# Maternal Docosahexaenoic Acid (DHA) Supplementation and Fetal DHA Accretion

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under the supervision of

**Professor Lawrence T. Weaver**

## DEDICATION

Dedicated to all the love and support I have received,
and to the memory of Dad and Uncle Charlie.

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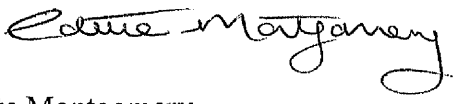
## AUTHOR'S DECLARATION

I declare that the work contained in this thesis is original. The recruitment of participants, the collection and analyses of samples, and the analysis and presentation of data were entirely undertaken by the author, unless otherwise stated. This thesis and the work contained herein was supervised by Professor Lawrence T. Weaver.



Colette Montgomery

## SUPERVISOR'S DECLARATION

I declare that the work contained in this thesis was performed by the author and that during the period of study, the requirements for the Degree of Doctor of Philosophy have been fulfilled.

Professor Lawrence T. Weaver

Samson Gemmell Chair of Child Health

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## LIST OF PRESENTATIONS AND PUBLICATIONS

Some of the work described in this dissertation has been presented or published elsewhere:

Berry, C., Montgomery, C., Sattar, N., Norrie, J. & Weaver, L.T. (2001) Fatty acid status of women of reproductive age. *European Journal of Clinical Nutrition*, **in press**.

Malcolm, C.A., Hamilton, R.H., McCulloch, D.L., Mactier, H., Montgomery, C. & Weaver, L.T. (2000) Flash visual evoked potentials (VEPS) in term infants born of mothers supplemented and unsupplemented with docosahexaenoic acid (DHA). *Investigative Ophthalmology and Visual Science*, **41 (4)**, s592. Presented at the Association for Research in Vision and Ophthalmology 2000 Annual Meeting, Fort Lauderdale Florida, 3 May 2000.

McCulloch, D.L., Malcolm, C.A., Hamilton, R.H., Montgomery, C. & Weaver, L.T. (2001) Maternal fish oil supplementation and maturation of visual evoked potentials (VEPs) in term infants. *Investigative Ophthalmology and Visual Science*, **in press.** Presented at the Association for Research in Vision and Ophthalmology 2000 Annual Meeting, Fort Lauderdale Florida, 29$^{th}$ April – 4$^{th}$ May 2001.

Montgomery, C., Berry, C., Sattar, N., Caine, N., McMillan, M., Norrie, J. & Weaver, L. (2000) Association between socio-economic status and fatty acid status in women of child-bearing age. *Proceedings of the Nutrition Society*, **59 OCA**, 37A. Presented at the Nutrition Society Summer Meeting, 29$^{th}$ June – 2$^{nd}$ July 1999, Glasgow.

Montgomery, C., Speake, B., Cameron, A., Sattar, N. & Weaver, L.T. (2001) Maternal Docosahexaenoic Acid Supplementation and Fetal Accretion. *Proceedings of the Nutrition Society*, **in press**. Presented at the Nutrition Society Scottish Section Meeting, 28-29$^{th}$ March 2001, Dundee.

Montgomery, C., Cameron, A., Sattar, N., Speake, B. & Weaver, L.T. (2001) DHA Supplementation of Mothers and Fetal Accretion. Presented at the 34$^{th}$ Annual Meeting of ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition), 9-12$^{th}$ May 2001, Geneva.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| αLA | alpha-linolenic acid |
| AA | arachidonic acid |
| CE | cholesterol ester |
| DHA | docosahexaenoic acid |
| DHGLA | dihomo-γ-linolenic acid |
| DPA | docosapentaenoic acid |
| EPA | eicosapentaenoic acid |
| FA | fatty acid |
| FAME | fatty acid methyl ester(s) |
| FFA | free fatty acid(s) |
| GC-MS | gas chromatography mass spectrometry |
| GLA | γ-linolenic acid |
| IUGR | intra-uterine growth retardation |
| LA | linoleic acid |
| LCPUFA | long chain polyunsaturated fatty acid(s) |
| LPL | lipoprotein lipase |
| MCSFA | medium chain saturated fatty acid(s) |
| MUFA | monounsaturated fatty acid(s) |
| NEFA | non-esterified fatty acid(s) |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PI | phosphatidylinositol |
| PIH | pregnancy-induced hypertension |
| PL | phospholipid |
| RBC | red blood cell |
| SFA | saturated fatty acid(s) |
| SM | sphingomyelin |
| TAG | triacylglycerol |
| TFA | total fatty acids |

## SUMMARY

### Background

Docosahexaenoic acid (DHA) (C22:6n-3) is a polyunsaturated fatty acid that is an essential constituent of cell membranes, particularly of the nervous system where it is found in relatively high concentrations in the brain and retina. DHA is obtained by endogenous synthesis, dependent on an adequate dietary intake of its parent essential fatty acid α-linolenic acid, or preformed in the diet, largely from fish, particularly oily fish. Levels of DHA (DHA status) can be determined by its measurement in blood and tissues. Circulating DHA status correlates with fish intake, even in the West of Scotland where consumption is low and intakes of DHA and oily fish are below current recommendations.

Fetal and neonatal DHA status is dependent on maternal DHA status. Maternal supplies of DHA are transferred to the fetus via the placenta, and to the neonate via breast-milk. There is evidence that maternal DHA is mobilized, particularly during the early stages of pregnancy, to ensure the fetus accretes sufficient DHA. This appears to be unsustainable, such that by term, maternal DHA status is lower relative to mid-pregnancy and to the fetus. Babies who are inadequately supplied with DHA either *in utero* or postnatally accumulate lower amounts in blood and tissue, and may be at a disadvantage with regards to neurodevelopment. Depletion of maternal DHA status may further affect subsequent pregnancies.

In this study expectant mothers were supplied with preformed DHA, at a dosage comparable with current dietary recommendations in a palatable and convenient form, to determine its effects on the nutritional status of both mother and fetus.

### Hypotheses

This study aimed to test the hypothesis that maternal DHA supplementation enriches maternal DHA status and thereby increases the amount available to the fetus and neonate, as reflected in:

(a) maternal red blood cells (RBC) and plasma

(b) placental and umbilical cord tissue

(c) (fetal) umbilical cord RBC and plasma

(d) breast milk

**Study Design**

A randomized double-blind, placebo-controlled trial was undertaken in which 100 expectant mothers were randomized to receive either fish oil (n=50) or placebo (n=50) capsules from 15 weeks gestation until term. The fish oil capsules contained 40% DHA (200mg/day); the placebo consisted of high oleic sunflower oil containing 81% oleic acid (400mg/day).

Maternal venous blood samples were obtained at 15 and 28 weeks gestation and at birth. Umbilical cord blood, umbilical cord tissue and placental tissue were collected at delivery. Breast milk was obtained soon after birth.

**Study Participants**

All pregnancies were singleton and delivered at term. Mothers were representative of the Glasgow population in terms of socio-economic status (SES). The two maternal groups were similar with regard to maternal age, anthropometry, SES, fish consumption, smoking and alcohol habits. Their infants were of comparable gestational age and anthropometry, and were matched in gender and feeding practice.

**Methods**

The fatty acid composition of total lipids in RBC, plasma, cord/placental tissue and breast milk was analysed. Fatty acids were extracted via a modified Folch extraction and derivatized with methanolic hydrochloric acid. Fatty acid methyl esters (FAME) were analysed by gas chromatography mass spectrometry (GC-MS) and quantified in both relative and absolute terms: results were expressed as % total fatty acids (% TFA) and as concentrations (nmol/ml for RBC, plasma and breast milk, nmol/g for placenta). Values were obtained for 19 individual fatty acids. In addition, the n-6 and n-3 PUFA classes were each summed (total n-6 and total n-3 fatty acids respectively) and expressed relative to each other (ratio n-6/n-3 fatty acids).

Differences between the fish oil and placebo groups were tested using Mann-Whitney 2-sample rank tests, with a chosen significance level of $p<0.05$. The longitudinal changes in maternal status and the differences between mother and fetus were assessed by one-sample Sign tests at a significance level of $p<0.01$.

**Results**

There were no significant differences between groups in baseline samples at 15 weeks. Both groups exhibited an increase in the concentration and % of DHA in maternal RBC and plasma between 15 and 28 weeks, followed by a decrease between 28 weeks and term. However, at 28 weeks, the concentration of DHA was 22% higher in plasma ($p=0.02$) and 13% higher in RBC ($p=0.02$) in the fish oil compared to the placebo group. At term, RBC DHA concentration remained 42% higher ($p=0.02$) in the fish oil supplemented group. DHA also accounted for a higher % TFA in RBC of fish oil supplemented mothers at 28 weeks ($p=0.003$) and at term ($p=0.01$). Total n-3 fatty acids were elevated, with a concomitant lower n-6/n-3 ratio, in the fish oil group at 28 weeks and at birth in both maternal RBC and plasma, for both relative and absolute measurements (all $p<0.05$). Thus, fish oil supplementation enhanced the overall maternal DHA and n-3 fatty acid status.

There were no significant differences between groups in DHA as a % TFA or concentration in cord blood, placental tissue, cord tissue, or breast milk. In both groups, DHA (% TFA and concentration) was higher in cord than maternal RBC and plasma at birth. The relative and absolute amounts of DHA in cord plasma and RBC were most similar to the maximal maternal DHA observed at 28 weeks.

The early increase in maternal DHA (between 15 and 28 weeks) was greater in the fish oil group; the subsequent decline in maternal status following mid-gestation (between 28 weeks and term) was less in the fish oil group. The materno-fetal difference was also less marked in the fish oil group. Fish oil supplementation thus enhanced maternal ability to provide the fetus with DHA during the second trimester, and limited the third trimester depletion of maternal stores and status, ensuring a more favourable maternal status by term such that the materno-fetal gradient was less severe.

**Conclusions**

Maternal DHA status is maximal in mid-trimester and declines to term. The extent of this decline is limited in supplemented compared to unsupplemented mothers. The relationship between mid-trimester maternal and term cord blood DHA suggests that timing of maternal supplementation is important, and is most likely to be beneficial if

it begins before mid-gestation. Maternal DHA supplementation enhances maternal DHA status and may aid preferential transfer of DHA from mother to fetus. Maintenance of a higher DHA status at term may also enhance maternal DHA status in subsequent pregnancies.

Chapter 1

# Fatty Acid Structure and Function

## 1.1 DEFINITIONS OF LIPIDS

### 1.1.1 Introduction

"Lipid" is a term applied to a group of substances that have in common not a chemical structure, but a "solubility characteristic" (Stedman's Dictionary 1997). Lipids are insoluble in water but soluble in nonpolar (organic) solvents, such as alcohols, ethers and chloroform, which are themselves immiscible with water (Gurr 1993, Jensen 1989a, Stedman's Dictionary 1997). Thus classes of lipids include heterogeneous substances, such as fatty acids, acylglycerols, phospholipids, glycolipids, sphingolipids, steroids and fat-soluble vitamins (A, D, E, K) (Gurr 1993, Jensen 1989a).

