum).

Costs Associated with Functional Foods

Functional foods are meant to replace their regular counterparts in your diet. For instance, omega-3 eggs or omega-3 liquid eggs can replace regular eggs in your diet with little additional cost. The researchers have previously assessed the potential additional costs associated with the consumption of 0.6 g of EPA + DHA per day through functional food strategies. This strategy does not substantially increase daily food cost ($9.87) as compared with a daily diet of similar products with no EPA and DHA ($8.96). It is important to note that since this cost analysis was performed, additional EPA + DHA functional foods have come on the market and it is becoming easier to add EPA and DHA to your diet in a more cost-effective manner.

Not all functional foods are created equally. The table on page 33 provides detailed information about the cost associated with getting 1 g of EPA+ DHA from each of the omega-3 functional foods on the market. Keep reading product labels to watch for new EPA+DHA-containing foods that appear on the market. While the researchers encourage you to substitute as many functional foods as possible into your diet, we also encourage you to pick the sources with the highest EPA + DHA content most often to maximize your intake and keep costs manageable over the long term.

RECIPES (Functional Foods)

The recipes shown below are intended to illustrate realistic ways to incorporate EPA + DHA functional foods into your typical diet. Substitute these functional foods into your favourite recipes in place of their regular counterparts.

For example, see http://www.eggs.ca/recipes/recipes.asp for more egg recipe ideas!

Western Omelette
(adapted from http://www.eggs.ca/recipes/Show_recipe.asp?actionID=392&NumPage=3)

INGREDIENTS

100mL	Naturegg, Breakfree liquid omega-3 eggs (or 2 omega-3 eggs)
2 tbsp	water
1 tsp	Becel, Omega-3 margarine with EPA+DHA
¼ cup	finely chopped lean ham
2 tbsp	chopped red and green pepper
1 tbsp	finely chopped onion
1	shredded or cubed Black Diamond Smart Growth Cheese String

Salt and pepper to taste

DIRECTIONS
1. Beat together eggs and water and season with salt and pepper.
2. Heat margarine over medium heat in an 8" (20 cm) non-stick pan.
3. Sauté ham, peppers and onion for 2 minutes or until tender.
4. Pour in egg and add cheese. As egg sets, gently lift cooked portion at edges with a spatula to allow uncooked egg to flow underneath.
5. Once cooked, fold omelette in half with spatula and serve

Makes 1 serving: ~545 mg EPA+DHA

Broccoli Pie
(adapted from http://www.eggs.ca/recipes/Show_recipe.asp?actionID=392&NumPage=3)

INGREDIENTS

	Becel, Omega-3 margarine with EPA+DHA
	Salt and pepper
½ cup	chopped green onions
1 clove	garlic, finely chopped
1 ½ cups	cooked chopped broccoli
½ cup	low fat cottage cheese
300 mL	Naturegg, Breakfree liquid omega-3 eggs (or 6 omega-3 eggs)
½ cup	Nielson, Dairy Oh! Milk
½ cup	variety baking mix (ex: Bisquick)
1 tbsp	each crumbled goat cheese or light cream cheese and grated Parmesan cheese (or try shredded Black Diamond, Smart Growth Cheese Strings)

DIRECTIONS (Broccoli Pie)
1. Grease 9" pie plate and a non-stick skillet with margarine.
2. Sauté green onions and garlic for 5 minutes or until transparent over medium heat.

3. Stir in cooked broccoli.
4. Layer broccoli mixture and cottage cheese in pie plate.
5. Whisk eggs with milk and baking mix and season with salt and pepper.
6. Pour egg mixture over broccoli and cottage cheese in pie plate.
7. Sprinkle top with goat and parmesan cheese.
8. Bake for 25 minutes at 350 degrees F or until a knife comes out clean.

Makes 4 servings: ~383 mg EPA+DHA per serving

Fudgy Brownies
(adapted from http://www.eggs.ca/recipes/Show_recipe.asp?actionID=392&NumPage=3)

INGREDIENTS
Brownies:

1 ¼ cups	all purpose flour
1 tsp	baking powder
½ tsp	salt
¾ cup	Becel, Omega-3 margarine with EPA+DHA
¾ cup	cocoa powder
1 cup	each packed brown sugar and white sugar
200 mL	Naturegg, Breakfree omega-3 liquid eggs
2 tsp	vanilla extract
1 cup	chopped walnuts (optional)

Icing:

2 tbsp	Becel, Omega-3 margarine with EPA+DHA
¼ cup	cocoa powder
½ tsp	vanilla extract
2 cups	sifted icing sugar
¼ cup	Nielson, Dairy Oh! Milk

DIRECTIONS
1. Blend together flour, baking powder and salt and set aside.
2. Melt margarine in microwave.
3. Stir cocoa into margarine and beat in brown sugar, white sugar, eggs and vanilla.
4. Stir in dry ingredients and walnuts.
5. Grease 9" square pan with Becel, Omega-3 margarine with EPA+DHA.
6. Spread batter into pan and bake for 40 minutes at 350 degrees F.
7. To make icing, melt margarine in microwave and stir in cocoa, vanilla, icing sugar and milk.
8. Beat until smooth.
9. Allow brownies to cool before spreading on icing.

Makes 16 servings: ~128 mg EPA+DHA per serving

Breakfast Smoothie
INGREDIENTS

½ cup	Nielson Dairy Oh! Milk
100 g	Danone Danino Omega-3 yogurt
½	banana
½ cup	any other fruit

DIRECTIONS

1. Blend in a blender until smooth.
2. Try adding or substituting in omega-3 ice cream, omega-3 orange juice or frozen fruit and/or ice cubes for a frozen smoothie, etc
3. Try freezing mixture in a popsicle maker for homemade popsicles.

~45mg EPA+DHA / smoothie

Orange Juice Muffins (adapted from http://www.cooksrecipes.com/diabetic/tangy_orange_juice_muffins_recipe.html)

INGREDIENTS

2 cups	all-purpose flour
3/4 cup	sugar
2 tsp	baking powder
1/8 tsp	salt
1 cup	President's Choice, Oh Mega J orange juice
4 tbsp	Becel, Omega-3 margarine with EPA+DHA, melted
50 mL	Naturegg, Breakfree omega-3 liquid eggs
1 tsp	grated orange peel

DIRECTIONS

1. Combine flour, sugar, baking powder and salt.
2. Stir in orange juice, melted margarine, egg and orange peel until all ingredients are just moist.
3. Spoon batter into muffin cups, filling 2/3 full.

Bake for 20-25 minutes at 375 degrees F or until wooden toothpick comes out clean from centre.

Makes 9 muffins: ~67mg EPA+DHA per muffin.

Indonesian Chicken (adapted from **http://allrecipes.com/Recipe/Erins-Indonesian-Chicken/Detail.aspx**)

INGREDIENTS

- 1 cup uncooked long grain brown rice
- 2 cups water
- 1 pound (~450g) fresh green beans, trimmed and snapped
- 2 teaspoons olive oil
- 1 pound (~450 g) skinless, boneless chicken breasts - cut into chunks
- 3/4 cup low-sodium chicken broth
- 1/3 cup Life Brand smooth peanut butter
- 2 teaspoons honey
- 1 tablespoon low sodium soy sauce
- 1 teaspoon red chili paste (optional)
- 2 tablespoons lemon juice
- 3 green onions, thinly sliced
- 2 tablespoons chopped peanuts (optional)

DIRECTIONS

1. Cook rice in water.
2. Steam green beans for 10 minutes, or until tender but crisp (or microwave beans).
3. Heat the oil in a skillet, and cook the chicken 5 minutes on each side, or until juices run clear.

4. Mix the chicken broth, peanut butter, honey, soy sauce, chili paste, lemon juice in a saucepan over medium heat. Cook and stir 5 minutes, until slightly thickened. Mix in the green beans. Serve over rice. Garnish with green onions and peanuts.

Makes 4 servings: ~75 mg EPA+DHA / serving

Sample Daily Diet

Meal	**Food**	**Amount**	**mg EPA+DHA**
Breakfast	Naturegg, Breakfree omega-3 liquid eggs	100g	500
	Becel, omega-3 margarine with EPA+DHA	1 tsp	25
	President's Choice, Oh Mega J orange juice	1 1/2 cup	75
	Wonder+, Headstart Bread with DHA	2 pieces	15
	Life Brand peanut butter with EPA+DHA	2 tbsp	50
	Banana	1 medium	
Snack	Danone, Danino omega-3 yogurt	100g pot	40
	Apple	1 medium	
Lunch	Wonder+, Headstart Bread with DHA	2 pieces	15
	Becel, omega-3 margarine with EPA+DHA	2 tsp	75
	Low sodium lean ham slices	1 ounce	
	Black Diamond, Smart Growth Cheese String	1	20
	Romaine lettuce	2 pieces	
	Tomato	3 slices	
	President's Choice, Oh Mega J orange juice	1.5 cup	75
Snack	Danone, Danino omega-3 yogurt	100g pot	40
	Baby carrots	10	
Dinner	Skinless chicken breast	3 ounces	
	Mixed green salad	1.5 cups	
	Oil and vinegar salad dressing	1.5 tbsp	
	Brown rice	1 cup	
	Becel, omega-3 margarine with EPA+DHA	1 tsp	25
	So Good Omega-3 DHA, soymilk	1 1/2 cup	48
TOTAL			**~1000mg (1g)**

Costs Associated with Functional Food Source of EPA + DHA

CATEGORY	BRAND	EPA+DHA (mg)/ serving	Serving Size	Cost at Various Locations			
				Sobey's	**Zehrs**	**Food Basics**	**Shoppers**
Milk	Nielson Dairy Oh! 1%, 2%	10	250 mL	6.29/ 4L	5.89/ 4L		
	Nielson Dairy Oh! Homo	10	250 mL	6.89/ 4L	4.59/ 4L		
	Silk Plus Omega-3, soymilk	30	250 mL	4.39/ 1.89L	4.29/ 1.89L		
	So Good Omega-3 DHA, soymilk	32	250 mL			3.77/ 1.89L	
Yogurt	Danone Danino	40	100 g	3.19/ 4x100g	2.99/ 4x100g	2.97/ 4x100g	
	Danone Danino Go yogurt drink	20	93 g	5.49/ 8x93g	4.99/ 8x93g	4.97/ 8x93g	
Margarine	Becel Omega-3 Plus Margarine (10g=2tsp)	50	10 g	4.79/ 680g	4.77/ 680g	4.77/ 680g	
Juice	Tropicana Essentials Omega-3	20	250 mL	4.49/ 1.89L		4.29/ 1.89L	
* froz. concentrate	Minute Maid Fruit Solutions Omega-3 (mango, orange, passion)	20	250 mL	3.7/ 1.89L	1.69/ 355mL*	1.67/ 355mL*	
	Lassonde Oasis Blueberry & Concord Grape	20	250 mL	4.29/ 1.89L		3.97/ 1.89L	
	PC Oh Mega J (orange juice)	50	250 mL		3.79/ 1.89L		
Eggs	Naturegg Breakfree omega-3 (liquid)	250	50 g	3.69/ 500g	3.69/ 500g	2.97/ 500g	
1 egg = 50 g	Naturegg Omega-3 eggs	75	50 g	3.69/ 12x50g	3.79/ 12x50g		
	Naturegg Omega-3 Pro eggs	125	50 g	3.99/ 12x50g	3.89/ 12x50g		
	Complements Omega-3	~75	50 g	3.59/ 12x50g			
	PC Omega-3	~75	50 g		3.49/ 12x50g		
	Gray Ridge large eggs	85	50 g			3.17/ 12x50g	
	Gray Ridge x-large eggs (chol=225mg)	102	60 g			3.37/ 12x60g	
Peanut Butter	Life Brand, Smooth Peanut Butter	25	30 g				2.99/ 500g
Cheese	Black Diamond, Smart Growth Cheese Strings	20	21 g	6.49/ 252g	6.29/ 252g		
Bread	Wonder+, Headstart Bread with DHA	15	75 g	$3.19/ 675g	$2.99/ 675g		

Costs presented above were collected August 2007/January 2008 and may change over time. These costs do not include any applicable taxes.
Keep checking your local grocery and health food stores for new products containing EPA and DHA.

Functional Foods Dietary Advice Package
Summary Sheet

Cardiovascular benefits associated with two omega-3 fatty acids:

- Eicosapentaenoic Acid (*EPA*) and Docosahexaenoic Acid (*DHA*)

Goal:

- **1 gram/day of EPA + DHA** from functional foods

How to accomplish goal:

- **Substitute** EPA+DHA-enriched functional foods into your regular diet in place of their regular counterparts (ex: omega-3 eggs substituted for regular chicken's eggs)
- Do not make other significant changes to your diet for 12 months
- Select functional foods with the highest EPA+DHA content most frequently
- Select EPA+DHA-enriched functional foods that best fit your food budget
- See Table p. 33 for the EPA+DHA content of various functional foods
- Become familiar with reading nutritional labels: 'omega-3' doesn't mean the product contains EPA+DHA
    - Omega-3 from flaxseed: no EPA+DHA
    - Omega-3 from a fish oil or algae source: look for EPA+DHA content on the label
- Check your local supermarket regularly for new EPA+DHA-enriched products

University of Waterloo

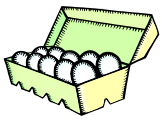
Men and Women Aged 35-50 Needed

For research on nutritional strategies involving omega-3 fatty acids

Benefits to the Participant:

- Receive individual diet counselling
- Individual dietary assessment (including caloric and nutrient analysis) and cardiovascular disease risk factor assessment
- Learn how to include omega-3 fatty acids into your diet
- Learn about the potential health benefits of omega-3 fatty acids

Up to $200 participant remuneration



Participant Procedures:

- Visit the University of Waterloo 7 times over 1 year
- Record all food consumed for 3-day intervals on 6 occasions
- Collect duplicate amounts of food consumed for 3-day interval once
- Provide blood samples collected by a certified technician on 6 visits

For more information, or to volunteer please contact:

The Laboratory of Nutraceutical and Nutritional Research
Department of Kinesiology
University of Waterloo
Phone – (519) 888-4567 ext 37873
Email – apatters@uwaterloo.ca

This project has been reviewed and received ethics clearance through the Office of Research Ethics, University of Waterloo, Waterloo. (519) 888-4567 ext. 36005

ADVERTISEMENT APPEARING IN KITCHENER-WATERLOO PENNYSAVER:

NUTRITION/ OMEGA-3 fatty acid research at the University of Waterloo. Participants aged 35-50 needed. Up to $200 remuneration. Participants must collect a diet sample, keep 6 food diaries, provide 6 blood samples to certified technicians over 7 visits over 1 year. Call (519)888-4567 x37873 or email apatters@uwaterloo.ca for details. This project was reviewed and received ethics clearance through the Office of Research Ethics, University of Waterloo.

INFORMATION AND CONSENT FORM

Laboratory of Nutritional & Nutraceutical Research
Department of Kinesiology
University of Waterloo

Title of Project: **Evaluation of the Adherence to Nutraceutical, Functional Food and Whole Food Strategies to Increase Omega-3 Fatty Acid Intakes in Men and Women at Risk for the Development of Cardiovascular Disease**

Principal Investigator: **Ken D. Stark**
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 37738

Co-Investigator: **Rhona Hanning**
University of Waterloo, Department of Health Studies
(519) 888-4567 Ext. 35685

Student Investigator: **Ashley Patterson**
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 37873

Online Information: Visit http://www.n3pufa.org or email apatters@uwaterloo.ca

Purpose of Study:

Omega-3 fatty acids, particularly the longer chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) usually found in fish can help prevent heart disease. Most Canadians do not eat fish and eat low amounts of these important fatty acids.

In the present study, you will receive dietary advice from a dietician or a nutritional science graduate student supervised by a licensed dietician about how to increase the amount of EPA and DHA in your diet by different strategies. You will be randomly assigned to advice based either on whole foods (foods naturally containing EPA and DHA such as fish), functional foods (new manufactured food products with EPA and DHA added), nutraceutical (encapsulated oils with EPA and DHA) or a combination of all strategies. We will compare the effectiveness of the advice over 1 year by determining how much EPA and DHA you have eaten. For us to determine your EPA and DHA intake, you will be asked to provide blood samples and complete dietary records and questionnaires at various time points over one year. You will also be asked to provide a 3-day food sample on one occasion so that omega-3 fatty acids can be directly measured as well as look at what other nutrients are associated with increased omega-3 intake.