The various classes of lipids differ in their biological functions. In general, lipids serve either an energy storage (acylglycerols) or a structural (phospholipid, cholesterol) role. Lipids may also be converted to compounds involved in cell signaling and regulation, such as prostaglandins, leukotrienes and steroid hormones. The role of lipids in health and disease has been the subject of numerous detailed reviews (Food and Agriculture Organization/FAO & World Health Organization/WHO 1980, Health Education Authority/HEA 1994).

The nature and structure of three specific classes of lipids – fatty acids, acylglycerols and phospholipids - are discussed further below, with attention to the nutritional relevance of these species.

### 1.1.2 Fatty Acids

Fatty acids are compounds of hydrocarbon chains which have at one end a methyl group ($CH_3$) and at the other, a carboxyl group (COOH) (Figure 1). The hydrocarbon chain allows lipid solubility, while the carboxyl moiety confers acidic properties on the fatty acid (British Nutrition Foundation/BNF 1992).

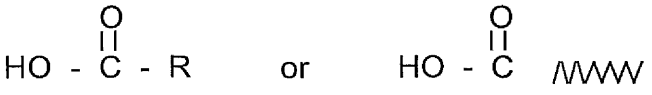



Figure 1. Basic structure of a fatty acid. The functional carboxyl group is shown. R or /\/\/\ denotes the hydrocarbon chain, including the methyl group, which varies in length, number and position of double bonds and so is notated without specification when depicting fatty acids in general.

1.1.2(a) Chain length

The hydrocarbon chain can contain 2-30 carbon atoms (C), but the most common naturally occurring fatty acids contain an even number of carbon atoms between 12 and 22 (C12-22) (BNF 1992, Gurr 1993, HEA 1994). The length of the hydrocarbon chain serves as the basis for the classification of fatty acids into short, medium or long (and occasionally, very long) species. There are, however, some discrepancies in the literature as to chain length classification: for example, medium-chain fatty acids have been defined as C8-10 (Gurr 1993), C8-12 (HEA 1994), C8-14 (Hamosh *et al* 1984), or as C10-14 (BNF 1992). Some authors also refer to fatty acids of chain length of C20+ as very long chain. On the basis that short-chain fatty acids are those of a volatile nature, and that chains of less than C16 are saturated, the classification below has been adopted for the purpose of this review:

| Short | Medium | Long |
|---|---|---|
| C1-6 | C8-14 | C16-22 |

1.1.2(b) Saturation

Fatty acid hydrocarbon chains may also vary in the number of bonds between constituent carbon atoms. Carbon atoms are capable of forming four covalent bonds. When each carbon atom (except the terminal ones) are bonded to adjacent carbon atoms via single covalent bonds, each C can also bond to two hydrogen atoms (H) (or three for terminal carbons), thus the four possible covalent bonds are complete. The bonding capacity of each C is "saturated" and the chain is "saturated" with hydrogen *i.e.* a saturated fatty acid. If two adjacent carbon atoms form an ethylenic double bond, they are only able to bond to one hydrogen each. The hydrocarbon chain as a whole has the capacity to bond more hydrogen should the double bond "break", thus the chain is not saturated with hydrogen *i.e.* an unsaturated fatty acid. When the hydrocarbon chain contains one double bond, the fatty acid is called monounsaturated; when it contains two or more double bonds, it is called polyunsaturated. The double bond sequence is usually methylene-interrupted.

The ethylenic double bond can itself give rise to variations of fatty acids, as it can adopt two geometrical configurations or isomers (BNF 1992) (Figure 2). When the

two hydrogen atoms bonded to the two carbons involved in the double bond are on the same side of the molecule as each other, there is a "kink" in the hydrocarbon chain. This is called the *cis* or Z configuration. When the hydrogen atoms are on opposite sides from each other, the chain has a straight configuration, called the *trans* or E configuration. The *cis* isomer occurs in naturally occurring lipids, while the *trans* isomer is "mechanically" introduced during the commercial production of fats (Bender 1997, BNF 1992).

(a) (b)

Figure 2. Fatty acid isomers in (a) *cis* configuration, (b) *trans* configuration.

1.1.2(c) Nomenclature

There is a systematic method of naming fatty acids: nomenclature encompasses chain length, saturation, isomerism, double bond position and metabolic relationships. The details of these conventions are discussed elsewhere (BNF 1992, FAO & WHO 1980, Salway 1994) and are summarized as follows (Figure 3):

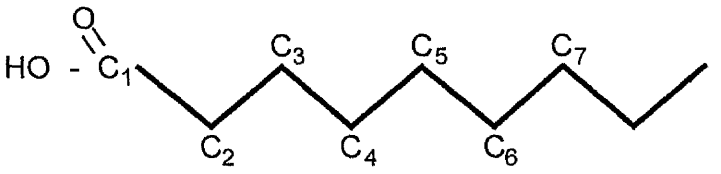
1. Systematic chemical name - derived from the name of the parent hydrocarbon, based on chain length. The final "e" is substituted with "anoic" for saturated fatty acids, "enoic" for monounsaturates, "dienoic" for polyunsaturates with two double bonds, "trienoic" for polyunsaturates with three double bonds, *etc.*
2. Identification of carbon atoms from the carboxyl end - by either
    (a) labeling the carboxyl carbon as $C_1$ and continuing numerically ($C_2$, $C_3$ *etc.*) along the chain until the methyl carbon (Figure 3(a))
    (b) labeling the carbon atom adjacent to the carboxyl carbon as the alpha carbon ($\alpha$), and continuing along the chain ($\beta$, $\gamma$ *etc.*) until the methyl carbon which is labeled the omega carbon ($\omega$) (Figure 3(b)). Convention has now adopted the symbol "n" to denote $\Omega$ (the upper case of $\omega$). In this system, the carboxyl carbon is not labeled, as it is part of a functional group rather than the hydrocarbon chain.
3. Position of double bonds - either

(a) relative to the carboxyl end, with the delta symbol Δ followed by a superscript number which denotes the first C of the pair in the double bond, as numbered from the carboxyl $C_1$

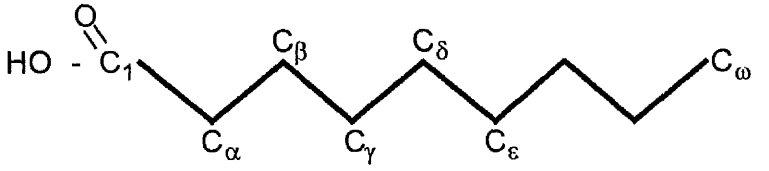
(b) relative to the methyl end, with the symbol ω (or n) followed by the number which denotes the first C of the pair in the double bond, as numbered from the methyl carbon (ω-C or n-C which is numbered ω-1 or n-1).

4. Identification of unsaturated fatty acids by isomerism - *cis* and *trans*, or *c* and *t*, before the double bond location, denotes the configuration of the fatty acid.
5. Metabolic relationships - since fatty acids are lengthened by adding carbon atoms at the carboxyl end, the actual position of the double bonds does not change relative to the methyl end, but differs relative to the total number of carbon atoms. If the ω-C at the methyl end is counted as ω-1 (n-1), the first carbon in the double bond can be denoted by numbering from the methyl end (*e.g.* n-3) (Figure 3(c)). Fatty acids can thus be considered related if their first double bond from the methyl end is in the same position relative to the ω-C. This allows fatty acids to be identified as belonging to a particular "family", *e.g.* n-3, n-6 and n-9 families.

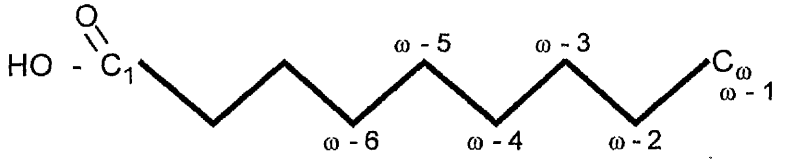
(a)




(b)




(c)




(d)

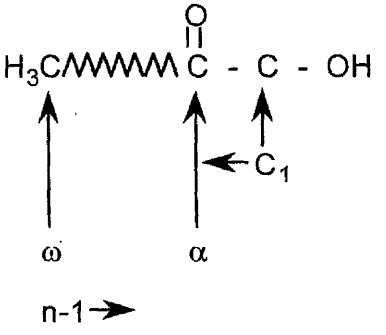



Figure 3. Fatty acid nomenclature. The various systems of classification shown are: (a) numbering the carbon atoms sequentially starting from C1, the carboxyl carbon, (b) labelling the carbon atoms sequentially starting from Cα, the carbon adjacent to the carboxyl carbon, (c) labelling the double bonds sequentially starting from Cω, the methyl carbon. The systems are summarised in (d).

Combinations of these conventions allow fatty acids are to be referred to by both their systematic chemical names and abbreviated formulae, as well as by common names. The full systematic nomenclature combines the type of isomer with the positions of the double bonds from the carboxyl end and the actual systematic name. Thus, for example, a fatty acid of 18C chain length with a double bond in the *cis* configuration between carbons 9 and 10 from the carboxyl end is denoted *cis*-9-octadecenoic. Although this system does not include the designation of family or type, it can be deduced since the length of the chain and position of the double bond are known. Thus, if the double bond occurs 9 to 10 carbons along from the carboxyl end in an 18C chain, it is therefore 9 carbons along from the methyl end, *i.e.* at n-9. Omission of the isomer type before the name implies that all double bonds present are in the *cis* configuration.

Shorthand notation of fatty acids consists of the number of carbons in the chain (x), followed by a colon, the number of double bonds (y), and then the position of the first double bond relative to the methyl end (n) to denote the family to which the fatty acid belongs (n-m), such that the general formula is x:yn-m (FAO & WHO 1980). By this notation, for example, *cis*-9-octadecenoic is denoted 18:1n-9.

The common name of a fatty acid does not impart any specific information regarding its size or type, and may be derived from other aspects such as the source of the fatty acid; for example *cis*-9-octadecenoic (18:1n-9) is derived from olive oil and hence referred to as oleic acid.