Specific details about the study procedures will be provided in the following section.

Procedures Involved in this Study:

As a participant in this study, you will be asked to complete the following:

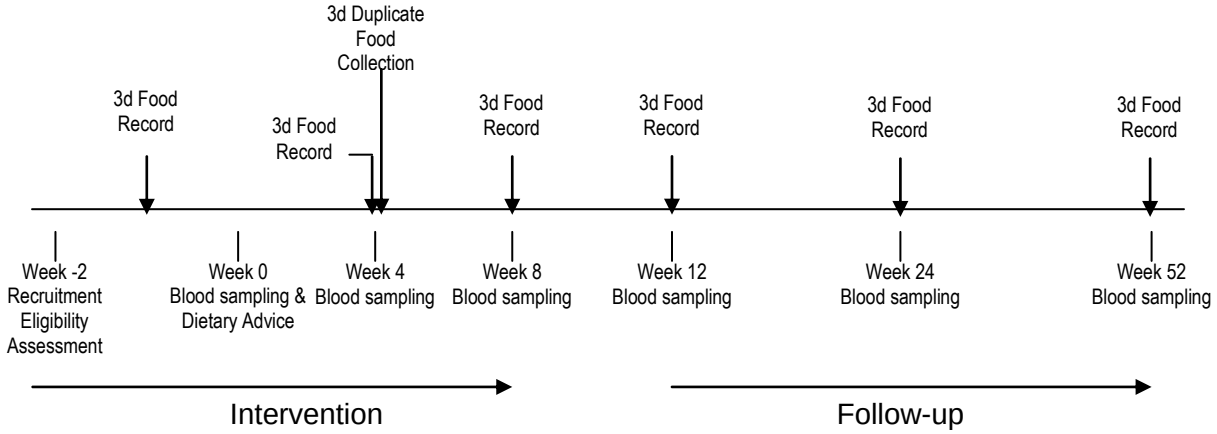
1. Fast overnight before providing a finger-prick blood sample to determine eligibility for this study. The fast includes no food or drink (water is allowed) 8-12 hours before the sample is collected.
2. Complete a questionnaire about your eating habits and a health screening form during the initial screening for study eligibility (2 weeks before study outset). The 3-day dietary record you receive at this time can be completed and returned to the investigators at week 0.
3. Have your height, weight, resting heart rate and blood pressure measured on each visit following initial eligibility assessment.
4. You are asked to advise the researchers of any changes to your medications or if you begin taking any medications during the study.
5. Refrain from donating blood or participating in any other study (ex: involving diet modification, stress, physical activity, etc) 14 days before Week 0 until week 52.
6. Fast overnight and then have about 20mL of venous blood taken (see details in explanation of procedure and risks). Have your resting heart rate, blood pressure height and weight measured on Week 0.
7. Receive dietary advice to increase omega-3 fatty acid intake on Week 0.
8. Follow dietary advice provided by the researchers over the subsequent 52 weeks. Since you will be assigned to one of four approaches to increase omega-3 fatty acid intake you will be asked to not make diet changes other than those assigned.
9. After fasting overnight, have venous blood sampling at weeks 4, 8, 12, 24 and 52. Completed 3-day dietary records will be collected by the researchers at these times. Resting heart rate, blood pressure and weight will also be measured.
10. Collect duplicates of all food consumed in provided containers over the course of 3 days. Please temporarily store the food in a refrigerator or freezer and bring with you on your week 4 visit.
11. Allow your blood to be analyzed for cardiovascular disease risk parameters and other biochemical markers including fatty acids, total and HDL cholesterol and glucose.

Further information about each of these tests is presented on the following sheets. All tests will be completed at our laboratory at the University of Waterloo.

Time Commitment:

The maximum time requirement for these tests will be 15-30 min in the laboratory on days when venous blood samples and completed dietary records are collected (Weeks 4, 8, 12, 24 and 52). The venous blood sample and completed dietary record collection as well as nutritional advice on Week 0 take approximately 45 minutes to 1 hour.

Study Timeline



Personal Benefits of Participation:

The purpose is to determine which of four strategies is most effective in increasing omega-3 intake and blood levels of omega-3 fatty acids to a desirable level. You will receive education including background information regarding omega-3 fatty acids, what foods contain them and the potential health benefits of eating omega-3 fatty acids. Information on this study, previous studies and omega-3 fatty acids can be obtained at http://www.n3pufa.org.

We will provide information regarding your personal omega-3 blood status as well as your blood lipid profiles, including cholesterol, in comparison to current health recommendations. You will receive this information at the completion of the study. You will also be asked to complete 3-day dietary assessments and food frequency questionnaires. These assessments, along with nutritional advice provided by the researchers, may help you to examine your eating behaviours and to assist you in making healthy food choices.

Detailed Explanation of Procedures and Risks:

Three-Day Dietary Records and Food Frequency Questionnaire – You will be asked to complete a 3-day dietary record and food frequency questionnaire before returning to the laboratory for dietary advice and blood sampling. Subsequent 3-day dietary records will also be requested at weeks 4, 8, 12, 24 and 52. Forms and detailed instructions are attached. The dietary record is a detailed list of everything you eat and drink for 3 days. This information will allow us to compare your diet to the results of the other tests. The food frequency questionnaire will pose 49 questions in a checkbox format about your usual eating patterns over the last 12 months to estimate your habitual intake of EPA and DHA fatty acids. This questionnaire will also assess the frequency of meals prepared at home versus purchased prepared meals and/or restaurant meals, your involvement in grocery purchases, food selections, as well as food preparation, and grocery shopping. Finally, the questionnaire will pose three sociodemographic-based questions including your cultural/racial background, your educational background and your annual household income.

Dietary Advice – Dietary advice strategies are assigned randomly. You will receive individual diet counseling by a licensed dietician or a nutrition graduate student supervised by a licensed dietician via structured interviews and a take home document about the benefits of omega-3 fatty

acids. Ashley Patterson will contact you at week 1 of the study to answer any questions you may have regarding the dietary advice. Ashley will also contact you on weeks 3 and 7 and 11 to answer any questions and confirm scheduling for return visits to the laboratory. For the visits at week 24 and 52, we will contact you 1 month in advance to schedule a visit and then call you the week before to remind you.

Venous Blood Sampling – This is similar to blood samples taken by your physician. Venous blood will be collected under sterile conditions with an 18-21 gauge needle from a vein in your arm and will be collected into sterile glass tubes. For each visit approximately 20 mL (~4 tsp) of blood will be collected. This procedure may result in slight bruising and bleeding. This can be minimized by the application of direct pressure to the point of needle entry into the vein. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Blood lipids (fats) levels including cholesterol and omega-3 fatty acids as well as blood glucose levels will be determined. Venous blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products.

Fingertip Prick Blood Sampling –This sample will be taken during the eligibility assessment to determine your baseline omega-3 fatty acid status. This process involves a small and sterile needle pricking the finger and blood being collected on a small strip of paper. There is a slight risk of bruising with this technique. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Fingertip prick blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products.

Encapsulated Fish Oil Consumption – Dietary advice strategies are assigned randomly. Some participants will be assigned to the nutraceutical or combined strategy that recommends that 2-3 fish oil capsules be eaten daily for the duration of the study. It is recommended that the capsules be consumed with meals throughout the day. Eating oil capsules may cause nausea and loose stools in some participants. Others may have an increased incidence of "burping" and minor stomach discomfort. Participants who have difficulty swallowing pills or capsules may not be able to complete the study.

Three-Day Food Duplicate Collection – You will be asked to collect duplicates of all the meals and snacks, including drinks, you consume over the course of three days. Detailed instructions are listed below. You will refrigerate or freeze this food collection at home until you return it to the lab during week 4. This food collection will be used to directly analyze the fatty acid content of your diet as well as the presence of any compounds associated with fish consumption.

INSTRUCTIONS FOR THREE-DAY FOOD DUPLICATE COLLECTION

1. Select two weekdays and one weekend day for your food duplicate collection. (Same three days as the food record returned on week 4)
2. Please do not change your normal diet on collection days.
3. Please store your food and beverage collection in large plastic containers. We request that for ALL food, drinks and snacks you eat (including condiments, dressings, etc) you place the same amount in the plastic containers. You may omit water in your collection.
4. Please store your containers in the refrigerator or freezer until you return them to the lab for your visit on week 4.
5. Please take care to be as accurate as possible when collecting food duplicates. For instance, if you do not finish a meal please only add the amount eaten to the plastic container. Using measuring cups, kitchen scales, etc will be helpful.
6. Please inform the researchers if there were any omissions or errors in your food collection.

Special Instructions:

Participants are asked to follow their normal diet until dietary advice is given by the researchers. Participants are asked to consume only the sources of omega-3 fatty acids advised by the researchers and avoid all other sources. You are asked to advise the researchers of any changes to your medications or if you begin taking any medications during the study. Please refrain from donating blood or participating in other experiments (ex: diet modification, stress or physical activity studies) during this study. Participants are asked to fast overnight (water allowed) and refrain from drinking alcohol in the 24-hour period immediately prior to testing.

Health Screening Form:

This questionnaire asks some questions about your health status. This information is used to guide us with your entry into the study and ensure your suitability for the study. If you have kidney problems, diabetes or cardiovascular disease including bleeding disorders, you will not be eligible to participate in the study.

Changing Your Mind about Participation:

Your participation is voluntary. You may withdraw from this study at any time without penalty. To do so, indicate this to the researcher or one of the research assistants by saying, "I no longer wish to participate in this study". Once you withdraw, we will ask if you are willing to provide more information about what motivated your decision to withdraw. If you agree to provide more information, we will ask you to identify the specific reason or reasons for your decision to withdraw. This can help us to identify problems in the study that we did not anticipate. You do not have to provide any answers regarding your decision to withdraw.

Confidentiality:

To ensure the confidentiality of individuals' data, each participant will be identified by an identification code known only to the principal investigator and his research assistants. All data including questionnaires will be entered and blood samples will be stored under this code. Once

data have been entered under an anonymous code and the feedback provided, the paper records and code sheet will be confidentially shredded.
Testing will be performed under private conditions where only laboratory personnel are present. No spectators will be allowed during the testing period. Blood samples will be stored at -80ºC in a secure location in the Department of Kinesiology. After biochemical analyses and data interpretation is complete, any remaining blood samples will be disposed in compliance with procedures of the Safety Office of the University of Waterloo.

Participant Feedback:

After the study is completed, you will be provided with a feedback sheet that will include your personal measurements and current recommendations based on scientific literature.

Remuneration:

You will receive a total of $200 remuneration for completing the study. Partial payment will be provided upon early withdrawal. The sum will be proportional to the length of time and the number of procedures you undertake. This is broken down into $25 per visit with blood sample collection (6 visits after eligibility assessment) and food diary collection and $50 for the food duplicate collection. This is provided to cover the cost of the food duplicate collection, transportation and time commitment of the participant.

Contact Information:

If you have any questions about the study at any time, please contact either Professor Ken Stark at his office (519) 888-4567 ext. 37738, Rhona Hanning at extension 35685 or Ashley Patterson at extension 37873, or the Lab Assistants at ext. 37873.

Concerns about Your Participation

I would like to assure you that this study has been reviewed and received ethics clearance through the Office of Research Ethics. However, the final decision about participation is yours. If you have any comments or concerns resulting from your participation in this study, you may contact the Director, Office of Research Ethics at (519) 888-4567 ext. 36005.

CONSENT FORM

I agree to take part in a research study being conducted by Professor Ken Stark, Professor Rhona Hanning and Ashley Patterson of the Department of Kinesiology, University of Waterloo. I have made this decision based on the information I have read in the Information letter. All the procedures, any risks and benefits have been explained to me. I have had the opportunity to ask any questions and to receive any additional details I wanted about the study. If I have questions later about the study, I can ask one of the researchers (Professor Ken Stark at his office (519) 888-4567 ext. 37738, Professor Rhona Hanning at extension 35685 or Ashley Patterson at extension 37873) or by emailing apatters@uwaterloo.ca.

I understand that I may withdraw from the study at any time without penalty by telling the researcher.

This project has been reviewed by, and received ethics clearance through, the Office of Research Ethics at the University of Waterloo. I am aware that I may contact this office (888-4567, ext. 36005) if I have any concerns or questions resulting from my involvement in this study.

Printed Name: _____

Local Address: _____

Phone #: _____ Birth Date: _____

Signature: _____

_____ _____

Dated at Waterloo, Ontario Witnessed

COMPLETING THE DIETARY RECORD

- Record all food and beverages consumed for 3 days (including one weekend day).
- Do not alter your intake during the recording period.
- Record if you are taking a vitamin and mineral supplement.
- Be sure to include beverages (non-alcoholic and alcoholic) and all snack foods.
- Record what you are eating <u>at the time</u> of the meal and not afterwards.
- Be sure to include brand names wherever possible.
- Remember: try to estimate less and measure more. The use of measuring cups/spoons will be helpful.
- Recipes may be attached. Please indicate how much of the recipe you ate (ex: 1/5 of a casserole recipe).
- Include the source of the meal (ex: home cooked, Burger King, etc.) in the appropriate column.

There is a wrong way	and a right way
2 hot dogs	2 enriched white hot dog buns (Wonder®) 2 (75g each) boiled all beef wieners (Shopsy's®) 2 tbsp ketchup 1 tbsp mustard
1 glass of milk	1 10 fl. oz. glass of 2% milk (Neilson, Dairy Oh!®)
1 large apple	1 Granny Smith apple (diameter 3")
1 small salad with ranch dressing	1 small salad: 1 cup of shredded iceberg lettuce ¼ cup chopped, raw carrots 4, ¼" thick slices of tomato 1" cube low fat cheddar cheese (Kraft®) 4 tbsp ranch dressing (Hidden Valley®)
1 yogurt	100g fat free strawberry yogurt, sweetened with aspartame (Astro®)

DIETARY ANALYSIS INTAKE FORM

Day 1
Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 2

Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
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24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 3

Date: _____ Name: _____

Please Consult your instruction sheets for directions on how to fill out this form

List of Foods

No.	Amount	Detailed Description of Food	Source
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
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32			

HEALTH SCREENING FORM
Evaluation of the Adherence to Nutraceutical, Functional Food and Whole Food Strategies to Increase Omega-3 Fatty Acid Intakes in Men and Women at Risk for the Development of Cardiovascular Disease

Past Health Problems:

Rheumatic Fever	( )	Epilepsy	( )
Heart Murmur	( )	Varicose Veins	( )
High Blood Pressure	( )	Disease of Arteries	( )
High Cholesterol	( )	Emphysema, Pneumonia,	
Congenital Heart Disease	( )	Asthma, Bronchitis	( )
Heart Attack	( )	Back Injuries	( )
Heart Operation	( )	Kidney and liver disease	( )
Diabetes (diet or insulin)	( )	Heartburn	( )
Ulcers	( )	Bleeding disorders	( )
Bleeding from Intestinal Tract	( )	Lipid Metabolism disorders	( )
Enteritis/colitis/diverticulitis	( )	Other (describe overleaf)	( )

Present Health:

List current problems:	List medications taken now or in last 3 months:
1.	1.
2.	2.
3.	3.

List Symptoms:

Irregular Heart Beat	( )	Fatigue	( )
Chest Pain	( )	Cough Up Blood	( )
Short of Breath	( )	Back Pain/Injury	( )
Persistent Cough	( )	Leg Pain-Injury	( )
Wheezing (asthma)	( )	Dizziness	( )

For females: Pregnant ____ Nursing ___

Are you currently taking any oral contraceptives? Yes ( ) No ( )

When was the last day of your menses? _____
How long does your cycle generally last? _____ days

Smoking:

Never ( ) Ex-smoker ( ) Regular ( ): Average # cigarettes/day ____

Birth Date (mm/dd/yy): _____

Dietary Habits:

Do you take vitamin-mineral or other supplements regularly? Yes ( ) No ( )
If so, please list:

Type (ex: multivitamin) **Brand** (ex: Centrum) **Frequency** (ex: daily) **Amount** (ex: 1, 500mg capsule)

1.
2.
3.

Do you follow a vegetarian, vegan or restricted diet? Yes ( ) No ( )
If yes, please list all foods restricted from your diet (ex: dairy, eggs, fish):

Do you have any food allergies/intolerances? Yes ( ) No ( )
If yes, please list:

Current Physical Activity Status:

I consider my physical activity status to be: High ( ), Average ( ), Low ( ).

List the activities that you do on a regular basis:

Type (ex: jogging)	**Frequency** (ex: 3x/week)	**Duration** (ex:30 min)
1.		
2.		
3.		
4.		

Signature of Subject:_____
Witness:_____
Date:_____

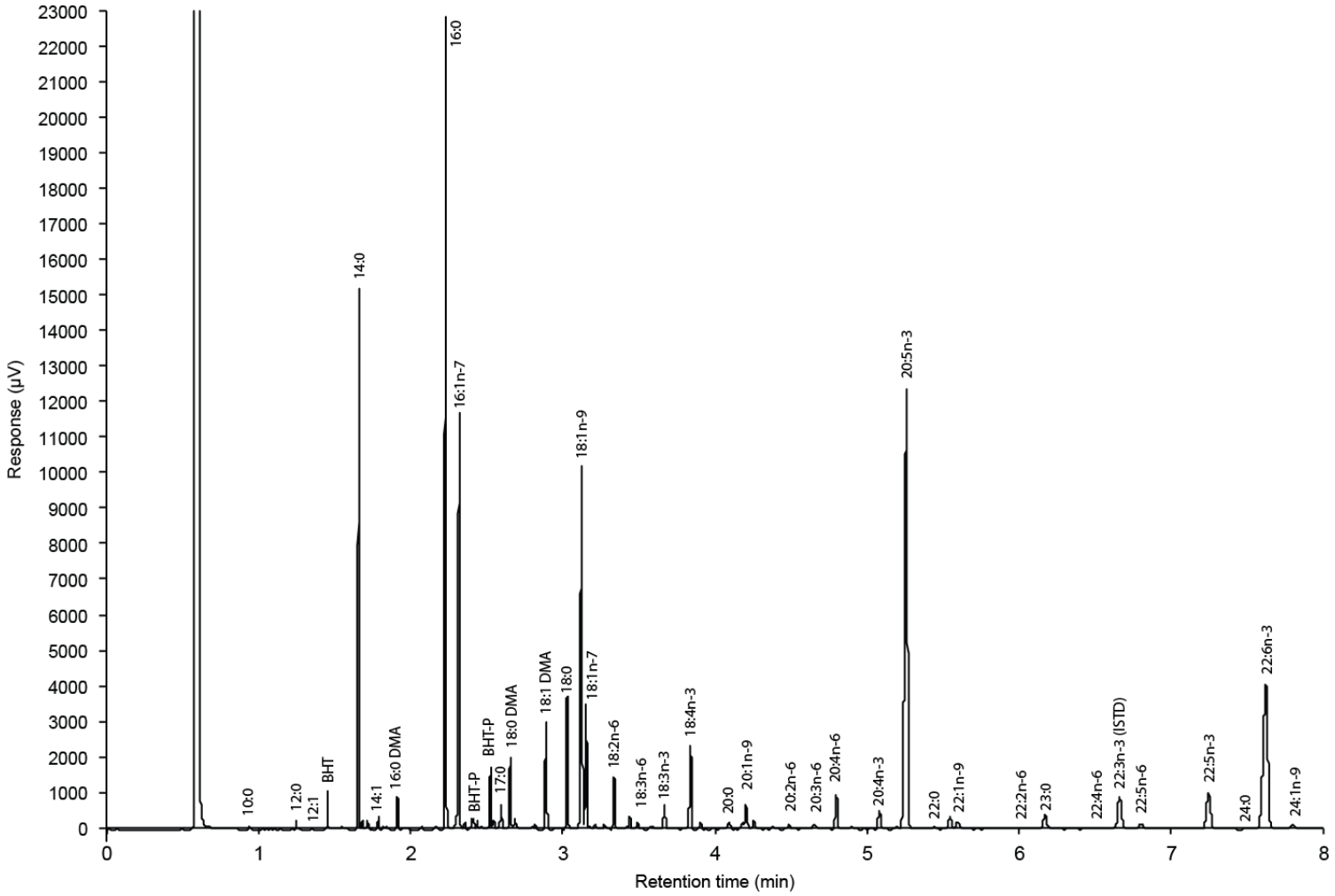
The current study has been identified as requiring medical clearance:
Yes_____ No___X__

APPENDIX C

Appendix Table C.1. Fatty acid composition of fish oil capsules for dose-response study[1]

Fatty Acid	Weight % of total fatty acids	Concentration (g/capsule)
Saturated Fatty Acids	29.8 ± 0.7	0.24 ± 0.01
14:0	7.3 ± 0.1	0.06 ± <0.01
16:0	15.7 ± 0.4	0.13 ± 0.01
16:0 dma	0.6 ± <0.1	<0.01 ± <0.01
17:0	0.7 ± <0.1	0.01 ± <0.01
18:0	3.6 ± 0.3	0.03 ± <0.01
18:0 dma	1.5 ± <0.1	0.01 ± <0.01
20:0	0.2 ± <0.1	<0.01 ± <0.01
22:0	0.1 ± <0.1	<0.01 ± <0.01
23:0	<0.1 ± <0.1	<0.01 ± <0.01
24:0	<0.1 ± <0.1	<0.01 ± <0.01
Monounsaturated Fatty Acids	24.8 ± 0.3	0.20 ± 0.01
14:1	<0.1 ± <0.1	<0.01 ± <0.01
16:1n-7	8.3 ± 0.1	0.07 ± <0.01
18:1 dma	2.5 ± 0.1	0.02 ± <0.01
18:1n-7	3.0 ± 0.1	0.02 ± <0.01
18:1n-9	9.5 ± 0.2	0.08 ± <0.01
20:1n-9	0.8 ± <0.1	0.01 ± <0.01
22:1n-9	0.3 ± <0.1	<0.01 ± <0.01
24:1n-9	0.3 ± <0.1	<0.01 ± <0.01
n-6 Polyunsaturated Fatty Acids	3.6 ± 0.1	0.03 ± <0.01
18:2n-6	1.3 ± <0.1	0.01 ± <0.01
18:3n-6	0.2 ± <0.1	<0.01 ± <0.01
20:2n-6	0.2 ± <0.1	<0.01 ± <0.01
20:3n-6	0.1 ± <0.1	<0.01 ± <0.01
20:4n-6	1.2 ± <0.1	0.01 ± <0.01
22:2n-6	<0.1 ± <0.1	<0.01 ± <0.01
22:4n-6	0.1 ± <0.1	<0.01 ± <0.01
22:5n-6	0.3 ± <0.1	<0.01 ± <0.01
n-3 Polyunsaturated Fatty Acids	36.4 ± 0.6	0.29 ± 0.01
18:3n-3	0.7 ± <0.1	0.01 ± <0.01
18:4n-3	2.4 ± 0.1	0.02 ± <0.01
20:3n-3	0.1 ± <0.1	<0.01 ± <0.01
20:4n-3	0.8 ± <0.1	0.01 ± <0.01
20:5n-3	19.2 ± 0.5	0.15 ± <0.01
22:5n-3	2.4 ± <0.1	0.02 ± <0.01
22:6n-3	10.8 ± <0.1	0.09 ± <0.01
Total	94.6 ± 0.5	0.75 ± 0.02

[1] Values are means ± sd, (n=3).



Appendix Figure C.1. Gas chromatogram of fish oil capsule fatty acid composition. Analysis on a DB-FFAP 15m capillary column with temperature program as follows: initial, 150°C with a 0.25 min hold; ramp: 35°C/min - 200°C, then by 8°C - 225°C with a 3.2 min hold, then by 80°C/min - 245°C with a 15 min hold. BHT: butylated hydroxytoluene, DMA: dimethyl acetal, ISTD: internal standard.

Men and Women Ages 18-35 Needed
For A Nutrition Research Study

Purpose: To improve how omega-3 fatty acid intakes are measured.

You may be eligible if you:

- Eat fish less than once a week and do not take fish oil supplements
- Do not have allergies or sensitivities to fish
- Do not have diabetes, cardiovascular disease or lipid metabolism disorders

Participants will be asked to:

- Attend a baseline screening (including a fingertip blood sample)
- Consume an omega-3 fatty acid dietary supplement daily for 2 months
  Supplements are commercially available and participants will be asked to consume two different doses over the 2 month period
- Fast from food for 8-12 hrs and provide a fingertip blood sample (0.2mL) on 6 visits over 2 months (visits will last up to 20 min each)
- Fast from food for 8-12 hrs and provide a venous blood sample (20mL) collected by certified technician on 3 visits (visits will last 30-45 minutes each)
- Record all food consumed for 3 days once and complete an omega-3 fatty acid intake questionnaire twice

Participants will receive up to $75 remuneration

Benefits to the participant:

- Receive individual dietary and heart disease risk factor feedback
- Learn about dietary nutrients and related health benefits

For more information, or to volunteer please contact:

The Laboratory of Nutraceutical and Nutritional Research
Department of Kinesiology, University of Waterloo
Email – apatters@uwaterloo.ca, Phone – (519) 888-4567 x37873

This project has been reviewed and received ethics clearance through the Office of Research Ethics, University of Waterloo, Waterloo.

Email Recruitment Script

Mailing Lists Targeted: Applied Health Science and Graduate Student Listservs
Email Subject Line: Seeking Participants for a Nutrition Research Study

Email Text:
The Laboratory of Nutritional and Nutraceutical Research in the Department of Kinesiology is looking for *male and female* participants between the *ages of 18 and 35* for a nutrition research study.

Study Description:
Dietary omega-3 fatty acids can help prevent heart disease. However, many Canadians do not eat enough omega-3 fatty acids to meet current recommendations. The Laboratory of Nutritional and Nutritional Research is conducting a research study to improve how omega-3 fatty acid intakes are measured.

You may be eligible if you:

- Eat fish less than once a week and do not take fish oil supplements
- Do not have allergies or sensitivities to fish
- Do not have diabetes, cardiovascular disease or lipid metabolism disorders

Participant will be asked to:

- Attend a baseline screening (including a fingertip blood sample)
- Consume an omega-3 fatty acid dietary supplement daily for 2 months
  Supplements are commercially available and participants will be asked to consume two different doses over the 2 month period
- Fast from food for 8-12 hrs and provide a fingertip blood sample (0.2mL) on 6 visits over 2 months (visits will last up to 20 min each)
- Fast from food for 8-12 hrs and provide a venous blood sample (20mL) collected by certified technician on 3 visits (visits will last 30-45 minutes each)
- Record all food consumed for 3 days once and complete an omega-3 fatty acid intake questionnaire twice

Participants will receive up to $75 remuneration

Benefits to the participant:

- Receive individual dietary and heart disease risk factor feedback
- Learn about dietary nutrients and related health benefits

For more information or to volunteer please contact:
The Laboratory of Nutraceutical and Nutritional Research
Department of Kinesiology, University of Waterloo
Phone – (519) 888-4567 ext 37873
Email – apatters@uwaterloo.ca
This project has been reviewed and received ethics clearance through the Office of Research Ethics, University of Waterloo, Waterloo.

INFORMATION AND CONSENT FORM

Laboratory of Nutritional & Nutraceutical Research
Department of Kinesiology
University of Waterloo

Title of Project: **Assessment of blood measures of omega-3 highly unsaturated fatty acids in response to current intake recommendations in men and women.**

Faculty Supervisor: **Professor Ken D. Stark**
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 37738

Student Investigators: **Ashley Patterson, PhD Student**
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 37873
apatters@uwaterloo.ca

Alan Chalil
University of Waterloo, Department of Kinesiology
(519) 888-4567 Ext. 37873

Purpose of Study:
Omega-3 fatty acids, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in seafood, can help prevent heart disease. It is currently recommended that healthy individuals consume 250-500 milligrams (mg) per day of EPA and DHA combined for optimal health. This amount of EPA and DHA is equal to 2 servings of oily fish (120g/4oz each) per week. Many Canadians do not regularly eat seafood or other EPA and DHA sources and do not meet these recommendations.

The amount of omega-3 fatty acids in an individual's diet is reflected by the amount of omega-3 fatty acids in their blood. The goal of this study is to examine how to use a blood sample to measure omega-3 fatty acid intakes in men and women.

Specific details about the study procedures are provided in the following section.

Procedures Involved in this Study:
As a participant in this study, you will be asked to:

Screening Procedures:

1. Fast overnight before providing a fingertip prick blood sample to determine eligibility for this study. The fast includes no food or drink (water is allowed) for 8-12 hours and no alcohol for 24 hours before the sample is collected.
2. Complete questionnaires about your omega-3 fatty acid intake and a health status during the initial screening for study eligibility.

Study Procedures:

3. During the study, continue to consume your usual diet and do not make changes. In particular, do not eat seafood or enriched foods / dietary supplements containing EPA and DHA, other than those provided by the researchers. An example of an enriched food is an omega-3 egg.
4. Follow your usual physical activity regime during the study and do not make significant changes.
5. Advise the researchers of any changes to your medications or if you begin taking any medications during the study.
6. Refrain from donating blood or participating in any other study (eg: involving diet modification, stress or physical activity) upon entry into the study.
7. Fast overnight and have approximately 20mL (4 tsp) of venous blood taken at baseline and at weeks 4 and 8 and a fingertip prick blood sample (0.2 mL) on weeks 1-3 and 5-7 (see details in explanation of procedure and risks).
8. At baseline, receive instructions for filling out a 3-day dietary record. Completed records can be returned to the investigators at the week 1 visit.
9. Have your height, weight, resting heart rate and blood pressure measured at baseline and at weeks 4 and 8. Blood pressure and heart rate will be measured using an automatic monitor.
10. Consume a total of 250 mg/d of EPA+DHA (1000 mg/d of fish oil) in the first 4 weeks and 500 mg/d of EPA+DHA (2000 mg/d of fish oil) in the second 4 weeks by consuming a daily, commercially available supplement.
11. Return any unused dietary supplements provided by the researchers at weekly visits.
12. Repeat the questionnaire measuring omega-3 fatty acid intake at the week 8 visit.
13. Allow blood samples taken to be analyzed for fatty acid composition.

Further information about each of these tests is presented on the following sheets. All tests will be completed at the Laboratory of Nutritional and Nutraceutical Research at the University of Waterloo.

Time Commitment:
The maximum time requirement for these tests will be 30-45 min on days when venous and fingertip prick blood samples are collected (baseline and weeks 4 and 8). The fingertip prick blood sample on weeks 1- 3 and 5-7 will take up to 20 minutes.

Study Timeline

Eligibility Screening

-Fingertip prick blood sample
-Health screening questionnaire
-Omega-3 fatty acid intake questionnaire

Eligible participants will be invited to participate in the following:

Baseline

-Venous blood sample (20 mL)
-Measurement of height, weight, resting heart rate and blood pressure
-Instructions for 3-day dietary record

Week 1

-Fingertip prick blood sample (0.2 mL)
-Return 3-day dietary record

Weeks 2-3

-Fingertip prick blood sample (0.2 mL)

Week 4

-Venous blood sample (20 mL)
-Measurement of height, weight, resting heart rate and blood pressure
-Return unused fish oil supplements from first month

Weeks 5-7

-Fingertip prick blood sample (0.2 mL)

Week 8

-Venous blood sample (20 mL)
-Omega-3 fatty acid intake questionnaire
-Measurement of height, weight, resting heart rate and blood pressure
-Return unused fish oil supplements from second month

MONTH 1

-Take daily fish oil supplement (250mg EPA+DHA/day)

MONTH 2

-Take daily fish oil supplement (500mg EPA+DHA/day)

Study Benefits to the Scientific Community & Society:

The results of this study will be useful for establishing blood-based markers of dietary EPA and DHA intake. Such markers could be used to screen for individuals with low EPA and DHA intakes and to monitor changes in dietary intake, particularly for individuals at risk for cardiovascular disease. The information will also show whether current intake recommendations are sufficient to allow men and women to meet blood targets for preventing cardiovascular disease.