The nomenclature and formulae of fatty acids are summarized in Tables 1-3.

| Shorthand | Common Name | Systematic Name | Chemical Formula | Molecular Weight | Sources |
|---|---|---|---|---|---|
| 1:0 | Formic | | $CH_2O_2$ | 46.03 | |
| 2:0 | Acetic | | $C_2H_4O_2$ | 60.05 | Vinegar |
| 3:0 | Propionic | | $C_3H_6O_2$ | 74.08 | |
| 4:0 | Butyric | Butanoic/Tetranoic | $C_4H_8O_2$ | 88.11 | Coconut oil, dairy products (butterfat, milk) |
| | | | $C_3H_7COOH$ | | |
| 6:0 | Caproic | Hexanoic | $C_6H_{12}O_2$ | | Coconut oil, dairy products (butterfat, milk) |
| | | | $C_5H_{11}COOH$ | | |
| 8:0 | Caprylic | Octanoic | $C_8H_{16}O_2$ | 144.2 | Coconut oil, dairy products (milk) |
| | | | $C_7H_{15}COOH$ | | |
| 10:0 | Capric | Decanoic | $C_{10}H_{20}O_2$ | 172.3 | Coconut oil, dairy products, palm oil |
| | | | $C_9H_{19}COOH$ | | |
| 12:0 | Lauric | Dodecanoic | $C_{12}H_{24}O_2$ | 200.3 | Coconut oil, dairy products |
| | | | $C_{11}H_{23}COOH$ | | |
| 14:0 | Myristic | Tetradecanoic | $C_{14}H_{28}O_2$ | 228.4 | Coconut oil, dairy products (butterfat, milk), animal fats, palm oil, nutmeg oil |
| | | | $C_{13}H_{27}COOH$ | | |
| 15:0 | Pentadecyclic | Pentadecanoic | $C_{15}H_{30}O_2$ | 242.4 | |
| 16:0 | Palmitic | Hexadecanoic | $C_{16}H_{32}O_2$ | 256.4 | Animal/Veg fat, palm oil, cottonseed oil, dairy products (milk, butter) |
| | | | $C_{15}H_{31}COOH$ | | |
| 17:0 | Margaric | Heptadecanoic | $C_{17}H_{34}O_2$ | 270.5 | |
| 18:0 | Stearic | Octadecanoic | $C_{18}H_{36}O_2$ | 284.49 | Animal/Veg fat, milk and dairy products, chocolate, hydrogenated fats |
| | | | $C_{17}H_{35}COOH$ | | |
| 20:0 | Arachidic/ Arachic | Eicosanoic | $C_{20}H_{40}O_2$ | 312.5 | Peanut oil, lard, nut and seed oils |
| | | | $C_{19}H_{39}COOH$ | | |
| 22:0 | Behenic | Docosanoic | $C_{22}H_{44}O_2$ | 340.6 | Peanut oil |
| | | | $CH_3(CH_2)_{20}COOH$ | | |
| 24:0 | Lignoceric | Tetracosanoic | $C_{24}H_{48}O_2$ | 368.6 | |
| | | | $CH_3(CH_2)_{22}COOH$ | | |
| 26:0 | Cerotic | Hexacosanoic | $C_{26}H_{52}O_2$ | 396.7 | |
| 28:0 | Montanic | Octacosanoic | $C_{28}H_{56}O_2$ | 424.7 | |

Table 1. Nomenclature of saturated fatty acids.

| Shorthand | Family | Common Name | Systematic Name | Chemical Formula | Molecular Weight | Source |
|---|---|---|---|---|---|---|
| 10:1 | n-1 | Caproleic | cis-9-Decenoic | $C_{10}H_{18}O_2$<br>$CH_2C{=}CH(CH_2)_7CO_2H$ | 170.25 | Butterfat |
| 12:1 | n-3 | Lauroleic | cis-9-Dodecenoic | $C_{12}H_{22}O_2$ | 198.31 | Butterfat |
| 14:1 | n-5 | Myristoleic | cis-9-Tetradecenoic | $C_{14}H_{26}O_2$ | 226.4 | Butterfat |
| 15:1 | | Pentadecenoic | cis-10-Pentadecenoic | $C_{15}H_{28}O_2$ | | |
| 16:1 | n-7 | Palmitoleic | cis-9-Hexadecenoic | $C_{16}H_{30}O_2$<br>$C_{15}H_{29}COOH$<br>$CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2H$ | 254.4 | Fish oils (cod liver oil), palm oil, meat fat |
| 17:1 | | | Heptadecenoic | | | |
| 18:1 | n-9 | Oleic/Elainic | cis-9-Octadecenoic | $C_{18}H_{34}O_2$<br>$C_{17}H_{33}COOH$<br>$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$ | 282.47 | Olive oil, peanuts, corn oil, nut and seed oils, safflower oil, marg., meat fat, butter, eggs, avocado, (most fats) |
| 18:1 | n-9 | Elaidic | trans-9-Octadecenoic | $C_{18}H_{34}O_2$ | 282.5 | Butter, beef and lamb fat, ruminant fats, hydrogenated fats |
| 18:1 | n-7 | Vaccenic | cis-11-Octadecenoic | $C_{18}H_{34}O_2$ | 282.47 | |
| 20:1 | n-11 | Gadoleic/ Eicosenic | cis-9-Eicosenoic | $C_{20}H_{38}O2$ | | Fish oils |
| 20:1 | n-9 | Gondoic | cis-11-Eicosenoic | $C_{20}H_{38}O_2$<br>$CH_3(CH_2)_7CH{=}CH(CH_2)_9CO_2H$ | 310.53 | Fish, peanut oil |
| 20:1 | n-7 | | Cis-13-Eicosenoic | $C_{20}H_{38}O_2$ | 310.5 | |
| 22:1 | n-9 | Erucic | cis-13-Docosenoic | $C_{22}H_{42}O_2$<br>$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}CO_2H$ | 338.6 | High erucic varieties of rapeseed oil and mustard oil |
| 22:1 | n-11 | Cetoleic | cis-11-Docosenoic | $C_{22}H_{42}O_2$ | | Fish oil |
| 24:1 | n-9 | Nervonic/ Selacholeic | cis-15-Tetracosenoic | $C_{24}H_{46}O_2$<br>$CH_3(CH_2)_7CH{=}CH(CH_2)_{13}CO_2H$ | 366.63 | |

Table 2. Nomenclature of monounsaturated fatty acids.

| Shorthand | Family | Common Name | Abbreviation | Systematic Name | Chemical Formula | Molecular Weight | Source |
|---|---|---|---|---|---|---|---|
| 16:2 | | | | Hexadecadienoic | | | |
| 16:4 | | | | Hexadecatetraenoic | | | |
| 18:2 | n-6 | Linoleic | LA | cis,cis-9,12-Octadecadienoic | $C_{18}H_{32}O_2$<br>$C_{17}H_{31}COOH$<br>$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_6CO_2H$ | 280.46 | Linseed, veg. oil, nuts sunflower, corn oil, lean meat, eggs, safflower oil |
| 18:3 | n-6 | γ-Linolenic | GLA | all cis-6,9,12-Octadecatrienoic | $C_{18}H_{30}O_2$ | 278.43 | Evening primrose oil, borage, blackcurrant seed |
| 18:3 | n-3 | α-Linolenic | αLA, ALA | all cis-9,12,15-Octadecatrienoic | $C_{18}H_{30}O_2$<br>$C_{17}H_{29}COOH$<br>$CH_3(CH_2CH{=}CHCH_2)_3(CH_2)_6CO_2H$ | 278.4 | Soybean oil, linseed oil, rapeseed oil |
| 18:4 | n-3 | Stearidonic | | all cis-6,9,12,15-Octadecatetrenoic | $C_{18}H_{28}O_2$ | 276.4 | |
| 20:2 | n-6 | | | cis-11,14-Eicosadienoic | $C_{20}H_{36}O_2$<br>$CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_8CO_2H$ | 308.51 | |
| 20:3 | n-6 | Dihomo-γ-linolenic | DHGLA | cis-8,11,14-Eicosatrienoic | $C_{20}H_{34}O_2$ | 306.5 | |
| 20:3 | n-9 | Mead | | all cis-5,8,11-Eicosatrienoic | $C_{20}H_{34}O_2$ | 306.5 | |
| 20:4 | n-6 | Arachidonic | AA | all cis-5,8,11,14-Eicosatetraenoic | $C_{20}H_{32}O_2$<br>$C_{19}H_{31}COOH$<br>$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2CO_2H$ | 304.5 | Lecithin, lard, meat, peanuts, eggs, offal, game, liver, brain |
| 20:4 | n-3 | | ETA | all cis-8,11,14,17-Eicosatetraenoic | | 302.46 | |
| 20:5 | n-3 | Timnodonic | EPA | all cis-5,8,11,14,17-Eicosapentaenoic | $C_{20}H_{30}O_2$ | 302.5 | Fish oils |
| 21:5 | | | | Heneicosapentaenoic | | | |
| 22:2 | n-6 | | | cis-13,16-Docosadienoic | $C_{22}H_{40}O_2$ | 336.6 | |
| 22:3 | | | | Docosatrienoic | | | |
| 22:4 | n-6 | Adrenic | | cis-7,10,13,16-Docosatetraenoic | $C_{22}H_{36}O_2$ | 332.5 | |
| 22:5 | n-6 | Osmond acid | DPA (n-6) | all cis-4,7,10,13,16-Docosapentaenoic | | | |
| 22:5 | n-3 | Clupanodonic | DPA (n-3) | cis-7,10,13,16,19-Docosapentaenoic | $C_{22}H_{34}O_2$ | 330.5 | Fish oils |
| 22:6 | n-3 | Cervonic | DHA | all cis-4,7,10,13,16,19-Docosahexaenoic | $C_{22}H_{32}O_2$ | 328.5 | Fish oils, eggs, liver, brain, offal |

Table 3. Nomenclature of polyunsaturated fatty acids.

1.1.2(d) Essentiality

The designation of unsaturated fatty acids as belonging to a particular family on the basis of the position of the first double bond relative to the methyl carbon (n) is of a greater biological importance than a system of nomenclature. The metabolism of fatty acids involves the addition of more carbons to the hydrocarbon chain (elongation) and more double bonds within the chain (desaturation), processes catalyzed by enzymes (see Chapter 3, Section 3.1.3 Fatty Acid Synthesis). The enzymes that introduce double bonds relative to the methyl carbon (n-1) at n-3, n-6 and n-9 are all present in plant cells, whereas animal cells only have enzymes capable of inserting double bonds after the n-6 position (*i.e.* not at n-3 or n-6). It is therefore essential that animals obtain fatty acids with double bonds in the n-3 and n-6 positions from their diet. However, animals do not need to obtain all n-3 and n-6 fatty acids preformed, as they are capable of elongating the chains and adding double bonds at positions other than at n-3 and n-6. The shortest hydrocarbon chain which can contain multiple double bonds is one of 18C. The first fatty acids in the n-3 and n-6 families are therefore α–linolenic acid (αLA, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively. From these, animals can derive longer and more unsaturated fatty acids in each family thus αLA and LA are required from the diet, *i.e.* are essential fatty acids (EFA). There is evidence that a lack of dietary LA gives rise to deficiency disorders, although this is controversial in the case of αLA, whose true essentiality may arise more from its role in development (Koletzko 1987) and as a source of DHA (Spector 1999).

1.1.2(e) Forms

Fatty acids can exist as individual moieties as free/non-esterified fatty acids (FFA/ NEFA), and as components of the other classes of lipids, including acylglycerols and phospholipids.

### 1.1.3 Acylglycerols

The carboxyl group of a fatty acid can form an ester linkage with glycerol (a three carbon alcohol) (Figure 4) (Bender 1997) to form an acyglycerol. The combination of one, two and three fatty acids with glycerol forms a monoacylglycerol, a diacylglycerol and a triacylglycerol respectively. These were previously referred to in older literature

as glycerides, but acylglycerols are the officially approved nomenclature (Gurr 1993, Manson & Weaver 1997).