Personal Benefits of Participation:
You will receive educational information including background regarding omega-3 fatty acids, dietary sources, recommended intakes and associated health benefits.
We will provide information regarding your personal omega-3 blood status in comparison to current health recommendations. You will also receive feedback of your overall dietary and omega-3 fatty acid intakes. You will receive this information at the completion of the study. During the study, you will also be asked to complete a 3-day dietary assessment and omega-3 fatty acid intake questionnaires. These assessments, along with omega-3 recommendations provided by the researchers, may help you to examine your eating behaviours and to assist you in making healthier food choices.

Detailed Explanation of Procedures and Risks:

Three-Day Dietary Records and Omega-3 Fatty Acid Intake Questionnaire – You will be asked to complete a 3-day dietary record and omega-3 fatty acid intake questionnaire at the beginning of the study. A subsequent questionnaire will be requested on week 8. Forms and detailed instructions are attached. The dietary record is a detailed list of everything you eat and drink for 3 days. This information will provide an overview of your daily dietary intake. The omega-3 fatty acid intake questionnaire will pose 48 questions in a checkbox format about your usual eating patterns over the past months to estimate your usual intake of EPA and DHA. This questionnaire will also assess the frequency of meals prepared at home versus purchased prepared meals and/or restaurant meals, your involvement in grocery purchases, food selections, as well as food preparation, and grocery shopping. Finally, the questionnaire will pose three sociodemographic-based questions including your cultural/racial background, your educational background and your annual household income.

Venous Blood Sampling – This is similar to blood samples taken by your physician. Venous blood will be collected under sterile conditions with an 18-21 gauge needle from a vein in your arm and will be collected into sterile glass tubes. For each visit approximately 20mL or 4tsp of blood will be collected. This procedure may result in slight bruising and bleeding. This can be minimized by the application of direct pressure to the point of needle entry into the vein. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Blood fatty acid levels will be determined. Venous blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products.

Fingertip Prick Blood Sampling –This sample will be taken during the eligibility assessment to determine your baseline omega-3 fatty acid status and throughout the study. This process

involves a small and sterile needle pricking the finger and blood being collected on a small strip of paper. There is a slight risk of bruising with this technique. The use of sterile gauze and alcohol swipes minimizes the risk of infection. Fingertip prick blood sampling will be performed by trained and competent laboratory personnel following universal guidelines for handling blood and blood products.

Encapsulated Fish Oil Consumption – You are asked to consume 250mg/day of EPA+DHA during the first month and 500mg/day of EPA+DHA during the second month by consuming a fish oil supplement. It is recommended that the fish oil capsules be consumed with meals. In addition to fish oil, the supplements are composed of a softgel capsule (gelatin, glycerin and water) and a low amount of vitamin E (less than 4.5 mg/capsule). Health Canada considers this level of supplementation safe for healthy adults.
Some individuals may experience nausea and loose stools after consuming the capsules. Others may have an increased incidence of "burping" and minor stomach discomfort. Participants who have difficulty swallowing pills or capsules should consider not participating in the study.

Special Instructions:

Participants are asked to follow their normal diet and in particular, refrain from eating seafood or enriched foods / dietary supplements containing EPA and DHA, other than those provided by the researchers, upon entry into the study. An example of an enriched food is an omega-3 egg. You are asked to advise the researchers of any changes to your medications or if you begin taking any medications during the study. Please refrain from donating blood or participating in other experiments (ex: diet modification, stress or physical activity studies) during this study. Participants are asked to fast overnight (water allowed) and refrain from drinking alcohol in the 24-hour period immediately prior to testing. Please return any unused dietary supplements provided by the researchers at week 4 and 8 visits.

Health Screening Form:

This questionnaire poses some questions about your health status. This information is used to guide us with your entry into the study and ensure your eligibility for the study. If you have diabetes or cardiovascular disease including bleeding disorders or lipid metabolism disorders, you will not be eligible to participate in the study.

Changing Your Mind about Participation:

Your participation is voluntary. You may withdraw from this study at any time without penalty. To do so, indicate this to the researcher or one of the research assistants by saying, "I no longer wish to participate in this study". Once you withdraw, we will ask if you are willing to provide more information about what motivated your decision to withdraw. If you agree to provide more information, we will ask you to identify the specific reason or reasons for your decision to withdraw. This can help us to identify problems in the study that we did not anticipate. You do not have to provide any answers regarding your decision to withdraw.

Confidentiality and Security of Data:

To ensure the confidentiality of individuals' data, each participant will be identified by an identification code known only to the principal investigator and his research assistants. All data including questionnaires will be entered and blood samples will be stored under this code. Once data have been entered under an anonymous code and the feedback provided, the paper records and code sheet will be confidentially shredded.
Testing will be performed under private conditions where only laboratory personnel are present. Blood samples will be stored at -80ºC in a secure location in the Department of Kinesiology. After biochemical analyses and data interpretation is complete, any remaining blood samples will be disposed in compliance with procedures of the Safety Office of the University of Waterloo.

Participant Feedback:

After the study is completed, you will be provided with a feedback sheet that will include your personal measurements and current recommendations based on scientific literature.

Remuneration:

You will receive a total of $75 remuneration for completing the study. Partial payment will be provided upon early withdrawal. The sum will be proportional to the number of procedures you undertake. This is provided to cover the cost of transportation and time commitment of the participant.

Contact Information:

If you have any questions about the study at any time, please contact either Professor Ken Stark at his office (519) 888-4567 ext. 37738 or Ashley Patterson at extension 37873.

Concerns about Your Participation

I would like to assure you that this study has been reviewed and received ethics clearance through the Office of Research Ethics. However, the final decision about participation is yours. If you have any comments or concerns resulting from your participation in this study, you may contact Dr. Susan Sykes, Director Office of Research Ethics at (519) 888-4567 ext. 36005, ssykes@uwaterloo.ca.

CONSENT FORM

I agree to take part in a research study being conducted by Professor Ken Stark and Ashley Patterson and Alan Chalil of the Department of Kinesiology, University of Waterloo.

I have made this decision based on the information I have read in the Information letter. All the procedures, any risks and benefits have been explained to me. I have had the opportunity to ask any questions and to receive any additional details I wanted about the study. If I have questions later about the study, I can ask one of the researchers (Professor Ken Stark at his office (519) 888-4567 ext. 37738 or Ashley Patterson at extension 37873) or by emailing apatters@uwaterloo.ca.

I am aware that I may withdraw from the study at any time without penalty by telling the researcher.

This project has been reviewed by, and received ethics clearance through, the Office of Research Ethics at the University of Waterloo. I am aware that I may contact Dr. Susan Sykes, Director of the Office of Research Ethics at (519) 888-4567 ext. 36005, ssykes@uwaterloo.ca, if I have any concerns or questions resulting from my involvement in this study.

Printed Name: _____

Local Address: _____

Phone #: _____ Birth Date: _____

With full knowledge of all foregoing, I agree, of my own free will, to participate in this study.

Signature: _____

_____ _____

Dated at Waterloo, Ontario Witnessed

COMPLETING THE DIETARY RECORD

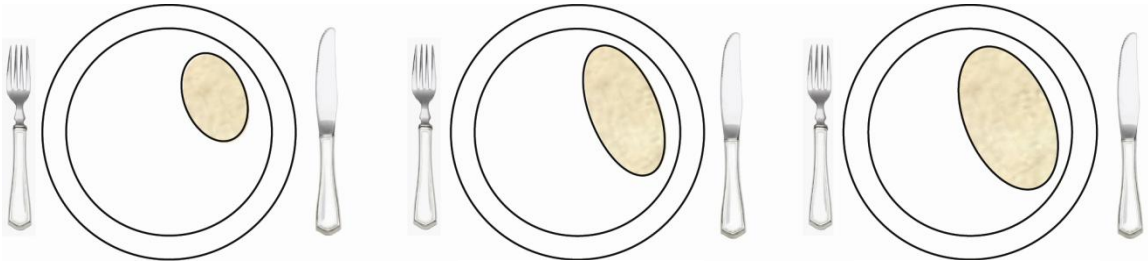
What to include:

- Record ALL food and beverages consumed for 2 weekdays and 1 weekend day:
  - Foods and beverages (non-alcoholic and alcoholic)
  - Snack foods
  - Condiments, spreads and sauces
  - Oils and seasonings used for cooking
  - Vitamin, mineral or other dietary supplements

- Include as much detail as possible:
  - Indicate the **source** of the meal (eg: home cooked, Burger King, etc.) or **brand** of purchased foods (eg: Wonder® bread) in the appropriate column.
  - Be **descriptive** and include relevant **cooking methods**.
    (eg: write '90g, lean ground beef, pan fried in 1 tsp olive oil' instead of just 'beef')

Measuring the correct amount:

- Use *measuring cups and spoons* or record amounts from food *package labels* (eg: label indicates 1 hamburger bun weighs 45g).
- Indicate whether the measurement is in **cooked or uncooked** form (eg: 1 cup uncooked macaroni versus 1 cup cooked)
- *Recipes* may be included for mixed foods (eg: baked goods, casseroles). Indicate total recipe yield and how much you ate (eg: recipe makes 6 portions, ate 1 portion).
- Guidelines for meat portions:



Deck of cards sized portion = 3oz / 90g

5oz / 150g

7oz / 200g

General Instructions:

- Do not alter your intake during the recording period.
- Record at the time of the meal instead of recalling intake afterwards.
- You are welcome to include package labels with your diet record.

The following diet record needs more detail and is an example of what NOT to do:

#	Measured Amount	Detailed Description of Food	Source/Brand
1	1	Multi-vitamin	Drug Store
2	1 bowl	Oatmeal	Homemade
3	1	Banana	
4	1	Egg	
5	1 small	Toss salad	Homemade
6	1 glass	Milk	
7	1	Steak	
8	1	Dinner roll	

Instead, please include following details:

#	Measured Amount	Detailed Description of Food	Source/Brand
1	1 capsule	Centrum Forte multi-vitamin	Centrum®
2	½ cup, *uncooked*	Original Quick Oats	Quaker®
	½ cup	Water (to prepare oatmeal)	tap
	1 tbsp (level)	Packed brown sugar	Redpath®
3	1	7" long banana	
4	1	Omega-3 egg, *pan fried*	President's Choice®
	1 tsp	Extra virgin olive oil	Bertolli®
5	1 cup	Shredded iceberg lettuce	Dole®
	4 slices (¼" thick)	Tomato	
	1 tbsp	Ranch salad dressing	Kraft®
6	320 mL	2% milk	Nielson®
7	5 oz	Rib eye steak, trim fat removed, *broiled*	Grocery deli counter
	2 tbsp	Original barbecue sauce	Kraft®
	1 dash	Table salt	Windsor®
8	1 (28g)	White dinner roll	Wonder®
	1 tsp	Salt-free margarine	Becel®

Recipe Example:

#	Measured Amount	Detailed Description of Food	Source/Brand
1	ate 1/4 of recipe below:	Stir Fry recipe:	Home made
2	4 (4 oz ea)	Boneless, skinless chicken breasts, *pan fried*	Maple Leaf®
3	1 tbsp	100% Canola oil	Canola Harvest®
4	1 cup	Diced green pepper	
5	1, 7oz can	Whole kernel sweet corn niblets	Green Giant®
6	2 tbsp	Low sodium soy sauce	Kikkoman®
7	4 cups *cooked* (1 cup *uncooked*)	Enriched, long grain, white rice	Uncle Ben's®
8	2 tsp	Salted butter	Gay Lea®

DIETARY ANALYSIS INTAKE FORM

Day 1

Date: _____

Please consult your instruction sheets for directions

#	Measured Amount	Detailed Description of Food	Source/Brand
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 2

Date: _____

Please consult your instruction sheets for directions

#	Measured Amount	Detailed Description of Food	Source/Brand
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

DIETARY ANALYSIS INTAKE FORM

Day 3

Date: _____

Please consult your instruction sheets for directions

#	Measured Amount	Detailed Description of Food	Source/Brand
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			

HEALTH SCREENING FORM

Comprehensive assessment of blood fatty acid response to sustained and variable supplementation with a low dose of omega-3 highly unsaturated fatty acids

Past Health Problems:

Rheumatic Fever	( )	Epilepsy	( )
Heart Murmur	( )	Varicose Veins	( )
High Blood Pressure	( )	Disease of Arteries	( )
High Cholesterol	( )	Emphysema, Pneumonia,	
Congenital Heart Disease	( )	Asthma, Bronchitis	( )
Heart Attack	( )	Back Injuries	( )
Heart Operation	( )	Kidney and liver disease	( )
Diabetes (diet or insulin)	( )	Heartburn	( )
Ulcers	( )	Bleeding disorders	( )
Bleeding from Intestinal Tract	( )	Lipid Metabolism disorders	( )
Enteritis/colitis/diverticulitis	( )	Other (describe overleaf)	( )

Present Health:

List current problems:	List medications taken now or in last 3 months:
1.	1.
2.	2.
3.	3.

List Symptoms:

Irregular Heart Beat	( )	Fatigue	( )
Chest Pain	( )	Cough Up Blood	( )
Short of Breath	( )	Back Pain/Injury	( )
Persistent Cough	( )	Leg Pain-Injury	( )
Wheezing (asthma)	( )	Dizziness	( )

Birth Date (mm/dd/yy): _____

Smoking:

Never ( ) Ex-smoker ( ) Regular ( ): Average # cigarettes/day ____

Alcohol Intake:

A standard drink is:
- -1 can/bottle of beer (355 ml/12 oz)
- -1 glass of wine (150 ml/5 oz)
- -1 shot of 40% hard liquor or spirits (44 ml/1.5 oz)

How often do you have 10 or more standard drinks on 1 occasion?
- ( ) Never
- ( ) Less than Monthly
- ( ) Monthly
- ( ) Weekly
- ( ) Daily or Almost Daily

Dietary Supplements:

Do you take vitamin-mineral or other supplements regularly? Yes ( ) No ( )

If yes, please list:

Type (eg: multivitamin)	**Brand** (eg: Centrum)	**Frequency** (eg: daily)	**Amount** (eg: 1, 500mg capsule)

The supplements contain the following ingredients:
- -fish oils (anchovy, mackerel, sardine and salmon)
- -gelatin
- -glycerin
- -purified water
- -vitamin E

Do you have any allergies or sensitivities these ingredients? Yes ( ) No ( )

Do you follow a vegetarian, vegan or restricted diet? Yes ( ) No ( )

If yes, please list all foods restricted from your diet (eg: fish):

Have you made any major changes to your diet over the past year? Yes ( ) No ( )
If yes, please list changes and when they took place:

Have you lost or gained weight in the past year? Yes ( ) No ( )
If yes, please describe changes and when they took place:

Current Physical Activity Status:

I consider my physical activity status to be: High ( ), Average ( ), Low ( ).

List the physical activities that you do on a regular basis:

Type (eg: jogging)	**Frequency** (eg: 3x/week)	**Duration** (eg:30 min)

Signature of Participant:_____
Witness:_____
Date:_____

Food Frequency Questionnaire

INSTRUCTIONS: Please answer the following background questions and subsequent 44 questions about your **usual** eating habits over the **past 12 months**.

Date you answered this questionnaire: _____

Age:

( ) Under 19 years	( ) 30-34 years	( ) 45-49 years	( ) 60-64 years
( ) 19-24 years	( ) 35-39 years	( ) 50-54 years	( ) 65 years and over
( ) 25-29 years	( ) 40-44 years	( ) 55-59 years	

Gender: ( ) Male ( ) Female

Sociodemographic

1. With which of the following racial/cultural groups do you most closely associate yourself?

( ) Caucasian
( ) Chinese
( ) South Asian (eg: East Indian, Pakistani, Sri Lankan)
( ) African
( ) Filipino
( ) Latin American
( ) Southeast Asian (eg: Cambodian, Indonesian, Laotian, Vietnamese)
( ) Arab
( ) West Asian (eg: Afghan, Iranian)
( ) Japanese
( ) Korean
( ) Aboriginal
( ) Other: _____

2. What is the highest level of education you have attained?

( ) Some High School
( ) High School Diploma
( ) Diploma/Certificate from trade, technical or vocational school or business college
( ) Diploma/Certificate from community college, CEGEP or nursing school
( ) Bachelor's undergraduate degree or teacher's college
( ) Master's degree
( ) Degree in Medicine, Dentistry, Veterinary Medicine, Optometry or Law
( ) Earned doctorate (eg: PhD)
( ) Other:
_____

3. What is your annual household income?

( ) less than $25,000
( ) $25,000 - $49,999
( ) $50,000 - $74,999
( ) $75,000 - $99,999
( ) more than $100,000

Diet

4. What type of milk do you usually use?

( ) I do not drink milk
( ) Regular cow's milk (non-omega-3)
( ) Regular soy milk (non-omega-3)
( ) *Nielson, Dairy Oh!* 1% or 2% milk
( ) *Nielson, Dairy Oh!* homogenized milk
( ) *Parmalat, Smart Growth* 2% milk
( ) *Parmalat, Smart Growth* homogenized milk
( ) Silk Plus, Omega-3 DHA soy milk
( ) So Good, Omega-3 DHA soy milk
( ) Other Omega-3 enriched milk
Please specify Brand:
_____

5. How much **omega-3** milk do you usually use **per day** (including milk added to tea, coffee, cereal, etc.)?

( ) None
( ) 1 tablespoon
( ) 2 tablespoons
( ) ¼ cup (62.5 mL)
( ) ½ cup (125 mL)
( ) 1 cup (250 mL)
( ) between 1-2 cups (250-500 mL)
( ) between 2-3 cups (500-750 mL)
( ) 3 cups (750 mL) or more

6. What kinds of margarine/spread do you usually use? (eg: On bread, vegetables, in cooking and baking, etc.)

( ) I don't use any margarine/spread
( ) Regular margarine (non-omega-3)
( ) *Becel*, *Omega-3 Plus* Margarine
( ) Other Omega-3 enriched margarine
Please specify brand:
_____

7. How much **omega-3** margarine/spread do you usually use **per day**?

( ) None
( ) 1-2 teaspoons
( ) 1 tablespoon
( ) 2 tablespoons
( ) 3 tablespoons
( ) 4 tablespoons
( ) 5-7 tablespoons
( ) 8 or more tablespoons

8. What kinds of eggs do you usually eat (include eggs in baked goods/cooking)?

( ) *Naturegg Breakfree* omega-3 *Pro* liquid eggs
( ) *Naturegg* Omega-3 eggs (in shell)
( ) *Naturegg* Omega-3 *Pro* eggs (in shell)
( ) *Complements* Omega-3 eggs
( ) *President's Choice* Omega-3 eggs
( ) *Gold Egg* Omega-3 eggs
( ) *Gold Egg* Omega Choice eggs
( ) *Conestoga Farms* Free Run Omega-3 eggs
( ) *Burnbrae Farms* Free Run Omega-3 eggs
( ) *Irresistables* Omega-3 eggs
( ) Regular Chicken eggs
( ) Organic eggs
( ) Other Omega-3 enriched eggs
Please specify brand:
_____

9. On average, how many eggs do you usually eat **per week (**including eggs in baked goods/cooking)?

( ) I don't eat eggs
( ) Less than 1 egg
( ) 1 to 2 eggs
( ) 3 to 5 eggs
( ) 6 or more eggs

10. What kind of juice do you usually drink?

( ) I don't drink juice
( ) Regular juice (non-omega-3)
( ) *President's Choice, Oh Mega J* orange juice
( ) *Lassonde Oasis*, omega-3 concord blueberry-grape juice
( ) *Lassonde Oasis*, omega-3 strawberry kiwi juice
( ) *Lassonde Oasis*, omega-3 orange juice
( ) Other Omega-3 enriched juice
Please specify brand:
_____

11. How much **omega-3** juice do you usually drink **per day**?

( ) None
( ) less than one cup (<250 mL)
( ) 1 cup (250 mL)
( ) between 1-2 cups (250-500 mL)
( ) between 2-3 cups (500-750 mL)
( ) 3 cups (750 mL) or more

12. What kind of yogurt do you usually eat?

( ) I don't eat yogurt
( ) Regular yogurt (non-omega-3)
( ) *President's Choice Blue Menu* strawberry yogurt smoothie
( ) *Li'l Ones* omega-3 yogurt
( ) Other Omega-3 enriched yogurt
Please specify brand:
_____

13. How much **omega-3** yogurt do you usually eat **per week**?

( ) none
( ) less than 1 single serve container
( ) 1, single serve container
( ) 2-3, single serve containers
( ) 4-5, single serve containers
( ) 6-7, single serve containers
( ) 8 or more single serve containers

14. What kind of sliced or brick cheese do you usually eat?

( ) I don't eat sliced or brick cheese
( ) Regular sliced or brick cheese (non-omega-3)
( ) *Silani* omega-3 mozzarella or provolone
( ) *Silani* omega-3 feta
( ) Other omega-3 sliced or brick cheese
Please specify brand:
_____

15. How many slices (15g / size of 2 dice) of omega-3 sliced or brick cheese do you usually eat? Include cheese in sandwiches.

( ) None
( ) Less than 1 slice per month
( ) 1-3 slices per month
( ) 1 slices per week
( ) 2-3 slices per week
( ) 4-6 slices per week
( ) 1 slice per day
( ) 2 or more slices per day

17. What kind of soft or grated cheese do you usually eat?

( ) I don't eat soft or grated cheeses
( ) Regular soft or grated cheese (not omega-3)
( ) *Silani* omega-3 grated parmesan
( ) *Silani* omega-3 ricotta
( ) *Silani* omega-3 mascarpone
( ) Other omega-3 soft or grated cheese
Please specify brand:
_____

18. How much omega-3 soft or grated cheese do you usually eat **per week**?

( ) None
( ) Less than 1 tablespoon
( ) 1-3 tablespoons
( ) 1/4-1/2 cup
( ) 1/2-1 cup
( ) 1-2 cups
( ) 3 or more cups

19. What kind of string cheese do you usually eat?

( ) I don't eat string cheese
( ) Regular string cheese (non-omega-3)
( ) *Black Diamond, Smart Growth* Cheese Strings with DHA
( ) Other omega-3 enriched string cheese
Please specify brand:
_____

17. How many omega-3 string cheese portions do you usually eat per week?

( ) None
( ) less than 1oz (less than 1 string cheese portion)
( ) 1 oz (1 string cheese portion)
( ) 2-3 oz (2-3 string cheese portions)
( ) 4-5 oz (4-5 string cheese portions)
( ) 6-7 oz (6-7 string cheese portions)
( ) 8 or more oz (8+ string cheese portions)

20. What kind of nut butter (eg: peanut) do you usually eat?

( ) I don't eat nut butter
( ) Regular nut butter (non-omega-3)
( ) *Life Brand*, Omega-3 Peanut Butter
( ) Other omega-3 enriched nut butter
Please specify brand:
_____

21. How much omega-3 nut butter do you eat per week?

( ) None
( ) less than 1 tablespoon (tbsp)
( ) 1 tbsp
( ) 2-3 tbsp
( ) 4-5 tbsp
( ) 6-7 tbsp
( ) 8 or more tbsp

22. What kind of biscuits do you usually eat?

( ) None
( ) Regular biscuits (non-omega-3)
( ) *President's Choice, Blue Menu* Blueberry Lemon with Flaxseeds whole grain biscuits
( ) Other omega-3 enriched biscuits
Please specify brand:
_____

23. How many omega-3 biscuits do you usually eat?

( ) None
( ) Less than 1 cookie per month
( ) 1-3 cookies per month
( ) 1 cookie per week
( ) 2-3 cookies per week
( ) 4-6 cookies per week
( ) 1 cookie per day
( ) 2 or more cookies per day

24. On average, how often do you eat fish paste? (eg: Surimi)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Specify the **type** of fish paste, **brand** and **amount** usually eaten (number of teaspoons)
Type of fish paste *Brand* *#tsp*
_____

25. On average, how often do you eat seaweed?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of seaweed and **amount** eaten on a typical occasion
Type of seaweed *Amount*
_____

26. On average, how often do you eat seafood-containing sauce? (eg: fish sauce, oyster sauce)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of seafood sauce, **brand** and **amount** usually eaten (# tsp)
Type of sauce *Brand* *#tsp*
_____

27. On average, how often do you eat fish-based broth (eg: fish broth-based soup)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

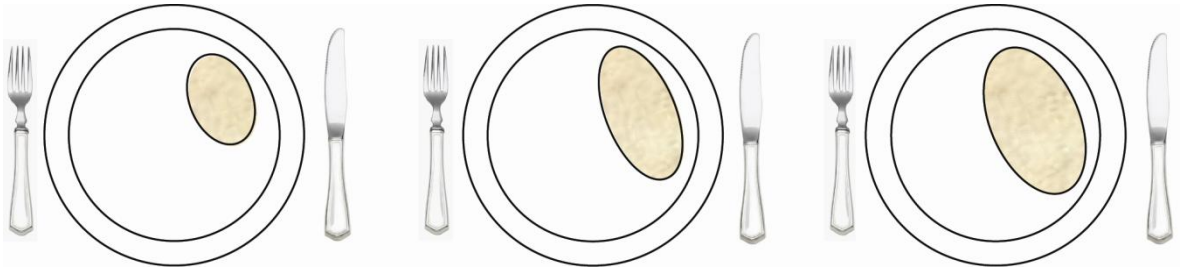
Please specify the **brand** (or 'homemade') and **amount** of fish broth usually eaten (eg: 1 cup)
Brand *Amount*
_____

28. How much of a fish oil supplement do you usually take **per day** (eg: capsules, chews, gummies, oil, powder)

( ) None
( ) Amount:
_____

Please Specify **brand**, **product name** and **type** of supplement
(eg: Jamieson, Omega-3 Complete, capsules)
Brand *Product Name* *Type*
_____
_____
_____
_____

29. When you eat meat or poultry, what size serving do you usually eat?



***A**: Deck of cards sized portion = 3oz / 90g*

***B**: 5oz / 150g*

***C**: 7oz / 200g*

( ) Less than **A**
( ) **A**
( ) Between **A** and **B**
( ) **B**
( ) Between **B** and **C**
( ) **C**
( ) More than **C**

30. On average, how often do you eat chicken or turkey?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **types** of chicken/turkey eaten most frequently and whether the **skin** is eaten (eg: Turkey thigh, with skin)

_____
_____

31. On average, how often do you eat duck or goose?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of duck/goose eaten most frequently and whether the **skin** in eaten. (eg: Duck breast, without skin)

_____
_____

32. How often do you usually eat beef?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of beef most often eaten (eg: Rump steak):

_____

33. On average, how often do you eat lamb?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of lamb most often eaten (eg: Loin chop):

_____

34. On average, how often do you eat veal?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of veal most often eaten (eg: Veal steak):

_____

35. On average, how often do you eat pork? (do not include ham or bacon)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** of pork most often eaten (eg: Pork chop):

_____

36. On average, how often do you eat bacon?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **number of slices** of bacon usually eaten on each occasion:

_____

37. On average, how often do you eat ham (not deli/sandwich meat)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **number of slices** of ham usually eaten on each occasion:

_____

38. On average, how often do you usually eat sliced deli meat (eg: luncheon meats or salami)?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** (eg: lean turkey breast), **brand** and the **number of slices** of sliced deli meat most often eaten:
Type *Brand* *# slices*

_____
_____

39. On average, how often do you eat sausages?

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** (eg: Beef, pork, turkey) and **number** of sausages usually eaten on each occasion:
Type *Number*

_____

40. On average, how often do you eat canned fish? (eg: canned tuna, salmon, sardines, etc.)

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify up to 4 **types** of canned fish that you regularly eat, including the **brand** and the **amount** typically eaten. (eg: White tuna in water, *Goldseal*, ½ 120g tin)

Type of Fish *Brand* *Amount*

_____
_____
_____
_____

41. On average, how often do you eat fresh or frozen fish? (Include fish meals at home, at a restaurant and take out).

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify up to 4 **types** of fresh or frozen fish that you regularly eat and the **amount** typically eaten. Include the **brand** for frozen fish. (eg: frozen Wild Atlantic Salmon, *Highliner*, 150g)

Type of Fish *Brand* *Amount*

_____
_____
_____
_____

42. How often do you eat fresh, frozen or canned shellfish? (eg: shrimp, oysters, scallops, crab, calamari, lobster, etc).

( ) Never
( ) less than once per month
( ) 1 to 3 times a month
( ) once per week
( ) 2 to 3 times per week
( ) 4 to 6 times per week
( ) once per day
( ) 2 or more times per day

Please specify the **type** and **amount** of shellfish eaten on a typical occasion:

Type of Shellfish *Amount*

_____
_____
_____

43. Are there any other meat, poultry or fish products that you eat regularly?

Please specify the **type, amount** and **how often** other meat or fish products are eaten

Type *Amount* *How often*

_____
_____
_____

44. Have you changed your diet in any significant way over the past year?

( ) Yes
( ) No

If yes, please specify the changes:

_____
_____
_____

Thank you for completing this survey

Appendix Table C.2. Whole blood fatty acid composition in the dose-response study (n=20)

	Week 0		Week 4		Week 8	
	Males	Females	Males	Females	Males	Females
14:0	0.75 ± 0.17	0.79 ± 0.24	0.81 ± 0.25	0.73 ± 0.20	0.80 ± 0.24	0.70 ± 0.09
16:0	21.28 ± 0.96	20.92 ± 1.45	21.13 ± 1.32	20.44 ± 1.72	20.68 ± 1.18	20.21 ± 1.10
16:0 dma[2]	1.08 ± 0.37	0.83 ± 0.07	0.86 ± 0.28	0.83 ± 0.13	1.02 ± 0.15	0.86 ± 0.11
17:0	0.31 ± 0.06	0.30 ± 0.04	0.31 ± 0.04	0.30 ± 0.02	0.31 ± 0.05	0.27 ± 0.08
18:0[1,3]	11.59 ± 1.30[a,b]	12.43 ± 0.96[a]	11.62 ± 1.52[a,b]	10.79 ± 0.62[b]	11.02 ± 0.83[a,b]	11.20 ± 1.36[a,b]
18:0 dma[1,2]	1.17 ± 0.13	0.97 ± 0.10	1.07 ± 0.47	0.98 ± 0.12	1.32 ± 0.22	1.08 ± 0.14
20:0	0.27 ± 0.05	0.30 ± 0.05	0.29 ± 0.05	0.29 ± 0.05	0.30 ± 0.06	0.38 ± 0.21
22:0	0.77 ± 0.13	0.79 ± 0.14	0.82 ± 0.24	0.88 ± 0.20	0.84 ± 0.10	0.85 ± 0.09
23:0	0.19 ± 0.02	0.19 ± 0.03	0.18 ± 0.05	0.21 ± 0.05	0.20 ± 0.03	0.21 ± 0.02
24:0	1.54 ± 0.24	1.50 ± 0.21	1.75 ± 0.84	1.69 ± 0.37	1.68 ± 0.16	1.74 ± 0.22
SFAs	39.23 ± 1.62	39.32 ± 2.07	39.14 ± 3.02	37.45 ± 1.11	38.78 ± 1.34	38.18 ± 2.26
14:1	0.03 ± 0.02	0.03 ± 0.02	0.04 ± 0.03	0.04 ± 0.02	0.05 ± 0.03	0.03 ± 0.01
16:1n-7	1.05 ± 0.48	0.85 ± 0.24	1.01 ± 0.40	0.87 ± 0.25	1.12 ± 0.40	0.87 ± 0.25
18:1 dma	0.31 ± 0.05	0.28 ± 0.06	0.28 ± 0.11	0.28 ± 0.04	0.35 ± 0.07	0.31 ± 0.05
18:1n-7	1.52 ± 0.08	1.49 ± 0.17	1.47 ± 0.11	1.44 ± 0.17	1.48 ± 0.14	1.43 ± 0.19
18:1n-9	14.54 ± 1.33	14.41 ± 1.92	14.71 ± 2.18	14.89 ± 1.57	14.98 ± 1.42	14.31 ± 1.59
20:1n-9[1]	0.22 ± 0.03	0.25 ± 0.05	0.21 ± 0.03	0.22 ± 0.03	0.21 ± 0.02	0.22 ± 0.04
22:1n-9[1,3]	0.28 ± 0.04[a,c]	0.34 ± 0.06[a]	0.16 ± 0.07[b]	0.19 ± 0.05[b,d]	0.24 ± 0.06[c,d]	0.22 ± 0.05[b,c]
24:1n-9	1.71 ± 0.21	1.69 ± 0.17	1.83 ± 0.62	1.92 ± 0.38	1.90 ± 0.29	2.00 ± 0.33
MUFAs	19.67 ± 1.65	19.36 ± 2.27	19.70 ± 1.85	19.86 ± 1.92	20.33 ± 1.55	19.39 ± 2.16
18:2n-6[2]	17.67 ± 1.86	19.37 ± 1.87	17.83 ± 3.71	21.73 ± 1.82	17.95 ± 1.58	19.76 ± 1.01
18:3n-6[1]	0.20 ± 0.05	0.16 ± 0.05	0.24 ± 0.13	0.21 ± 0.09	0.26 ± 0.09	0.19 ± 0.07
20:2n-6[1,2,3]	0.25 ± 0.03[a]	0.27 ± 0.05[a]	0.26 ± 0.10[a]	0.39 ± 0.14[b]	0.27 ± 0.03[a]	0.29 ± 0.05[a,b]
20:3n-6[1]	1.80 ± 0.53	1.72 ± 0.28	1.68 ± 0.48	1.48 ± 0.24	1.64 ± 0.40	1.55 ± 0.18
20:4n-6[1]	11.33 ± 1.49	10.38 ± 1.54	10.37 ± 1.32	9.41 ± 0.92	10.20 ± 1.39	9.60 ± 0.69
22:2n-6	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.01
22:4n-6[1]	1.68 ± 0.41	1.51 ± 0.22	1.62 ± 0.58	1.30 ± 0.14	1.43 ± 0.37	1.32 ± 0.09