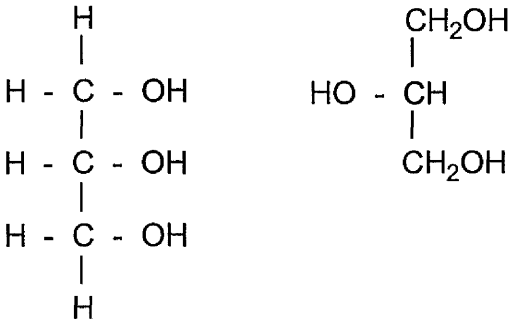


Figure 4. Structure of glycerol.

Most of the lipid obtained from the diet is in the form of triacylglycerols. In addition, lipid is stored in adipose tissue as triacylglycerols. Hence, the commonly used term "fat" usually refers to triacylglycerols. Triacylglycerols are hydrophobic.

1.1.3(a) Nature and Fatty Acid Composition

The fatty acids on a triacylglycerol are not necessarily the same and adopt specific distributions (Figure 5) (BNF 1992, Manson & Weaver 1997) (see Chapter 3, Section 3.1.4 Triacylglycerol Synthesis, Table 14). The position of each fatty acid on the glycerol moiety of a triacylglycerol is denoted by stereospecific numbering, abbreviated to *Sn* (Manson & Weaver 1997) or *sn* (Jensen 1989a). The distribution of fatty acids on the glycerol molecule may not always be random, and certain fatty acids are preferentially located at a specific position. This has implications for the absorption and metabolism of dietary fats, particularly in neonates (see Chapter 3, Section 3.1.2 Infant Fat Digestion and Absorption) (Amate, Ramirez & Gil 1999, Carnielli *et al* 1995, Nelson & Innis 1999).

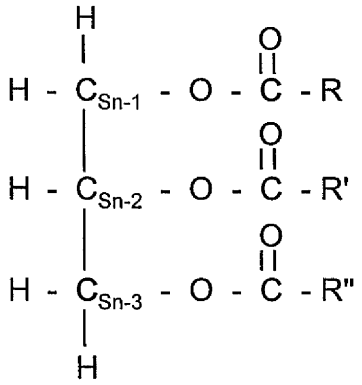


Figure 5. Structure of a triacylglycerol. R, R' and R" denote different fatty acyl chains at the *Sn*-1, 2 and 3 positions respectively. *Sn* denotes stereospecific numbering.

The fatty acids present in a triacylglycerol determine its characteristics and the nature of the fat of which it is a component. If the majority of the fatty acids are saturated, the fat is referred to as saturated fat; such fats are largely derived from animal sources and are generally solid at room temperature. Triacylglycerols rich in *trans* unsaturated fatty acids are also solid at room temperature and are obtained from partially hydrogenated fats produced by processing. The presence of *cis* unsaturated fatty acids imparts certain characteristics, including lowering the melting point of the fatty acid. Such unsaturated fats therefore tend to be liquid at room temperature (the definition of an oil). Unsaturated fats are commonly obtained from non-animal foods, such as vegetable sources (vegetable fats or oils) or fish (fish oils). Unsaturated fats or oils may differ further in the nature of the predominant fatty acids, which may be either monounsaturated or polyunsaturated; the polyunsaturates may themselves be derived from a particular family, principally the n-3 or n-6 families. Thus, oils can contain more or less of certain fatty acids and so be a source of the discrete fatty acid classes.

The triacylglycerols stored in the body by adipocytes are subject to dietary influences, and their fatty acid composition and hence nature are reliant on that of dietary triacylglycerols.

### 1.1.4 Phospholipids

Of the other compounds classified as lipids, most have a more structural role (Gurr 1993), particularly as components of cell membranes. They can be obtained from the

diet in much smaller quantities than triacylglycerols, in the cell membranes of the food ingested. Such structural fats account for 5-8% of body weight and specific organs, notably the brain, have a high content of structural fat (Health Education Authority/HEA 1994).

The most abundant lipids in membranes are phospholipids, which consist of two fatty acids esterified to a molecule of glycerol, the third hydroxyl group of which is esterified to phosphate, which is itself esterified to a hydrophilic base (Figure 6). The base (or alcohol) group may be choline, ethanolamine, serine or inositol, giving rise to the phospholipids, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) respectively (Bender 1997, Houslay & Stanley 1982).

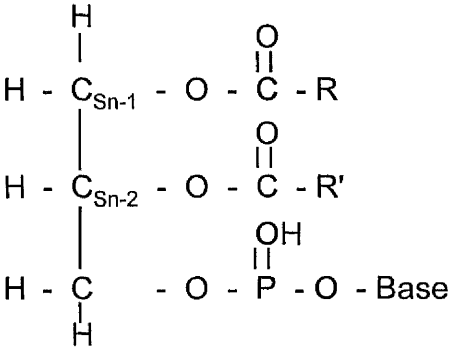



Figure 6. Structure of a phospholipid. R and R' denote different fatty acyl chains at the *Sn*-1 and 2 positions respectively. *Sn* denotes stereospecific numbering.

Phospholipids are amphiphilic/ambiphilic/amphipathic as they contain a polar/charged hydrophilic base and nonpolar aromatic/alipathic hydrophobic fatty acids (BNF 1992, Gurr 1993, Houslay & Stanley 1982).

1.1.4(a) Membrane Structure and Function

In aqueous solution, the hydrophobic fatty acids of phospholipids aggregate to expel water molecules. They "turn in" to one another to form a bilayer, in which their acyl chains form a hydrophobic core (Figure 7). Van der Waal's forces between the acyl chains of adjacent phospholipids allow them to pack closely together (Stryer 1988).

The hydrophilic phosphorylated alcohol unit (polar head group) of each phospholipid exists on the surface of such bilayers.

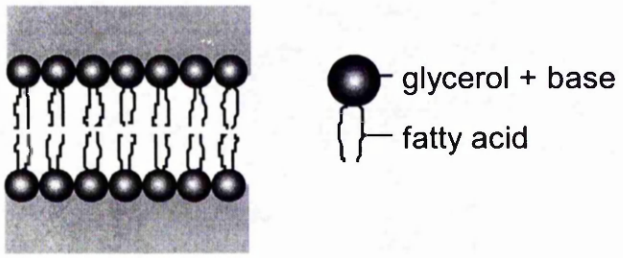



Figure 7. Schematic representation of a phospholipid and a phospholipid bilayer. The hydrophobic acyl chains of the fatty acids and the hydrophilic phosphorylated base are shown.

These lipid membranes form permeability barriers into which proteins can insert; such proteins are involved in cell signaling and recognition processes. Lipid and protein molecules are mobile along the plane of the membrane (Gurr 1993, Houslay & Stanley 1982, Stryer 1988). Such lateral diffusion (Stryer 1988) gives rise to different constituents on either side of the membrane and hence to membrane asymmetry. In this type of asymmetrical bilayer sheet (Singer and Nicolson fluid mosaic model of biological membranes), the function of the membrane proteins is influenced by the fatty acid composition and cholesterol content, which affect membrane fluidity (Houslay & Stanley 1982, Stryer 1988). Tissue specific patterns of the fatty acid content of membrane phospholipids are observed and thus implicated in physiological functions. Moreover, phospholipid composition is subject to less dietary influence than that of triacylglycerols, with extreme conditions required to produce change in phospholipid fatty acids (FAO & WHO 1980).

## 1.2 FUNCTIONS OF LIPIDS

The functions of lipids and their constituent fatty acids have been defined as energy storage, energy-providing, metabolic and structural (BNF 1992). The mechanisms and biochemistry by which they achieve these essential functions are beyond the scope of this discussion. The various roles of lipids are summarized, with emphasis on their contribution to neural function, below.

### 1.2.1 Lipids in Energy and Metabolism

Lipids can be stored as triacylglycerols in adipose tissue and breast milk, which can then be mobilized and oxidized to obtain energy (see Chapter 3, Section 3.1.5 Fatty Acid Oxidation). As with all tissues, adipose possesses unique characteristics (Pond 1999), which are outwith the scope of this discussion; the lipids of breast milk are discussed later (see Chapter 5, Section 5.2 Fatty Acid Status of Breast Milk).

The metabolic role of lipids pertains to their contribution to eicosanoid metabolism and to cell signaling via the inositol cycle and generation of second messengers. Both n-6 and n-3 families are precursors of eicosanoids (prostaglandins, prostacyclins, thromboxanes, leukotrienes, lipoxins), their absolute and relative amounts determinants of eicosanoid metabolism (Rubin & Laposata 1992). Eicosanoids are physiologically active in the inflammatory response, platelet aggregation, vasodilation/constriction and muscle function. The role of fatty acids in eicosanoid metabolism and its relevance to pregnancy (Ogburn Jr. 1998) and infancy (Sellmayer & Koletzko 1999) has been discussed in detail elsewhere. The myriad of effects mediated by lipids within the immune (Various 1998) and cardiovascular (Leaf & Weber 1988, Marckmann & Grønbæk 1999) systems have been reviewed, as has the evidence for their beneficial effects (de Deckere *et al* 1998, Simopoulos 1999a, Uauy & Valenzuela 2000).

### 1.2.2 Lipids in Cell Membrane Structures

Lipids have a definite structural role as components of cell membranes, exerting an influence on membrane conformation and stability. This has led to the implication of membrane lipids as determinants of membrane nature or fluidity (Manku *et al* 1983); the more correct interpretation, however, may be of membrane "molecular order and mobility" (Watts 1997). Phospholipids account for approximately 80% of membrane lipids, with phosphatidylcholine and phosphatidylethanolamine the predominant classes of phospholipids. Specific fatty acids adopt selective positioning within phospholipid molecules, which are in turn preferentially incorporated into membranes.

The shape of the phospholipid molecule is determined by the size of both its base group (choline, ethanolmine *etc.*) and constituent fatty acids; phospholipid shape can

itself influence membrane structure and function (Watts 1997). In addition, the degree of unsaturation within a fatty acid contributes to the "packing" of the lipids within the membrane bilayer. Unsaturated fatty acids do not adopt a linear conformation and introduce "local deformations" (Watts 1997) to the bilayer, which affect membrane permeability. Moreover, lipids are not static membrane components, but are capable of a high degree of diffusion within the bilayer, conferring both fluidity and asymmetry on the membrane.

By determining much of the membrane's conformation and nature, lipids influence the environment of membrane proteins. Lipids and proteins are also capable of interaction and lipids may not only provide the optimal environment for protein but may also facilitate protein activity and function (BNF 1992). Membrane lipids may thus be as important as their protein neighbours in the regulation of membrane and hence cellular processes (Futerman, Ghidoni & van Meer 1998).

Such functionality dictates the need for selectivity by cell membranes of both lipid classes and their constituent fatty acids. This gives rise to the characteristic patterns of fatty acid composition of cell membranes, particularly in neural tissues such as the brain and retina (see Chapter 3, Section 3.2.5 Fetal Lipid Accretion), where a high concentration of DHA is implicated in neurotransmission (Cockburn 1997).