22:5n-6[1]	0.38 ± 0.04	0.36 ± 0.06	0.36 ± 0.08	0.33 ± 0.04	0.33 ± 0.03	0.31 ± 0.06
n-6	33.37 ± 1.87	33.82 ± 2.05	32.40 ± 3.10	34.90 ± 1.89	32.13 ± 2.07	33.08 ± 1.44
18:3n-3	0.43 ± 0.14	0.44 ± 0.19	0.41 ± 0.15	0.46 ± 0.17	0.47 ± 0.12	0.42 ± 0.11
20:3n-3	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
20:5n-3[1,3]	0.55 ± 0.15[a]	0.38 ± 0.06[a]	0.78 ± 0.13[b]	0.77 ± 0.07[b]	1.19 ± 0.17[c]	1.33 ± 0.16[c]
22:5n-3[1,2]	1.53 ± 0.18	1.18 ± 0.18	1.59 ± 0.31	1.11 ± 0.14	1.66 ± 0.20	1.42 ± 0.16
22:6n-3[1,2]	2.38 ± 0.37	2.73 ± 0.14	2.55 ± 0.46	2.76 ± 0.29	2.97 ± 0.32	3.57 ± 0.46
n-3[1,3]	4.92 ± 0.48[a]	4.76 ± 0.30[a]	5.36 ± 0.58[b]	5.13 ± 0.41[a]	6.31 ± 0.42[a]	6.77 ± 0.68[b]
PUFAs	38.29 ± 1.84	38.59 ± 1.95	37.76 ± 2.85	40.03 ± 1.90	38.44 ± 2.28	39.84 ± 1.71
HUFAs[1,2]	19.68 ± 1.58	18.30 ± 1.73	18.98 ± 2.10	17.18 ± 1.14	19.45 ± 1.63	19.12 ± 0.92
EPA+DHA[1,2,3]	2.93 ± 0.46[a]	3.11 ± 0.13[a,b]	3.33 ± 0.44[b]	3.53 ± 0.26[b]	4.16 ± 0.36[c]	4.90 ± 0.48[d]
n-6:n-3[1,3]	6.85 ± 0.78[a]	7.14 ± 0.75[a]	6.12 ± 0.92[b]	6.84 ± 0.71[a,b]	5.10 ± 0.35[c]	4.93 ± 0.49[c]
HUFA Score[1,3]	22.80 ± 1.97[a]	23.87 ± 2.84[a,b]	26.10 ± 1.80[b,c]	27.21 ± 2.03[c,d]	30.10 ± 1.43[d,e]	33.14 ± 2.56[e]
DHA:EPA[1,2,3]	4.51 ± 1.12[a]	7.36 ± 1.50[c]	3.41 ± 1.03[b]	3.63 ± 0.60[a,b]	2.53 ± 0.44[b]	2.73 ± 0.49[b]
DHA:DPAn-3[1,2]	1.57 ± 0.29	2.37 ± 0.38	1.63 ± 0.27	2.50 ± 0.34	1.80 ± 0.21	2.53 ± 0.30
n-3 HUFA[1,3]	4.49 ± 0.54[a]	4.33 ± 0.22[a]	4.95 ± 0.67[a]	4.67 ± 0.34[a]	5.84 ± 0.46[b]	6.34 ± 0.61[b]
n-6 HUFA[1,2]	15.19 ± 1.29	13.97 ± 1.82	14.02 ± 1.56	12.51 ± 1.02	13.60 ± 1.26	12.78 ± 0.77
Total [FA][1]	164.16 ± 30.91	139.20 ± 27.95	208.18 ± 44.89	182.75 ± 39.69	203.64 ± 31.25	211.39 ± 33.96

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100uL whole blood.

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: % n-3 HUFA in total HUFA, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, [FA]: fatty acid concentration

Significant main effect of time[1], sex[2] and sex*time[3] by the linear mixed models procedure (LMM) with time, sex and interaction as factors. Different letters represent a significant sex x time interaction by Bonferroni's post-hoc test, following a significant F-value by the LLM procedure.

Appendix Table C.3. Erythrocyte fatty acid composition in the dose-response study (n=20)

	Week 0		Week 4		Week 8	
	Males	Females	Males	Females	Males	Females
14:0[1,3]	0.50 ± 0.09[a]	0.69 ± 0.11[b,c]	0.79 ± 0.09[b]	0.71 ± 0.18[b,c]	0.50 ± 0.11[a]	0.58 ± 0.10[a,c]
16:0	21.37 ± 0.67	21.74 ± 0.83	21.23 ± 0.89	21.09 ± 1.08	20.55 ± 1.20	21.48 ± 0.88
16:0 dma[1]	1.78 ± 0.60	1.66 ± 0.10	1.33 ± 0.15	1.22 ± 0.48	1.69 ± 0.23	1.47 ± 0.18
17:0	0.33 ± 0.03	0.32 ± 0.03	0.31 ± 0.03	0.30 ± 0.03	0.32 ± 0.04	0.32 ± 0.03
18:0[1]	13.27 ± 0.69	12.53 ± 0.68	12.87 ± 1.00	13.32 ± 0.91	13.65 ± 0.62	13.79 ± 1.29
18:0 dma[1]	2.70 ± 0.93	2.33 ± 0.22	1.70 ± 0.25	1.64 ± 0.73	2.22 ± 0.61	2.03 ± 0.39
20:0[1]	0.28 ± 0.05	0.31 ± 0.04	0.29 ± 0.04	0.31 ± 0.03	0.33 ± 0.04	0.33 ± 0.05
22:0	1.14 ± 0.17	1.12 ± 0.13	1.15 ± 0.20	1.14 ± 0.15	1.15 ± 0.21	1.11 ± 0.13
23:0[1]	0.19 ± 0.02	0.18 ± 0.03	0.17 ± 0.03	0.16 ± 0.03	0.19 ± 0.03	0.18 ± 0.03
24:0	3.06 ± 0.44	3.22 ± 0.44	3.13 ± 0.65	3.26 ± 0.44	3.18 ± 0.36	3.21 ± 0.40
SFAs[1]	44.87 ± 1.48	44.36 ± 0.70	43.18 ± 1.96	43.38 ± 1.28	44.05 ± 1.02	44.72 ± 1.14
14:1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.04	0.01 ± 0.01
16:1n-7	0.30 ± 0.13	0.25 ± 0.06	0.35 ± 0.24	0.24 ± 0.04	0.27 ± 0.08	0.24 ± 0.05
18:1 dma[1]	0.67 ± 0.25	0.64 ± 0.11	0.44 ± 0.09	0.46 ± 0.19	0.59 ± 0.16	0.60 ± 0.13
18:1n-7[1]	1.28 ± 0.10	1.26 ± 0.11	1.23 ± 0.14	1.18 ± 0.12	1.20 ± 0.12	1.17 ± 0.11
18:1n-9[1]	12.21 ± 0.95	12.28 ± 1.03	12.30 ± 1.05	11.87 ± 1.00	11.63 ± 1.14	11.74 ± 0.77
20:1n-9[2]	0.24 ± 0.03	0.27 ± 0.04	0.23 ± 0.03	0.27 ± 0.04	0.23 ± 0.04	0.25 ± 0.02
22:1n-9[1]	0.13 ± 0.02	0.14 ± 0.02	0.12 ± 0.03	0.10 ± 0.01	0.16 ± 0.06	0.16 ± 0.08
24:1n-9	3.10 ± 0.43	3.37 ± 0.46	3.20 ± 0.52	3.38 ± 0.27	3.25 ± 0.52	3.31 ± 0.41
MUFAs[1]	17.93 ± 1.04	18.22 ± 1.57	17.89 ± 0.90	17.50 ± 1.28	17.37 ± 1.37	17.48 ± 1.07
18:2n-6	9.97 ± 1.04	10.49 ± 1.07	10.49 ± 2.74	10.18 ± 0.92	9.29 ± 0.70	9.85 ± 0.82
18:3n-6[2]	0.05 ± 0.02	0.04 ± 0.01	0.06 ± 0.06	0.03 ± 0.01	0.05 ± 0.02	0.03 ± 0.01
20:2n-6	0.23 ± 0.03	0.26 ± 0.05	0.23 ± 0.04	0.26 ± 0.06	0.26 ± 0.05	0.26 ± 0.05
20:3n-6[1]	1.61 ± 0.46	1.51 ± 0.22	1.54 ± 0.44	1.45 ± 0.23	1.45 ± 0.44	1.34 ± 0.17
20:4n-6[1]	13.68 ± 1.19	13.10 ± 0.93	12.82 ± 1.21	12.84 ± 0.78	12.80 ± 0.84	12.29 ± 0.66

22:2n-6[2]	0.06 ± 0.02	0.08 ± 0.01	0.06 ± 0.02	0.08 ± 0.01	0.06 ± 0.03	0.07 ± 0.02
22:4n-6[1]	3.12 ± 0.63	3.27 ± 0.36	2.82 ± 0.72	3.07 ± 0.31	2.79 ± 0.75	2.75 ± 0.33
22:5n-6[1]	0.56 ± 0.06	0.56 ± 0.08	0.51 ± 0.07	0.52 ± 0.06	0.49 ± 0.09	0.45 ± 0.07
n-6[1]	29.28 ± 1.19	29.31 ± 0.87	28.53 ± 1.93	28.44 ± 0.61	27.18 ± 1.38	27.04 ± 1.11
18:3n-3[1]	0.15 ± 0.04	0.15 ± 0.05	0.17 ± 0.08	0.17 ± 0.03	0.20 ± 0.04	0.21 ± 0.04
20:3n-3	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.01
20:5n-3[1,3]	0.51 ± 0.14[a,b]	0.38 ± 0.04[a]	0.68 ± 0.15[c]	0.62 ± 0.05[b,c]	1.00 ± 0.14[a]	1.02 ± 0.10[d]
22:5n-3[1,2]	2.27 ± 0.21	1.85 ± 0.20	2.30 ± 0.33	1.98 ± 0.24	2.60 ± 0.33	2.19 ± 0.19
22:6n-3[1,2]	3.05 ± 0.47	3.71 ± 0.43	3.17 ± 0.41	3.89 ± 0.46	3.69 ± 0.35	4.22 ± 0.59
n-3[1]	5.99 ± 0.49	6.11 ± 0.51	6.34 ± 0.50	6.68 ± 0.61	7.52 ± 0.45	7.67 ± 0.78
PUFAs	35.27 ± 1.16	35.42 ± 1.12	34.87 ± 1.79	35.12 ± 0.85	34.70 ± 1.67	34.70 ± 1.49
HUFAs	24.82 ± 1.01	24.40 ± 1.03	23.86 ± 1.48	24.40 ± 0.82	24.84 ± 1.68	24.28 ± 1.12
EPA+DHA[1,2]	3.56 ± 0.53	4.09 ± 0.43	3.85 ± 0.45	4.51 ± 0.46	4.69 ± 0.37	5.25 ± 0.63
n-6:n-3[1]	4.92 ± 0.49	4.83 ± 0.39	4.54 ± 0.56	4.29 ± 0.40	3.62 ± 0.20	3.56 ± 0.37
HUFA Score[1]	23.56 ± 1.81	24.47 ± 2.28	25.87 ± 1.84	26.71 ± 2.43	29.49 ± 1.41	30.68 ± 2.63
DHA:EPA[1,2,3]	6.24 ± 1.36[a]	9.93 ± 1.51[d]	4.86 ± 1.08[b,d]	6.35 ± 0.83[a,b]	3.75 ± 0.63[c]	4.15 ± 0.59[c,d]
DHA:DPAn-3[3]	1.36 ± 0.29[a]	2.03 ± 0.33[a]	1.41 ± 0.28[a]	1.99 ± 0.30[b]	1.44 ± 0.24[b]	1.93 ± 0.21[b]
n-3 HUFA[1]	5.85 ± 0.50	5.96 ± 0.48	6.17 ± 0.53	6.52 ± 0.59	7.32 ± 0.46	7.46 ± 0.78
n-6 HUFA[1,3]	18.98 ± 0.92	18.44 ± 1.16	17.69 ± 1.26	17.88 ± 0.92	17.52 ± 1.38	16.82 ± 0.90
Total [FA]	190.08 ± 14.07	184.15 ± 12.16	188.18 ± 10.84	200.63 ± 33.05	189.10 ± 17.22	191.95 ± 12.19

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100mg erythrocytes.

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: % n-3 HUFA in total HUFA, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, [FA]: fatty acid concentration

Significant main effect of time[1], sex[2] and sex*time[3] by the linear mixed models procedure (LMM) with time, sex and interaction as factors.

Different letters represent a significant sex x time interaction by Bonferroni's post-hoc test, following a significant F-value by the LLM procedure.

Appendix Table C.4. Plasma phospholipid fatty acid composition in the dose-response study (n=20)

	Week 0		Week 4		Week 8	
	Males	Females	Males	Females	Males	Females
14:0[1]	0.50 ± 0.08	0.49 ± 0.12	0.52 ± 0.15	0.54 ± 0.16	0.52 ± 0.13	0.70 ± 0.17
16:0[3]	25.41 ± 1.26	24.77 ± 1.27	25.43 ± 1.35	23.78 ± 2.58	24.68 ± 1.93	26.07 ± 2.12
16:0dma	0.48 ± 0.13	0.37 ± 0.19	0.37 ± 0.25	0.38 ± 0.27	0.43 ± 0.21	0.37 ± 0.24
18:0	14.44 ± 0.96	14.95 ± 0.98	15.38 ± 2.81	14.75 ± 3.39	14.42 ± 2.66	15.37 ± 3.95
18:0dma	0.27 ± 0.05	0.18 ± 0.10	0.21 ± 0.16	0.22 ± 0.16	0.29 ± 0.13	0.27 ± 0.07
20:0[1]	0.50 ± 0.08	0.52 ± 0.08	0.47 ± 0.08	0.47 ± 0.07	0.39 ± 0.05	0.45 ± 0.07
22:0[1]	1.29 ± 0.21	1.41 ± 0.31	1.13 ± 0.29	1.08 ± 0.35	0.85 ± 0.17	0.95 ± 0.21
23:0[1,3]	0.57 ± 0.11	0.53 ± 0.11	0.46 ± 0.11	0.38 ± 0.11	0.32 ± 0.05	0.38 ± 0.07
24:0[1]	1.18 ± 0.21	1.28 ± 0.31	1.07 ± 0.23	0.95 ± 0.33	0.79 ± 0.18	0.89 ± 0.19
SFAs	44.98 ± 1.00	44.79 ± 1.39	45.47 ± 3.44	43.19 ± 6.51	43.02 ± 4.03	46.00 ± 4.48
14:1	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.02	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.02
16:1n-7[3]	0.42 ± 0.19[a]	0.38 ± 0.07	0.54 ± 0.14[b]	0.37 ± 0.06	0.45 ± 0.14[a,b]	0.38 ± 0.10
18:1dma	0.13 ± 0.04	0.11 ± 0.05	0.10 ± 0.07	0.10 ± 0.06	0.12 ± 0.04	0.13 ± 0.03
18:1n-7	1.65 ± 0.11	1.62 ± 0.21	1.61 ± 0.21	1.61 ± 0.37	1.63 ± 0.28	1.60 ± 0.22
18:1n-9[1,3]	8.59 ± 1.18[a]	9.08 ± 1.32	9.38 ± 2.06[a]	13.31 ± 6.82	13.59 ± 3.95[b]	10.93 ± 5.28
20:1n-9[3]	0.17 ± 0.03	0.23 ± 0.04	0.17 ± 0.03	0.26 ± 0.14	0.24 ± 0.08	0.20 ± 0.05
22:1n-9	0.32 ± 0.14	0.30 ± 0.09	0.28 ± 0.10	0.38 ± 0.29	0.39 ± 0.16	0.36 ± 0.06
24:1n-9[1]	2.12 ± 0.36	2.18 ± 0.49	1.72 ± 0.51	1.51 ± 0.63	1.17 ± 0.25	1.38 ± 0.32
MUFAs[1,3]	13.41 ± 1.13	13.92 ± 1.77	13.84 ± 2.29	17.56 ± 7.08	17.65 ± 4.33	15.02 ± 5.21
18:2n-6	18.62 ± 2.36	20.15 ± 1.17	18.15 ± 2.56	19.86 ± 2.14	17.83 ± 1.54	18.71 ± 1.99
18:3n-6	0.11 ± 0.04	0.08 ± 0.05	0.10 ± 0.05	0.08 ± 0.06	0.09 ± 0.05	0.10 ± 0.06
202:n-6[1]	0.34 ± 0.05	0.38 ± 0.09	0.30 ± 0.04	0.28 ± 0.08	0.24 ± 0.04	0.28 ± 0.05
20:3n-6[1]	3.32 ± 0.87	3.05 ± 0.47	2.93 ± 0.91	2.23 ± 0.59	2.54 ± 0.69	2.42 ± 0.52
20:4n-6[1]	11.78 ± 2.00	10.34 ± 2.04	9.56 ± 1.80	7.88 ± 1.52	8.48 ± 1.47	8.07 ± 1.54
22:2n-6[3]	0.05 ± 0.02	0.04 ± 0.01	0.03 ± 0.02	0.06 ± 0.05	0.05 ± 0.04	0.03 ± 0.01

22:4n-6[1,2]	0.46 ± 0.07	0.41 ± 0.05	0.35 ± 0.06	0.27 ± 0.06	0.25 ± 0.04	0.23 ± 0.04
22:5n-6[1]	0.32 ± 0.05	0.33 ± 0.06	0.23 ± 0.05	0.20 ± 0.05	0.15 ± 0.03	0.18 ± 0.04
n-6[1]	34.99 ± 1.82	34.78 ± 1.72	31.65 ± 3.03	30.86 ± 1.90	29.64 ± 2.07	30.02 ± 2.87
18:3n-3[1,3]	0.28 ± 0.20[a]	0.27 ± 0.16[a]	0.28 ± 0.34[a]	0.95 ± 1.09[a,b]	1.12 ± 0.71[b]	0.28 ± 0.09[a]
20:3n-3[1]	0.05 ± 0.02	0.05 ± 0.02	0.03 ± 0.02	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
20:5n-3[1,2]	0.73 ± 0.23	0.48 ± 0.11	1.08 ± 0.16	0.91 ± 0.17	1.58 ± 0.30	1.62 ± 0.23
22:5n-3[2]	1.10 ± 0.21	0.79 ± 0.17	1.09 ± 0.18	0.73 ± 0.20	1.09 ± 0.16	0.95 ± 0.14
22:6n-3[2,3]	2.95 ± 0.48[a]	3.36 ± 0.45[a,b]	3.09 ± 0.59[a]	2.96 ± 0.63[a]	2.92 ± 0.27[a]	3.83 ± 0.56[b]
n-3[1]	5.12 ± 0.66	4.94 ± 0.60	5.57 ± 0.53	5.58 ± 0.63	6.74 ± 0.54	6.73 ± 0.84
PUFAs[1]	40.11 ± 1.80	39.72 ± 2.07	37.23 ± 3.30	36.44 ± 1.92	36.37 ± 1.73	36.74 ± 3.54
HUFAs[1,2,3]	20.71 ± 2.11[a]	18.81 ± 2.50[a,c]	18.37 ± 2.05[b,c]	15.21 ± 2.66[a,c]	17.04 ± 1.40[b]	17.34 ± 2.46[b,c]
EPA + DHA[1,3]	3.68 ± 0.62[a]	3.84 ± 0.52[a,b]	4.17 ± 0.59[b]	3.86 ± 0.72[a,b]	4.50 ± 0.42[a,b]	5.46 ± 0.69[c]
n-6:n-3[1]	6.94 ± 0.95	7.10 ± 0.69	5.71 ± 0.59	5.61 ± 0.79	4.44 ± 0.62	4.49 ± 0.40
HUFA Score[1,2]	23.29 ± 2.44	25.01 ± 2.74	28.79 ± 1.62	30.49 ± 2.20	33.10 ± 2.54	37.41 ± 3.61
DHA:EPA[1,2,3]	4.31 ± 1.16[a]	7.28 ± 1.85[a,b,c]	2.95 ± 0.84[c]	3.32 ± 0.70[d]	1.91 ± 0.37[a,b]	2.38 ± 0.37[b,c]
DHA:22:5n-3[2]	2.72 ± 0.50	4.39 ± 0.79	2.87 ± 0.57	4.23 ± 1.09	2.74 ± 0.56	4.10 ± 0.68
n-3 HUFA[1,3]	4.83 ± 0.79[a,b]	4.68 ± 0.64[a,b]	5.29 ± 0.68[a,c]	4.63 ± 0.83[a,b]	5.62 ± 0.38[b]	6.44 ± 0.78[c]
n-6 HUFA[1,2]	15.87 ± 1.57	14.13 ± 2.15	13.07 ± 1.46	10.58 ± 1.93	11.42 ± 1.27	10.89 ± 1.97
Total [FA][1,3]	105.49 ± 22.60[a,b]	104.69 ± 17.97[a,b]	109.21 ± 21.76[c]	132.07 ± 23.03[a]	141.66 ± 29.27[b,c]	110.48 ± 5.75[a,b]

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100uL plasma.

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, HUFA: highly unsaturated fatty acids, HUFA Score: % n-3 HUFA in total HUFA, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, [FA]: fatty acid concentration.

Significant main effect of time[1], sex[2] and sex*time[3] by the linear mixed models procedure (LMM) with time, sex and interaction as factors. Different letters represent a significant sex x time interaction by Bonferroni's post-hoc test, following a significant F-value by the LLM procedure.

Appendix Table C.5. Subgroup whole blood fatty acid composition in the dose-response study (n=5)

	Week 0	Week 4	Week 8	Week 12
14:0	0.77 ± 0.24	0.76 ± 0.36	0.75 ± 0.17	0.50 ± 0.11
16:0[1]	20.97 ± 1.27[a]	20.49 ± 1.61[a]	20.64 ± 1.42[a]	18.55 ± 0.44[b]
16:0 dma	1.21 ± 0.54	0.86 ± 0.26	1.02 ± 0.18	1.14 ± 0.14
17:0	0.31 ± 0.03	0.32 ± 0.04	0.32 ± 0.04	0.32 ± 0.05
18:0	11.70 ± 1.34	11.05 ± 0.76	11.63 ± 1.49	10.21 ± 0.65
18:0 dma[1]	1.17 ± 0.10[a]	1.09 ± 0.45[a]	1.34 ± 0.24[a]	0.43 ± 0.06[b]
20:0	0.28 ± 0.06	0.27 ± 0.03	0.41 ± 0.30	0.25 ± 0.04
22:0	0.79 ± 0.14	0.71 ± 0.12	0.87 ± 0.14	0.72 ± 0.12
23:0[1]	0.20 ± 0.01[a,b]	0.16 ± 0.03[a]	0.23 ± 0.03[b]	0.17 ± 0.03[a]
24:0[1]	1.45 ± 0.08[a,b]	1.32 ± 0.16[a,b]	1.61 ± 0.17[a]	1.27 ± 0.20[b]
SFA[1]	39.15 ± 1.63[a]	37.35 ± 2.19[a,b]	39.53 ± 2.50[a]	33.86 ± 1.20[b]
14:1	0.04 ± 0.04	0.05 ± 0.04	0.03 ± 0.02	0.03 ± 0.01
16:1n-7	0.98 ± 0.67	0.99 ± 0.36	0.98 ± 0.59	0.84 ± 0.24
18:1 dma[1]	0.32 ± 0.06[a]	0.31 ± 0.14[a,b]	0.37 ± 0.07[a]	0.17 ± 0.04[b]
18:1n-7	1.53 ± 0.07	1.49 ± 0.19	1.42 ± 0.24	1.50 ± 0.19
18:1n-9	14.51 ± 1.14	15.41 ± 0.27	14.26 ± 1.85	15.28 ± 1.51
20:1n-9	0.22 ± 0.03	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.03
22:1n-9[1]	0.27 ± 0.04[a]	0.14 ± 0.02[b]	0.25 ± 0.09[a]	0.24 ± 0.04[a]
24:1n-9	1.75 ± 0.25	1.54 ± 0.19	1.96 ± 0.42	1.50 ± 0.28
MUFAs[1]	19.63 ± 1.63[a,b]	20.12 ± 0.49[a]	19.47 ± 2.58[b]	19.75 ± 1.11[a]
18:2n-6[1]	16.89 ± 1.64[a]	19.51 ± 2.40[a]	18.10 ± 1.35[a]	22.43 ± 3.16[b]
18:3n-6	0.20 ± 0.08	0.31 ± 0.16	0.29 ± 0.11	0.23 ± 0.05
20:2n-6[1]	0.23 ± 0.03[a,b]	0.21 ± 0.03[a]	0.27 ± 0.03[b]	0.20 ± 0.03[a]
20:3n-6[1]	1.62 ± 0.40[a]	1.45 ± 0.24[a,b]	1.47 ± 0.24[a,b]	1.19 ± 0.24[b]
20:4n-6[1]	12.41 ± 1.46[a]	10.80 ± 0.92[b]	10.33 ± 0.98[b]	10.07 ± 0.46[b]
22:2n-6	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02
22:4n-6[1]	1.87 ± 0.50[a]	1.61 ± 0.50[a,b]	1.51 ± 0.49[b]	1.17 ± 0.39[c]
22:5n-6[1]	0.39 ± 0.04[a]	0.33 ± 0.04[a]	0.32 ± 0.07[a]	0.23 ± 0.04[b]
n-6	33.65 ± 2.97	34.27 ± 3.30	32.34 ± 2.13	35.58 ± 2.62
18:3n-3	0.36 ± 0.03	0.40 ± 0.10	0.41 ± 0.09	0.47 ± 0.07
20:3n-3	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
20:5n-3[1]	0.49 ± 0.09[a]	0.79 ± 0.10[a]	1.25 ± 0.14[b]	1.81 ± 0.28[c]
22:5n-3	1.42 ± 0.18	1.40 ± 0.18	1.54 ± 0.27	1.54 ± 0.31
22:6n-3[1]	2.38 ± 0.09[a]	2.47 ± 0.15[a]	2.87 ± 0.27[b]	3.26 ± 0.20[b]
n-3[1]	4.69 ± 0.11[a]	5.09 ± 0.17[a]	6.09 ± 0.42[b]	7.10 ± 0.59[c]
PUFAs[1]	38.34 ± 2.94[a]	39.35 ± 3.35[a,b]	38.43 ± 2.43[a]	42.68 ± 2.04[b,c]
HUFAs	20.62 ± 1.47	18.88 ± 0.98	19.30 ± 1.64	19.30 ± 1.21

EPA + DHA[1]	2.88 ± 0.16[a]	3.26 ± 0.17[a]	4.12 ± 0.22[b]	5.07 ± 0.37[c]
n-6:n-3[1]	7.18 ± 0.72[a]	6.74 ± 0.63[a]	5.32 ± 0.29[b]	5.07 ± 0.84[b]
HUFA Score[1]	21.11 ± 1.79[a]	24.91 ± 1.55[b]	29.47 ± 1.19[c]	34.35 ± 1.63[d]
DHA:EPA[1]	4.96 ± 0.79[a]	3.20 ± 0.51[b]	2.34 ± 0.47[b,c]	1.84 ± 0.28[c]
DHA:22:5n-3[1]	1.70 ± 0.25[a]	1.79 ± 0.28[a]	1.90 ± 0.30[a]	2.17 ± 0.34[b]
n-3 HUFA[1]	4.33 ± 0.13[a]	4.69 ± 0.13[a]	5.68 ± 0.39[b]	6.63 ± 0.58[c]
n-6 HUFA[1]	16.28 ± 1.49[a]	14.18 ± 1.00[b]	13.62 ± 1.30[b]	12.66 ± 0.77[b]
Total [FA]	173.38 ± 35.96	210.90 ± 32.91	209.85 ± 52.47	217.96 ± 42.15

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100uL whole blood.
SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, HUFA; highly unsaturated fatty acids, HUFA Score; %n-3 HUFA in total HUFA, FA: fatty acids

Significant main effect of time[1] by the linear mixed models procedure ($p < 0.05$).
Different letters represent a significant difference between time points by Bonferroni's post-hoc test, following a significant F-value by the linear mixed models procedure.