The human brain, from various ages of the human spectrum, displays specific patterns of fatty acid composition (Svennerholm 1968). Brain FA are distributed between the major PL in a specific manner, and within each PL species in a tissue specific manner between cerebral gray and white matter, with white matter containing less PUFA. Moreover, levels of PUFA from the n-6 and n-3 families vary with age; n-6 PUFA increase until adulthood then decrease, with concomitant increase in n-3 PUFA (Svennerholm 1968).

The conservation of brain DHA (& AA) patterns between species suggests a prerequisite for brain function. Brain size and development may therefore have been determined by the availability of DHA in the evolutionary past, implicating DHA in distinguishing humans from other species (Broadhurst, Cunnane & Crawford 1998a,

Crawford *et al* 1992 & 1999), a theory not without controversy (Broadhurst, Cunnane & Crawford 1998b, Gurr 1998).

DHA co-exists in the outer rod cells of the retina with the photoreceptor rhodopsin. The function of rhodopsin appears to be dependent on the presence of DHA, which may facilitate the optimal spatial environment within the bilayer for rhodopsin to function (Dratz & Holte 1992, Watts 1997).

The precise reason(s) for the specific presence and requirement of DHA in the membranes of neural tissues is unknown. It has been postulated that DHA interacts with membrane proteins in a specific manner, that it forces the membrane to adopt a certain conformation which induces stability and/or it is involved in neuronal signaling (Kim & Edsall 1999, Kurlak & Stephenson 1999). The continual diffusion of lipids within the membrane bilayer is, however, cited as evidence that protein-lipid interactions would be transient and contribute little to modulating protein function (Crawford *et al* 1999). Regulation of gene expression may offer an alternative mechanism for the functionality of DHA (Uauy, Mena & Rojas 2000). Whatever the mode of action, since a lack of DHA (cis-4,7,10,13,16,19-docosahexaenoic) leads to incorporation of 22:5n-6 DPA (cis-4,7,10,13,16-docosapentaenoic), it has been postulated that the molecular requirement for DHA resides in its methyl end and n-3 double bond (Spector 1999).

Chapter 2

# N-3 PUFA Intakes and Requirements

## 2.1 INTRODUCTION

The sources of each fatty acid in the human diet were summarised in Tables 1-3 (Chapter 1, Section 1.1.2(c) Fatty Acid Nomenclature). Although the parent n-3 EFA, α-linolenic acid (αLA, 18:3n-3) can be derived from plant sources (vegetable seed oils), its longer and more unsaturated derivatives, such as EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3), can only be found in appreciable amounts in fish oils. The sources of and requirements for n-3 PUFA in the human diet are outlined and discussed.

## 2.2 CLASSIFICATION OF FISH AND DEFINITION OF FISH OILS

Fish can be classified in various ways according to whether they are found in fresh or sea water, whether they are bony or cartilaginous, and/or whether they are pelagic (occur near the surface of the water) or demersal (deep in the water). However, the most common classification when considering dietary fish categorises fish as either white and lean, or oily. Essentially the pelagic/demersal classification translates into oily and lean respectively (Sargent 1997).

Fish oils are the edible fats found in fish; they are referred to as oils because they are liquid at room temperature. Fish contain these oils mainly in the form of phospholipids and triacylglycerols (Stansby 1973). The phospholipids are generally intracellular, occurring in the cell membranes of tissue and flesh, and have structural and functional roles. The triacylglycerols are stored as energy reserves and since fish have no adipose tissue, are deposited either in the flesh or in the liver. Fish which contain these triacylglycerol depots in their liver have very few fats, other than the phospholipids, in their flesh (*i.e.* their flesh is lean), while 50-80% of the weight of the liver may be accounted for by oil (Stansby 1973). Thus it is the oils found in their livers (*i.e.* fish liver oil) which are a rich source of n-3 fatty acids. Species of lean fish include cod, haddock, plaice and whiting. In contrast, some fish store reserve triacylglycerols in their flesh and up to 20% of their wet weight is comprised of oil (Sargent 1997), hence they are referred to as oily, fatty or oil-rich fish. The composition of fish oils varies with season, species and diet (Sargent 1997, Stansby 1973), but all fish oils share the characteristic of high n-3 levels. Thus the distinction between oily and lean fish is made on the basis of where their oil is stored (flesh or liver) and hence the relevance to the human diet, since humans consume the flesh

rather than the livers of fish. The content of selected n-3 PUFA in various species of fish is known; when considering the human diet, the types of fish commonly consumed, the ways in which they are prepared and the portion size consumed must be taken into account (Table 4).

## 2.3 ORIGINAL SOURCES OF N-3 PUFA OBTAINED FROM FISH OILS

Fish obtain n-3 LCPUFA from their own diet. Marine phytoplankton (microscopic plants) are the source of long chain n-3 products in the food chain, containing large amounts in their chloroplast membrane lipids. Species of phytoplankton differ in which PUFA they synthesise; the reason for DHA production is unknown but may pertain to the motility and photostatic (light seeking) behaviour of the phytoplankton (Sargent 1997). Marine zooplankton (microscopic animals) feed on phytoplankton and store the lipids obtained in their oils. Fish then ingest the zooplankton and so obtain preformed n-3 LCPUFA. Manipulation of the diet of farmed fish by providing an adequate supply of marine oils, and therefore preformed n-3 PUFA, can thus ensure fish destined for human consumption contain adequate amounts of n-3 PUFA (Sargent 1997).

| Food | αLA (18:3n-3) g per 100g[a] | EPA (20:5n-3) g per 100g[a] | DHA (22:6n-3) g per 100g[a] | Average Portion Size (g)[b] | αLA (18:3n-3) g per Portion | EPA (20:5n-3) g per Portion | DHA (22:6n-3) g per Portion |
|---|---|---|---|---|---|---|---|
| bass, sea, raw | 0 | 0.1 | 0.4 | | | | |
| caviare, bottled in brine, drained | trace | 0.5 | 0.8 | 19 | | 0.10 | 0.15 |
| clams, canned in brine, drained | trace | 0.1 | trace | | | | |
| cockles, boiled | 0 | trace | trace | 4 | 0.00 | | |
| cockles, bottled in vinegar, drained | 0 | 0.1 | trace | 25 | 0.00 | 0.03 | |
| cod, coated in batter, frozen, baked | 0.1 | 0 | 0.1 | 100 | 0.10 | 0.00 | 0.10 |
| cod, raw | trace | 0.1 | 0.2 | | | | |
| cod, steamed | trace | 0.1 | 0.2 | 120 | | 0.12 | 0.24 |
| crab, boiled | trace | 0.5 | 0.5 | 40 | | 0.20 | 0.20 |
| eel, jellied | 0.1 | 0.2 | 0.2 | 70 | 0.07 | 0.14 | 0.14 |
| fish cakes, frozen | 0.2 | trace | 0.1 | 50 | 0.10 | | 0.05 |
| fish fingers, cod, frozen | 0.1 | 0.1 | 0.1 | 28 | 0.03 | 0.03 | 0.03 |
| fish paste | 0 | 0 | 0 | 10 | 0.00 | 0.00 | 0.00 |
| haddock raw | trace | 0.1 | 0.1 | | | | |
| haddock, steamed | trace | 0.1 | 0.1 | 120 | | 0.12 | 0.12 |
| herring, grilled | 0.2 | 0.7 | 0.8 | 119 | 0.24 | 0.83 | 0.95 |
| herring, raw | 0.2 | 0.8 | 1 | | | | |
| kipper, grilled | 0.3 | 1.3 | 1.5 | 130 | 0.39 | 1.69 | 1.95 |
| kipper, raw | 0.3 | 1.1 | 1.3 | | | | |
| lemon sole, steamed | trace | 0 | 0.1 | 170 | | 0.00 | 0.17 |
| lobster, boiled | 0.1 | 0.2 | 0.1 | 85 | 0.09 | 0.17 | 0.09 |
| mackerel, grilled | 0.2 | 0.8 | 1.2 | 160 | 0.32 | 1.28 | 1.92 |
| mackerel, raw | 0.2 | 0.7 | 1.1 | | | | |
| monkfish, raw | trace | trace | 0.1 | | | | |
| mussels, boiled | trace | 0.4 | 0.2 | 40 | | 0.16 | 0.08 |
| mussels, canned/bottled, drained | trace | 0.3 | 0.1 | 139 | | 0.42 | 0.14 |
| mussels, raw | trace | 0.3 | 0.1 | | | | |
| pilchards, canned in tomato sauce | 0.1 | 1.2 | 1.2 | 110 | 0.11 | 1.32 | 1.32 |
| plaice, frozen, steamed | trace | 0 | 0.1 | 130 | | 0.00 | 0.13 |
| plaice, grilled | trace | 0 | 0.1 | 130 | | 0.00 | 0.13 |
| plaice, raw | trace | 0 | 0.1 | | | | |
| prawns, boiled | trace | 0.1 | 0.1 | 60 | | 0.06 | 0.06 |
| prawns, frozen, raw | trace | 0.1 | trace | | | | |
| roe, cod, hard, raw | trace | 0.2 | 0.3 | | | | |
| roe, herring, soft, raw | trace | 0.2 | 0.4 | | | | |
| salmon, pink, canned in brine, drained | 0.1 | 0.5 | 0.8 | 45-100 | 0.05-0.1 | 0.23-0.5 | 0.36-0.8 |
| salmon, raw | 0.2 | 0.5 | 1.3 | | | | |
| salmon, red, canned in brine, drained | 0.1 | 0.7 | 0.9 | 45-100 | 0.05-0.1 | 0.32-0.7 | 0.41-0.9 |
| sardines, canned in oil, drained | 0.4 | 0.9 | 0.8 | 100 | 0.40 | 0.90 | 0.80 |
| sardines, canned in tomato sauce | 0.2 | 0.9 | 0.7 | 100 | 0.20 | 0.90 | 0.70 |
| sardines, grilled | 0.1 | 1 | 1.2 | 86 | 0.09 | 0.86 | 1.03 |
| sardines, raw | 0.1 | 0.9 | 1.1 | | | | |
| shrimps, boiled | trace | 0.4 | 0.3 | 50 | | 0.20 | 0.15 |
| shrimps, canned in brine, drained | trace | 0.2 | 0.1 | 50 | | 0.10 | 0.05 |
| taramasalata | 5.2 | 0.2 | trace | 45 | 2.34 | 0.09 | |
| trout, rainbow, grilled | 0.1 | 0.2 | 0.7 | 155 | 0.16 | 0.31 | 1.09 |
| trout, rainbow, raw | 0.1 | 0.2 | 0.8 | | | | |
| tuna, canned in brine, drained | trace | trace | 0.1 | 45-100 | | | 0.05-0.1 |
| tuna, canned in oil, drained | 0.9 | 0.1 | 0.3 | 45-100 | 0.41-0.9 | 0.05-1.0 | 0.14-0.3 |
| tuna, raw | 0 | 0.3 | 1.1 | | | | |
| whelks, boiled | trace | 0 | 0.1 | 30 | | 0.00 | 0.03 |
| whiting, raw | trace | 0 | 0.1 | | | | |
| whiting, steamed | trace | 0 | 0.1 | 85 | | 0.00 | 0.09 |
| winkles, boiled | 0.1 | 0.1 | trace | | | | |

Table 4. Selected species of fish and their n-3 PUFA content. [a] Data from Holland, Brown & Buss (1993); [b] portion sizes from Ministry of Agriculture, Fisheries and Food (MAFF) (1993). Values for PUFA will vary with season, and are approximate only.