Appendix Table C.6. Subgroup erythrocyte fatty acid composition in the dose-response study (n=5)

	Week 0	Week 4	Week 8	Week 12
14:0[1]	0.53 ± 0.08[a]	0.78 ± 0.10[b]	0.55 ± 0.05[a]	0.43 ± 0.04[a]
16:0	20.97 ± 1.27[a]	20.49 ± 1.61[a]	20.64 ± 1.42[a]	18.55 ± 0.44[b]
16:0 dma	1.21 ± 0.54	0.86 ± 0.26	1.02 ± 0.18	1.14 ± 0.14
17:0	0.31 ± 0.03	0.32 ± 0.04	0.32 ± 0.04	0.32 ± 0.05
18:0	11.70 ± 1.34	11.05 ± 0.76	11.63 ± 1.49	10.21 ± 0.65
18:0 dma[1]	1.17 ± 0.10[a]	1.09 ± 0.45[a]	1.34 ± 0.24[a]	0.43 ± 0.06[b]
20:0	0.28 ± 0.06	0.27 ± 0.03	0.41 ± 0.30	0.25 ± 0.04
22:0	0.79 ± 0.14	0.71 ± 0.12	0.87 ± 0.14	0.72 ± 0.12
23:0[1]	0.20 ± 0.01[a,b]	0.16 ± 0.03[a]	0.23 ± 0.03[b]	0.17 ± 0.03[a]
24:0[1]	1.45 ± 0.08[a,b]	1.32 ± 0.16[a,b]	1.61 ± 0.17[a]	1.27 ± 0.20[b]
SFA[1]	39.15 ± 1.63[a]	37.35 ± 2.19[a,b]	39.53 ± 2.50[a]	33.86 ± 1.20[b]
14:1	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
16:1n-7	0.98 ± 0.67	0.99 ± 0.36	0.98 ± 0.59	0.84 ± 0.24
18:1 dma[1]	0.32 ± 0.06[a]	0.31 ± 0.14[a,b]	0.37 ± 0.07[a]	0.17 ± 0.04[b]
18:1n-7	1.53 ± 0.07	1.49 ± 0.19	1.42 ± 0.24	1.50 ± 0.19
18:1n-9	14.51 ± 1.14	15.41 ± 0.27	14.26 ± 1.85	15.28 ± 1.51
20:1n-9	0.22 ± 0.03	0.20 ± 0.04	0.19 ± 0.04	0.19 ± 0.03
22:1n-9[1]	0.27 ± 0.04[a]	0.14 ± 0.02[b]	0.25 ± 0.09[a]	0.24 ± 0.04[a]
24:1n-9	1.75 ± 0.25	1.54 ± 0.19	1.96 ± 0.42	1.50 ± 0.28
MUFA[1]	19.63 ± 1.63[a,b]	20.12 ± 0.49[a]	19.47 ± 2.58[b]	19.75 ± 1.11[a]
18:2n-6[1]	16.89 ± 1.64[a]	19.51 ± 2.40[a]	18.10 ± 1.35[a]	22.43 ± 3.16[b]
18:3n-6	0.20 ± 0.08	0.31 ± 0.16	0.29 ± 0.11	0.23 ± 0.05
20:2n-6[1]	0.23 ± 0.03[a,b]	0.21 ± 0.03[a]	0.27 ± 0.03[b]	0.20 ± 0.03[a]
20:3n-6[1]	1.62 ± 0.40[a]	1.45 ± 0.24[a,b]	1.47 ± 0.24[a,b]	1.19 ± 0.24[b]
20:4n-6[1]	12.41 ± 1.46[a]	10.80 ± 0.92[b]	10.33 ± 0.98[b]	10.07 ± 0.46[b]
22:2n-6	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02
22:4n-6[1]	1.87 ± 0.50[a]	1.61 ± 0.50[a,b]	1.51 ± 0.49[b]	1.17 ± 0.39[c]
22:5n-6[1]	0.39 ± 0.04[a]	0.33 ± 0.04[a]	0.32 ± 0.07[a]	0.23 ± 0.04[b]
n-6 PUFA	33.65 ± 2.97	34.27 ± 3.30	32.34 ± 2.13	35.58 ± 2.62
18:3n-3	0.36 ± 0.03	0.40 ± 0.10	0.41 ± 0.09	0.47 ± 0.07
20:3n-3	0.03 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
20:5n-3[1]	0.49 ± 0.09[a]	0.79 ± 0.10[b]	1.25 ± 0.14[c]	1.81 ± 0.28[d]
22:5n-3	1.42 ± 0.18	1.40 ± 0.18	1.54 ± 0.27	1.54 ± 0.31
22:6n-3[1]	2.38 ± 0.09[a]	2.47 ± 0.15[a]	2.87 ± 0.27[b]	3.26 ± 0.20[c]
n-3 PUFA[1]	4.69 ± 0.11[a]	5.09 ± 0.17[a]	6.09 ± 0.42[b]	7.10 ± 0.59[c]
PUFA[1]	38.34 ± 2.94[a]	39.35 ± 3.35[a,b]	38.43 ± 2.43[a]	42.68 ± 2.04[b,c]
HUFA	20.62 ± 1.47	18.88 ± 0.98	19.30 ± 1.64	19.30 ± 1.21

EPA + DHA[1]	2.88 ± 0.16[a]	3.26 ± 0.17[a]	4.12 ± 0.22[b]	5.07 ± 0.37[c]
n-6:n-3[1]	7.18 ± 0.72[a]	6.74 ± 0.63[a]	5.32 ± 0.29[b]	5.07 ± 0.84[b]
HUFA Score[1]	21.11 ± 1.79[a]	24.91 ± 1.55[b]	29.47 ± 1.19[c]	34.35 ± 1.63[d]
DHA:EPA[1]	4.96 ± 0.79[a]	3.20 ± 0.51[b]	2.34 ± 0.47[b,c]	1.84 ± 0.28[c]
DHA:22:5n-3[1]	1.70 ± 0.25[a]	1.79 ± 0.28[a]	1.90 ± 0.30[a]	2.17 ± 0.34[b]
n-3 HUFA[1]	4.33 ± 0.13[a]	4.69 ± 0.13[a]	5.68 ± 0.39[b]	6.63 ± 0.58[c]
n-6 HUFA[1]	16.28 ± 1.49[a]	14.18 ± 1.00[b]	13.62 ± 1.30[b]	12.66 ± 0.77[b]
Total [FA][1]	173.38 ± 35.96[a]	210.90 ± 32.91[a,b]	209.85 ± 52.47[a,b]	217.96 ± 42.15[b]

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100mg erythrocytes.
SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, HUFA; highly unsaturated fatty acids, HUFA Score; %n-3 HUFA in total HUFA, FA: fatty acids.

Significant main effect of time[1] by the linear mixed models procedure ($p < 0.05$).
Different letters represent a significant difference between time points by Bonferroni's post-hoc test, following a significant F-value by the linear mixed models procedure.

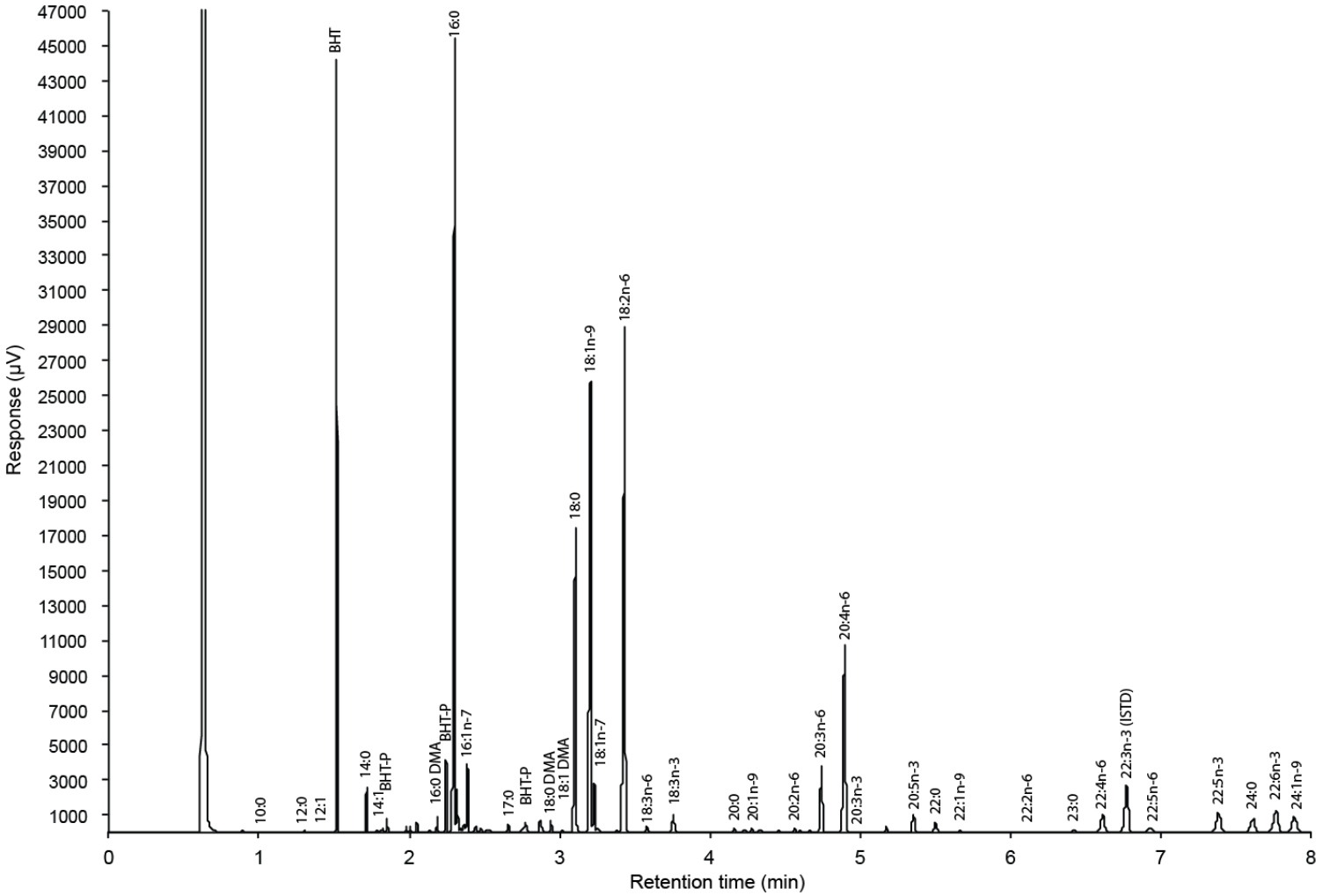
Appendix Table C.7. Subgroup plasma phospholipid fatty acid composition in the dose-response study (n=5)

	Week 0	Week 4	Week 8	Week 12
14:0	0.52 ± 0.11	0.50 ± 0.17	0.53 ± 0.14	0.67 ± 0.07
16:0	25.27 ± 1.23	23.47 ± 1.47	24.24 ± 2.26	25.71 ± 0.72
16:0 dma[1]	0.44 ± 0.18[a,b]	0.33 ± 0.30[a]	0.50 ± 0.15[a,b]	0.73 ± 0.10[b]
18:0	14.82 ± 0.38	14.41 ± 1.34	16.40 ± 5.08	17.86 ± 2.22
18:0 dma[1]	0.26 ± 0.06	0.18 ± 0.16	0.33 ± 0.14	0.44 ± 0.04
20:0	0.52 ± 0.10	0.47 ± 0.08	0.40 ± 0.09	0.49 ± 0.07
22:0[1]	1.31 ± 0.31[a]	1.18 ± 0.33[a,b]	0.77 ± 0.13[b]	0.97 ± 0.13[a,b]
23:0[1]	0.60 ± 0.12[a]	0.50 ± 0.13[a,b]	0.33 ± 0.07[b]	0.43 ± 0.04[a,b]
24:0[1]	1.16 ± 0.27[a]	1.09 ± 0.24[a,b]	0.71 ± 0.15[b]	0.94 ± 0.10[a,b]
SFAs	45.29 ± 1.13	42.51 ± 2.99	44.52 ± 7.22	48.66 ± 2.90
14:1	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.02
16:1n-7	0.43 ± 0.28	0.45 ± 0.16	0.39 ± 0.20	0.31 ± 0.03
18:1 dma[1]	0.11 ± 0.03[a]	0.09 ± 0.08[a]	0.13 ± 0.02[a,b]	0.21 ± 0.03[b]
18:1n-7	1.66 ± 0.04	1.77 ± 0.18	1.56 ± 0.33	1.55 ± 0.21
18:1n-9	9.00 ± 1.48	11.65 ± 4.96	13.59 ± 5.23	7.14 ± 1.08
20:1n-9	0.18 ± 0.05	0.22 ± 0.07	0.23 ± 0.10	0.15 ± 0.02
22:1n-9	0.32 ± 0.21	0.25 ± 0.05	0.41 ± 0.16	0.36 ± 0.11
24:1n-9[1]	2.25 ± 0.47[a]	1.91 ± 0.67[a,b]	1.14 ± 0.23[b]	1.51 ± 0.26[a,b]
MUFAs	13.97 ± 1.18	16.35 ± 4.36	17.50 ± 5.79	11.25 ± 1.04
18:2n-6	17.30 ± 1.94	17.81 ± 1.94	17.07 ± 1.46	17.60 ± 2.40
18:3n-6	0.13 ± 0.06	0.10 ± 0.08	0.09 ± 0.06	0.14 ± 0.02
20:2n-6[1]	0.30 ± 0.04[a]	0.26 ± 0.05[a,b]	0.22 ± 0.04[b]	0.24 ± 0.02[b]
20:3n-6[1]	2.86 ± 0.71[a]	2.50 ± 0.57[a,b]	2.13 ± 0.47[a,b]	1.99 ± 0.42[b]
20:4n-6[1]	12.75 ± 2.03[a]	10.53 ± 1.40[a,b]	8.20 ± 0.58[b]	9.56 ± 0.96[b]
22:2n-6	0.05 ± 0.03	0.03 ± 0.02	0.03 ± 0.01	0.04 ± 0.02
22:4n-6[1]	0.45 ± 0.02[a]	0.33 ± 0.05[b]	0.22 ± 0.02[c]	0.20 ± 0.03[c]
22:5n-6[1]	0.36 ± 0.05[a]	0.23 ± 0.04[b]	0.14 ± 0.04[c]	0.16 ± 0.03[c]
n-6[1]	34.21 ± 1.63[a]	31.80 ± 1.80[a,b]	28.11 ± 1.71[c]	29.91 ± 2.11[b,c]
18:3n-3	0.33 ± 0.27	0.51 ± 0.81	1.18 ± 0.87	0.17 ± 0.04
20:3n-3	0.05 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
20:5n-3[1]	0.64 ± 0.15[a]	1.03 ± 0.14[a,b]	1.63 ± 0.26[b,c]	2.10 ± 0.45[c]
22:5n-3	1.02 ± 0.14	1.07 ± 0.18	1.11 ± 0.17	1.03 ± 0.08
22:6n-3[1]	2.73 ± 0.47[a]	3.37 ± 0.51[a,b]	2.86 ± 0.30[a]	4.06 ± 0.16[b]
n-3[1]	4.77 ± 0.40[a]	6.02 ± 0.20[b]	6.80 ± 0.87[b,c]	7.41 ± 0.58[c]
PUFAs[1]	38.98 ± 1.64[a]	37.81 ± 1.67[a,b]	34.91 ± 2.15[b]	37.32 ± 2.30[a,b]
HUFAs[1]	20.87 ± 2.14[a]	19.09 ± 2.22[a,b]	16.32 ± 1.02[b]	19.13 ± 1.45[a,b]
EPA + DHA[1]	3.37 ± 0.43[a]	4.40 ± 0.57[a]	4.49 ± 0.47[a]	6.16 ± 0.55[b]

n-6:n-3[1]	7.21 ± 0.68[a]	5.30 ± 0.44[b]	4.19 ± 0.58[c]	4.06 ± 0.41[c]
HUFA Score[1]	21.28 ± 1.37[a]	28.79 ± 1.17[b]	34.42 ± 1.63[c]	37.83 ± 1.92[d]
DHA:EPA[1]	4.53 ± 1.48[a]	3.30 ± 0.59[a,b]	1.77 ± 0.27[b]	2.01 ± 0.42[b]
DHA:22:5n-3[2]	2.70 ± 0.49	3.18 ± 0.26	2.63 ± 0.51	3.95 ± 0.31
n-3 HUFA[1]	4.44 ± 0.52[a]	5.50 ± 0.73[a]	5.63 ± 0.57[a]	7.23 ± 0.59[c]
n-6 HUFA[1]	16.43 ± 1.72[a]	13.58 ± 1.54[b]	10.70 ± 0.53[c]	11.90 ± 1.06[b,c]
Total [FA]	114.82 ± 34.41	113.72 ± 45.28	143.15 ± 43.42	128.90 ± 35.30

Values are means ± standard deviation. Values are wt% of total fatty acids with the exception of total fatty acid concentration expressed as fatty acids (ug)/100uL plasma.
SFA; saturated fatty acids, MUFA; monounsaturated fatty acids, PUFA; polyunsaturated fatty acids, HUFA; highly unsaturated fatty acids, HUFA Score; %n-3 HUFA in total HUFA, FA: fatty acids.

Significant main effect of time[1] by the linear mixed models procedure ($p < 0.05$).
Different letters represent a significant difference between time points by Bonferroni's post-hoc test, following a significant F-value by the linear mixed models procedure.



Appendix Figure C.2. Gas chromatogram of whole blood fatty acid composition. Analysis on a DB-FFAP 15m capillary column with temperature program as follows: initial, 150°C with a 0.25 min hold; ramp: 35°C/min - 200°C, then by 8°C - 225°C with a 3.2 min hold, then by 80°C/min - 245°C with a 15 min hold. BHT: butylated hydroxytoluene, DMA: dimethyl acetal, ISTD: internal standard.