## 2.4 CURRENT N-3 PUFA INTAKES IN THE HUMAN DIET

The contribution of the various food groups to total fat and fatty acid intake in the diet of British adults is summarised in Table 5. No significant differences in intakes of total fat or classes of fatty acids exist between women in the various regions of the U.K. (Gregory *et al* 1990). Fish contributes an average of 3% of the total daily fat intake; n-3 PUFA account for an average of 0.7% total energy. Of the average daily n-3 PUFA intake, 14% is obtained from fish, with oily fish accounting for 7% of daily n-3 intake. Current intakes of total n-3 are approximately 1.7g/day (Gregory *et al* 1990). Thus if oily fish consumption provides 7% of a total 1.7g n-3 intake, the mean intake of n-3 LCPUFA (obtainable only from fish) is 0.1g/day; this intake is elevated in regular consumers of oily fish/once per week, to 0.2-0.3g/day or 2g/week.

| | Average daily fat intake in g (% of total intake) | | | | | |
|---|---|---|---|---|---|---|
| | | ←——— Fatty Acids ———→ | | | | |
| | Total Fat | Saturated | Trans | MUFA | PUFA | |
| | | | | | n6 | n3 |
| Milk and milk products | 13.4 (15) | 8.5 (23) | 0.5 (10) | 3.3 (12) | 0.2 (2) | 0.1 (6) |
| Fat spreads (including butter and margarine) | 14.1 (16) | 6.2 (17) | 1.4 (30) | 3.0 (11) | 2.4 (20) | 0.3 (15) |
| Meat and meat products | 21.5 (24) | 83 (23) | 0.9 (18) | 8.3 (31) | 2.0 (17) | 0.3 (19) |
| Fish and fish dishes | 2.5 (3) | 0.6 (2) | 0.1 (1) | 0.9 (3) | 0.5 (4) | 0.2 (14) |
| Eggs and egg dishes | 3.6 (4) | 1.1 (3) | 0.1 (2) | 1.4 (5) | 0.5 (4) | 0.0 (2) |
| Cereal products | 16.9 (19) | 6.6 (18) | 1.3 (27) | 4.7 (18) | 2.6 (22) | 0.3 (17) |
| Vegetables (including roast and fried) | 9.7 (11) | 2.4 (6) | 0.3 (6) | 3.2 (12) | 2.8 (24) | 0.4 (22) |
| Fruit and nuts | 0.7 (1) | 0.1 (0) | 0.0 (0) | 0.3 (1) | 0.2 (2) | 0.0(1) |
| Sugar, confectionery and preserves | 2.3 (3) | 1.3 (4) | 0.2 (3) | 0.6 (2) | 0.1 (1) | 0.0 (1) |
| Beverages | 0.2 (0) | 0.2 (0) | 0.0 (0) | 0.0 (0) | 0.0 (0) | 0.(0) |
| Other | 3.1 (3) | 1.2 (3) | 0.1 (2) | 0.9 (3) | 0.5 (5) | 0.0 (2) |
| Total | 88 | 36.5 | 4.8 | 26.7 | 11.7 | 1.6 |

Table 5. The contribution of food groups to daily intake of fat and fatty acids by adults. Reproduction of Table 3.7 from the BNF Task Force Report (BNF 1992), using data from Gregory *et al* 1990.

However, fish intake in the adult population of Britain is low at approximately 150g per week (21g per day) (Gregory *et al* 1990). Most of this (60%) is accounted for by white fish, with oily fish contributing only 23% of total fish intake (Gregory *et al* 1990). Approximately one third of British adults consume one portion (average 135g) of oily fish per week, although an equal number of men and women do so (Gregory *et al* 1990).

## 2.5 REQUIREMENTS FOR N-3 PUFA

The definition of "requirements", and the rationale and criteria for establishing them is problematic, and further complicated by the way in which they are expressed, for example as a population "average", minimum/maximum, or acceptable daily intake. In addition, the units in which the requirements are expressed vary, and include expression in units relative to energy intake (% total energy intake) or in absolute weight (g/day, g/week). These issues and the difficulties in comparing reference values from various Reports are considered in more detail elsewhere (BNF 1992, Roche 1999).

There are two main reference sources for recommended levels of PUFA intake applicable to the U.K. The 1991 Department of Health (DoH) Committee on Medical Aspects of Food Policy (COMA) report on dietary reference values (Department of Health/DoH 1991) based its considerations solely on EFA levels required to prevent EFA deficiency, and therefore set values primarily for minimum requirements, stating that there was no evidence that higher levels were any more beneficial. The COMA panel recommended a minimum intake of n-3 PUFA as 18:3n-3 amounting to 0.2% of total energy. It made no recommendations for PUFA other than the EFA.

In comparison, the BNF Task Force Report on unsaturated fatty acids (UFA) (BNF 1992) considered all UFA and their roles in various aspects of health and disease, and made recommendations for infants, pregnant and lactating women, and the general adult population. The report considered EFA separately from their LCPUFA derivatives; under the term n-3 LCPUFA, it includes 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3 and defines total n-3 PUFA as 18:3n-3 in combination with the LCPUFA. It therefore produced more comprehensive recommendations than the COMA report, including minimum and maximum recommended intakes for 18:3n-3, n-3 LCPUFA and total PUFA (Table 6). The Task Force report included a higher minimum recommended intake for 18:3n-3 (0.5% total energy) than the 1991 COMA report, resulting in recommended total n-3 PUFA intakes ranging from 0.5-4.5% total energy. The maximum requirement for 18:3n-3 was based on toxicity studies; the maximum LCPUFA intake recommended (2% total energy) could be obtained on regular consumption of oily fish. For n-3 LCPUFA, no lower limit was set as the n-3 status of vegetarians appears adequate, although whether this is due to a low

arachidonic acid intake and/or metabolic adaptation is unknown. Assuming daily total energy intakes of 2550kcal and 1940kcal for males and females respectively (*i.e.* the estimated average requirement/EAR for energy set by the 1991 COMA report), the Task Force provided both relative and absolute recommendations (Table 7).

| | 1991 COMA | 1992 BNF Task Force |
|---|---|---|
| Average population intake of MUFA | 12.0 | 12.0 |
| Minimum individual intake of MUFA | Not given | 0 |
| Maximum individual intake of MUFA | Not given | 20.0 |
| | | |
| Average population intake of n-6 PUFA as 18:2n-6 | 6.0 | 6.0 |
| Minimum individual intake of n-6 PUFA as 18:2n-6 | 1 | 3 |
| Maximum individual intake of n-6 PUFA | 10 | 10 |
| | | |
| Average population intake of n-3 PUFA as 18:3n-3 | Not given | 1.0 |
| Minimum individual intake of n-3 PUFA as 18:3n-3 | 0.2 | 0.5 |
| Maximum individual intake of n-3 PUFA as 18:3n-3 | Not given | 2.5 |
| Average population intake of long chain n-3 PUFA | Not given | 0.5 |
| Minimum individual intake of long chain n-3 PUFA | Not given | Not given |
| Maximum individual intake of long chain n-3 PUFA | Not given | 2.0 |
| Minimum individual intake of total n-3 PUFA | 0.2 | 0.5 |
| Maximum individual intake of total n-3 PUFA | Not given | 4.5 |
| | | |
| Average population intake of total PUFA | 6.0 | 7.5 |
| Minimum individual intake of all PUFA | 1.2 | 3.5 |
| Maximum individual intake of all PUFA | 10 | 14.5 |

Table 6. Comparison of recommended intakes of unsaturated fatty acids for healthy adults made by the COMA Report (1991) and the BNF Task Force (1992). Values are for fatty acids as % total energy. Adapted from Table 22.6 from the BNF Task Force Report (BNF 1992).

| Fatty Acid | Average Population Intake (% Total Energy) | ←——— Safe Ranges ———→ | | |
|---|---|---|---|---|
| | | | ←——— g/day ———→ | |
| | | % Total energy | Men | Women |
| 18:2n-6 | 6 | 3-10 | 8-26 | 6-20 |
| 18:3n-3 | 1 | 0.5-2.5 | 1-6 | 1-5 |
| n-3 LCPUFA | 0.5 | 0-2.0 | 0-5 | 0-4 |
| All PUFA | 7.5 | 3.5-14.5 | 9-38 | 7-29 |
| MUFA | 12 | 0-20 | 0-51 | 0-39 |

Table 7. BNF Task Force recommended intakes of unsaturated fatty acids for healthy adults. Assumed intakes of 2550kcal/d and 1940kcal/d for men and women respectively. Reproduction of table 22.5 from the BNF Task Force Report (BNF 1992).

Recommendations for infants were based on the average contribution of PUFA to the total energy of human breast milk summarised in Table 8. Assuming a 6 month old

infant weighs 6kg and consumes 800ml milk per day, which has a fat content of 4.1g/100ml and an energy content of 69kcal/100ml, the Task Force also derived absolute values for PUFA (Table 9). Thus, milk deriving 0.4% of its energy from 18:3n-3 and 0.2% of its energy from DHA would provide 40mg of αLA and 20mg DHA per kg body weight of the infant. The Task Force considered two opposing arguments: (a) that there is evidence that all infants require preformed DHA, assuming that infants have minimal ability to elongate/desaturate αLA to DHA; (b) that there is no evidence of developmental problems in children receiving formulae with no or minimal DHA and that "it is impossible to know how many of these never achieved their full intellectual or visual potentials". The Task Force concluded by advising that DHA should be present in all formulae in equivalent amounts to that in breast milk (0.2% total energy) (Table 9).

| | **Linoleic acid 18:2n-6** | **α-Linolenic acid 18:3n-3** | **Arachidonic acid 20:4n-6** | **DHA 22:6n-3** |
|---|---|---|---|---|
| **All UK omnivores** | 4.4 | 0.39 | 0.18 | 0.21 |
| **All UK vegetarians** | 12.4 | 0.87 | 0.25 | 0.10 |

Table 8. Fatty acid composition of human milk in the U.K. Values are for fatty acids as % total energy. Reproduction of Table 22.3 from the BNF Task Force Report (BNF 1992).

Similarly, the opposing arguments regarding pregnant and lactating women concerned the apparent need for an increased intake versus the metabolic adaptations of pregnancy which may limit PUFA oxidation and/or increase accumulation, followed by enhanced mobilisation during lactation. The recommendations were thus that counselling should be offered pre-conceptionally, during pregnancy and during lactation to ensure intakes similar to the recommended average intake for the general population (Table 9).

| | | | |
|---|---|---|---|
| *Women* | | | |
| Pre-pregnant, pregnant and lactating | No specific increased recommendation for PUFA but counselling to ensure that recommendations for average population intakes are met, *i.e.* average total PUFA intakes of at least 7.5% to be achieved. | | |
| *Infants and children* | | | |
| Pre-term and full-term infants not on human milk | Formulae should contain quantities of linoleic acid, arachidonic acid, alpha linolenic acid and DHA to replicate the amounts found in human milk: | | |
| | Linoleic acid (18:2n-6) | 4% of total energy | (377mg/kg body weight with safe range of 280 to 1130mg/kg body weight) |
| | Arachidonic acid (20:4n-6) | 0.2% of total energy | (20mg/kg body weight) |
| | Alpha linolenic acid (18:3n-3) | 0.4% of total energy | (40mg/kg body weight) |
| | DHA(22:6n-3) | 0.2% of total energy | (20mg/kg body weight) |
| *Children on mixed diets* | The polyunsaturated fatty acid composition of the diet should replicate that of human milk until more information is forthcoming | | |

Table 9. Summary of BNF Task Force (1992) recommendations for PUFA intakes in special groups. Reproduction of table 22.7 from the BNF Task Force Report (BNF 1992).

## 2.6 ACHIEVING N-3 PUFA RECOMMENDATIONS

When considering the role of PUFA in the aetiology and prevention of cardiovascular disease, the DoH COMA Report of 1994 (DoH 1994) recommended that the average intake of n-3 LCPUFA, mainly EPA and DHA, be increased to 0.2g/day (1.5g/week), *i.e.* levels achieved by those eating oily fish once per week and approximating to 0.1% total energy. The DoH thus recommends the consumption of two portions of fish per week, of which one should be oily. Adoption of such recommendations would effectively double the number consuming oily fish per week in the population.

To meet the higher BNF Task Force recommendations for n-3 LCPUFA, males would need to consume approximately 1-3 portions (150-480g) per week and females 1-3 portions (120-380g) per week of oil-rich fish (herring, salmon, mackerel, pilchards, sardines) (British Nutrition Foundation/BNF 1993) (see Table 4 for n-3 PUFA composition and portion sizes of dietary fish).

However, there has been public concern regarding the level of environmental contaminants, such as polychlorinated dibenzo-*p*-dioxins/dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCB), in fish and fish products. Although fat content is only one factor which influences dioxin and PCB accumulation in fish (Ministry of Agriculture, Fisheries and Food/MAFF &

Department of Health/DOH 1999), oily fish have been found to contain slightly higher concentrations of these chemicals (MAFF & DOH 1999). Exposure to such contaminants for an adult consuming a high level of oily fish ($97.5^{th}$ percentile) remains within U.K. and WHO recommended limits (MAFF & DOH 1999). Nonetheless, DoH recommendations are that adults should consume one portion of oily fish per week, since this will comply with COMA recommendations based on health benefits without approaching U.K. or WHO limits for dioxins and PCBs (DOH 1999). The contaminant methylmercury has been found to increase with maternal consumption of seafood, and to be associated with an increased risk to neurodevelopment in the neonate, although this was observed in a population with a high component of marine food including whale in the diet (Steuerwald *et al* 2000). As a result, it is difficult to persuade an already reluctant population to consume even one portion of oily fish and hence comply with recommended n-3 PUFA intakes.

Such contaminants can be removed in the industrial preparation of purified fish oils. As an alternative to dietary fish, the BNF (1993) acknowledges the use of fish oil capsules (or liquids) and suggests consumption of 4 or 5 1g capsules per day to achieve BNF Task Force (1992) n-3 LCPUFA recommendations. Fish oil capsules available to the general public in retail outlets vary greatly in composition. Typically they are 1g in size and provide a total of 0.25-0.3g n-3 fatty acids per capsule (BNF 1993). The highest dosage available appears to be in capsules containing 1000mg of fish oil, with a DHA content of up to 250mg and a maximum EPA content of 350mg (The Boots Company PLC, Nottingham, U.K.). Most capsules are intended to be consumed once per day, although it is possible that they are actually consumed in greater quantities.

The manipulation and enrichment of foods, and the potential use of biotechnology and algal sources may provide novel methods of increasing n-3 intake (Borod *et al* 1999, Kris-Etherton *et al* 2000, Sanders 2000, Simopoulos 1999b), but their contribution is, as yet, negligible.

## 2.7 POSSIBLE ADVERSE EFFECTS OF N-3 PUFA INTAKE

Despite concerns regarding toxic contaminants, increased risk of lipid peroxidation, and an antithrombotic effect, the consumption of high levels of fish oil have not been

unequivocally associated with adverse effects (Eritsland 2000). Since environmental contaminants are removed on refinement of fish oils, the use of purified supplements may actually be more desirable than high levels of fish consumption in heavily industrialised areas. There has been no evidence to suggest that increased fish intake induces auto-oxidation and produces carcinogenic effects (Leaf & Weber 1988). In addition, plasma antioxidant levels are unaffected by a high fish intake (Anttolainen *et al* 1996). The inclusion of a suitable antioxidant within a refined fish oil product may alleviate any potential risk (Turley *et al* 1998); supplements on general sale are generally made from oils derived from fish flesh rather than fish livers, and so are low in vitamins A and D (BNF 1992).

The suggestion that the antithrombogenesis induced by high n-3 PUFA intakes, while preventing ischaemic heart disease, may increase the risk of haemorrhagic stroke (Pedersen *et al* 1999) has not been substantiated. Although high doses of n-3 PUFA (3-4g EPA per day with or without DHA) are associated with prolonged bleeding time, blood loss in supplemented surgical patients was not increased, and there is no evidence that this is clinically significant (Simopoulos 1991). Moreover, the ingestion of aspirin produces a greater increase in bleeding time than does the ingestion of fish oils, and no incidences of bleeding during clinical trials of fish oils have been reported (Leaf & Weber 1988). Supplementation studies do however, generally exclude participants prescribed drugs with antithrombocytic functions, such as non-steroidal anti-inflammatories, to prevent supplement-drug interaction. Similarly, in supplementation studies during pregnancy, women with a history or risk of abruption or post-partum haemorrhage are excluded.

It is worth noting that "no epidemiological data indicate that the intake of a reasonable quantity of fats of marine origin carries a specific risk factor for human health" (FAO & WHO 1980).

## 2.8 POSSIBLE IMPLICATIONS OF N-3 INTAKE FOR DURATION AND OUTCOME OF PREGNANCY

The role of EFA and LCPUFA in pregnancy and fetal growth has recently been reviewed (Jumpsen, Van Aerde & Clandinin 1997, Sattar, Berry & Greer 1998). The putative relationship between n-3 PUFA, gestational length and birth weight was first

considered following the association between a high marine diet and RBC n-3 levels in the Faroese population, and the observation that both gestational length and birth weight were greater in the Faroe Islands compared to Denmark. Since dietary PUFA can modulate the metabolism of prostaglandins, which are implicated in the onset of labour, the hypothesis arose that n-3 PUFA intake and status could be associated with the length of gestation and the subsequent weight attained (Olsen *et al* 1986).

In Danish mothers, the frequency of fish consumption in the month prior to 36 weeks gestation was not related to gestational length (Olsen, Olsen & Frische 1990). In non-smokers, however, fish consumption did correlate with placental weight, birth weight and head circumference, *i.e.* indices of fetal growth. However, when intakes of n-3 PUFA in Danish mothers ranged from 0-0.75g/d, no associations were found between intake or maternal RBC status at 30 weeks gestation and gestational length or birth weight (Olsen *et al* 1995b). Frequent consumption of oily fish throughout pregnancy (up to 55g/d oily fish and 2.9g/d n-3) did not affect gestational length (Sanjurjo, Matorras & Perteagudo 1995). Supplementation with n-3 fatty acids during the last trimester (mean 2.6g/d, range 0.9-3.1g/d n-3 during weeks 26-35) has been shown to be safe and to have no effect on gestational length (Connor, Lowensohn & Hatcher 1996). A similar dose and duration of supplementation (2.7g/d n-3 during weeks 24-38) in high risk pregnancies also failed to affect gestation and birth weight (Onwude *et al* 1995).

Conversely, the n-3/n-6 ratio in maternal RBC total lipids at term has not been correlated with gestational age in Faroese mothers, but has been positively so in Danish mothers. The question was subsequently raised as to whether this was due to statistical error or a dose-response relationship (Olsen *et al* 1991). However, gestation was significantly longer in Danish women supplemented with fish oil (2.7g/d n-3) from 30 weeks gestation compared to those receiving olive oil or no supplement (Olsen *et al* 1992). Since the effect was greatest in those mothers with the lowest baseline intake of fish, it has been suggested that there is indeed a dose-response relationship between n-3 intake and effect, which reaches saturation level on high intakes, such as in Faroese women (Olsen *et al* 1992).

There has been some suggestion that the high LA intake, and hence potential synthesis of AA, in vegetarian women may shorten gestation (Reddy, Sanders & Obeid 1994), but evidence for such an effect is lacking (Sanders 1999). Supplementation with LA (3g/d), in combination with a vitamin-mineral supplement, during the second and third trimesters did not affect gestation or infant anthropometry, including birth weight, suggesting that maternal nutrition affects fetal development much earlier in pregnancy (Doyle *et al* 1992).

Other outcomes of pregnancy possibly related to n-3 intake include complications or adverse outcomes. Within the Canadian Inuit population, those communities with a high marine diet have significantly lower blood pressure during labour, and a lower incidence of pregnancy-induced hypertension (PIH) (Gerrard *et al* 1990). However, supplementation with 3g/d EPA from the first trimester until delivery did not reduce the incidence of either PIH or intra-uterine growth retardation (IUGR) in a high risk population (Bulstra-Ramakers, Huisjes & Visser 1994).

Several studies have supplemented mothers with 2.7g/d n-3 in the last trimester and monitored the effect on various outcomes. At this dose and time-point, fish oil supplementation exerted no significant effects on blood lipids, urate, coagulation or fibrinolysis (Sørensen *et al* 1994). Similarly no effects on the normal third trimester rise in blood pressure (Salvig, Olsen & Secher 1996), or on the incidence of either PIH or IUGR (Onwude *et al* 1995) have been observed. Moreover, supplementation with 2.7g/d from 20 weeks did not reduce recurrence, nor did 6.1g/d from 33 weeks reduce risk, of either IUGR or PIH (Olsen *et al* 2000).

Chapter 3

# Fatty Acid Metabolism

## 3.1 ASSIMILATION AND METABOLISM OF FAT

90% of the dietary fat ingested by the adult, and 98% of that ingested by the breast-fed infant is in the form of triacylglycerol. In both cases, the remainder of the total fat consumed is cholesterol, cholesteryl esters, phospholipids and free fatty acids. The reactions, control and aberrations of lipid metabolism have been reviewed extensively (Carey & Hernell 1992, Hamosh 1998, Stanley 1998a, Wolfe 1998). The processes involved in fat digestion are essentially the same in both the adult and infant, although newborns, because of their special diet (milk) and immaturity of exocrine pancreatic function, cope with fats in a manner unique to neonatal life. For this reason, the digestive and absorptive processes of the adult and infant will be reviewed separately, to draw attention to the differences between maternal and neonatal metabolism. Intermediary lipid metabolism – synthesis and oxidation – is also discussed, with emphasis on the implications for and the fate of LCPUFA.

### 3.1.1 Adult/Maternal Fat Digestion and Absorption

3.1.1(a) Gastric Digestion

On ingestion, the hydrophobic nature of triacylglycerols prevents them from mixing with the water phase of the gastric contents. Digestive enzymes are hydrophilic and therefore soluble in an aqueous solution. Only minimal digestion of ingested fat can occur in the stomach, without mixing of the triacylglycerol substrates with the required enzymes. The peristaltic action of the stomach increases the dispersion of the fat molecules in the aqueous environment, thereby increasing contact between the enzymes and substrates.

Lingual lipase (Table 10) is secreted by the serous glands of the tongue and can function in the acidic environment of the stomach, which is devoid of bile salts (BNF 1992, Gurr 1993). Lingual lipase catalyses the slow hydrolysis of the *Sn*-3 fatty acid on the triacylglycerol. The concurrent action of gastric lipase, secreted from the gastric mucosa, hydrolyses short- (C4-6) and medium- (C8-14) chain saturated, and long-chain unsaturated (C16-22) fatty acids at the *Sn*-3 position (Embleton & Pouton 1997, Hamosh *et al* 1992). Saturated fatty acids are not released on lipolysis in the stomach (Hamosh *et al* 1992). The net result of lipase action in the stomach is the production of unsaturated 1,2-diacylglycerol and non-esterified fatty acids (Figure 8). However,

most ingested triacylglycerol escapes hydrolysis in the stomach and enters the duodenum still fully esterified.

| Lipase | Site of origin | Site of action | pH | Bile salts | Substrate | Action | *Sn*- position | Product |
|---|---|---|---|---|---|---|---|---|
| Lingual (preduodenal) | Serous glands | Stomach | 2.3-6.5 | No | Dietary TAG | Release of UFA | *Sn*-3 | 1,2-DAG, NEFA |
| Gastric (predoudenal) | Gastric mucosa | Stomach | 2.3-6.5 | No | Dietary TAG | Release of UFA | *Sn*-3 | 1,2-DAG, NEFA |
| Pancreatic | Pancreatic exocrine cells | Small intestine | 6.5-8 | Yes | Dietary TAG | Release of C18 UFA | *Sn*-1 and 3 | 2-MAG, NEFA |
| Bile salt stimulated lipase (BSSL) | Mammary glands | Small intestine | 3-8? | Yes | Dietary TAG, DAG, MAG | Aids other lipases | *Sn*-1,2,3 | MAG, Glycerol, NEFA |
| Lipoprotein lipase (LPL) | Exrahepatic tissue | Endothelial cell surface | | No | Chylomicrons, VLDL | Lipoprotein degradation to allow FA uptake | *Sn*-1,2,3? | Glycerol, NEFA |
| Hormone sensitive lipase (HSL) | Adipocytes | Adipocytes | | No | Stored TAG | Degradation of TAG stored in adipocytes | *Sn*-1,2,3? | Glycerol, NEFA |

Table 10. Classification and characteristics of lipases. TAG – triacylglycerol; UFA – unsaturated fatty acids; FA – fatty acids; 1,2-DAG – 1,2-diacylglycerol; NEFA – non-esterified fatty acids; *Sn* – stereospecific numbering.

3.1.1(b) Intestinal Digestion

The mucosal cells of the duodenum and jejunum secrete the hormone cholecystokinin in response to luminal fats. Cholecystokinin slows gastric motility, thus decreasing the rate at which chyme enters the intestine. Secretin is also produced by duodenal mucosal cells, stimulating the secretion of bicarbonate from the pancreas, which neutralizes the pH of chyme. Thus optimal "time" and pH conditions are created in the duodenum to facilitate lipolysis.

However, the immiscibility of the hydrophobic lipids maintains their separation from the water phase of intestinal contents and therefore from the digestive enzymes. For efficient digestion, the fats must be emulsified, that is, dispersed and stabilized in aqueous solution. The release of bile from the gallbladder, in response to cholecystokinin stimulation, helps to achieve this.

Bile acids are synthesized in the liver (Table 11). Chenodeoxycholic acid and cholic acid are derived from cholesterol and conjugated to the amino acids taurine and glycine, producing the primary bile acids glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycocholic acid and taurocholic acid. Secondary bile acids, lithocholic and deoxycholic acids, are produced when intestinal bacteria metabolize primary bile acids, and are recirculated in bile (Bender 1997). The sterol portion of bile is lipophilic, while the amino portion is hydrophilic. Thus bile acids can bind to the lipid molecules while remaining stable in the water, drawing the lipid molecules into solution. Any long chain unsaturated fatty acids released on triacylglycerol digestion by gastric lipase also help to stabilize the enzyme-bile-fat complex (Embleton & Pouton 1997, Hernell & Bläckberg 1992).

| Bile Acid | Amino Acid | Primary bile acid (salt) | Secondary bile acid (salt) |
|---|---|---|---|
| Chenodeoxycholic acid | Glycine | Glycochenodeoxycholic (-ate) | }Lithocholic (-ate) |
| | Taurine | Taurochenodeoxycholic (-ate) | |
| Cholic acid | Glycine | Glycocholic (-ate) | }Deoxycholic (-ate) |
| | Taurine | Taurocholic (-ate) | |

Table 11. Classification of bile acids and salts.

The emulsified lipids are susceptible to the action of a variety of enzymes, all of which are secreted by pancreatic exocrine cells in response to cholecystokinin. Pancreatic lipase (Table 10), which is dependent on and stabilized by its cofactor colipase, acts at the *Sn*-1 and *Sn*-3 positions of triacylglycerol to produce 2-monoacylglycerol and non-esterified fatty acids (Embleton & Pouton 1997, Hamosh *et al* 1992, Manson & Weaver 1997, Tso & Weidman 1987). Non-enzymatic isomerization of 2-monoacylglycerol to 1-monoacylglycerol (Embleton & Pouton 1997) provides a suitable substrate for pancreatic lipase, allowing more complete hydrolysis of dietary triacylglycerols (Figure 8). *In vitro* studies have suggested that selective hydrolysis by pancreatic lipase depends not only on the *Sn*- position, but on the fatty acid itself. Pancreatic lipase hydrolyses the essential C18 fatty acids more efficiently than their longer chain derivatives. To this end, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are resistant to pancreatic lipase hydrolysis, regardless of their *Sn*- position (Hernell & Bläckberg 1992).

Pancreatic cholesteryl ester hydrolase (cholesterol esterase) hydrolyses cholesterol esters to cholesterol and non-esterified fatty acids. The C2 fatty acid of phospholipids are removed by phospholipase $A_2$, producing a lysophospholipid whose C1 fatty acid is subsequently removed by lysophospholipase to produce a glycerylphosphorylbase which is either absorbed or excreted (Tso & Weidman 1987).

3.1.1(c) Jejunal Absorption

Lipids then enter the jejunum, where absorption occurs. Glycerol, short- (C4-6) and medium-chain (C8-10) fatty acids are readily absorbed across the brush border of the intestinal mucosa (BNF 1992, Gurr 1993). Fatty acids of chain length longer than C12, cholesterol, 2-monoacylglycerols and bile salts form mixed micelles, which aid absorption of lipid moieties. The bile salt components of the micelles pass into the ileum where they are absorbed. Via the enterohepatic circulation, portal blood transports them to the liver, where they are re-secreted into bile and once again enter the duodenum.

The initial phase of lipid absorption is a passive process, down a concentration gradient across the apical membrane of enterocytes (BNF 1992, Tso & Weidman 1987). This is

achieved in two steps: firstly, by binding of fatty acids (possibly in the preferred order of long chain polyunsaturated to long/medium/short-chain saturated fatty acids) to fatty acid binding protein (FABP); secondly, by re-esterification of free fatty acids into phospholipids and triacylglycerols within the cell.

Rates of lipid absorption vary, according to their fatty acid composition. Animal studies suggest that the total amount of dietary fat and its degree of saturation influence the rate of absorption (BNF 1992). In humans, the rate and efficiency of fatty acid absorption can be considered dependent on both the site and nature of the fatty acid. The *Sn-* position of each fatty acid on the triacylglycerol molecule influences preferential lipase digestion, and hence absorption. The type of fatty acid can also affect absorption (BNF 1992, Ling & Weaver 1997). Unsaturated fatty acids (UFA) are absorbed in preference to saturated fatty acids (SFA) across the intestinal mucosa, probably due to increased gastric lipolysis and/or emulsification by bile salts (Ling & Weaver 1997), and/or their binding by FABP and re-esterification (BNF 1992).

3.1.1(d) Plasma Circulation

Once absorbed, the fates of the different classes of lipid in the intestinal mucosa differ. Glycerol, short- and medium-chain fatty acids are released directly into the portal bloodstream and transported to the liver, where they are oxidized.

Long-chain fatty acids are converted into fatty acyl CoA derivatives by fatty acyl CoA synthetase. Specific acyltransferases then re-esterify these derivatives to monoacylglycerols which can then be reassembled into triacylglycerols. Cholesterol and lysophospholipids are reacylated to similarly reform their original cholesteryl esters and phospholipids respectively (BNF 1992, Tso & Weidman 1987).

The reconstituted triacylglycerols and cholesteryl esters are water-insoluble and thus need to be in a form in which they can enter the circulation. To achieve this, proteins, phospholipids and cholesterol accumulate around the triacylglycerols and cholesteryl esters, forming lipoprotein vesicles. There are various classes of lipoprotein (Table 12), chylomicrons being those responsible for the transport of dietary lipids from the intestine to peripheral tissues, including muscle, adipose, renal and cardiac tissues

(Nestel 1987, Gaw *et al* 1995). The chylomicrons, on release from the cells of the intestinal mucosa, enter the lymph system via intestinal lacteals. Lymph transports the chylomicrons to the thoracic duct, where they join the left subclavian vein and enter the bloodstream. The selective absorption and incorporation into chylomicrons of fatty acids may account for the similarities and variations observed between the fatty acid composition of the diet compared to adipose tissue (Summers *et al* 2000).

| Lipoprotein | Composition | Density (g/ml) | Apolipoproteins | Origin | Function |
|---|---|---|---|---|---|
| Chylomicron | 2% Protein<br>83% TAG<br>8% Cholesterol<br>7% Phospholipid | < 0.95 | A-I, $B_{48}$, C-II, E | Intestine | Transport of dietary TAG |
| Very low density lipoprotein (VLDL) | 7% Protein<br>50% TAG<br>22% Cholesterol<br>20% Phospholipid | 0.95-1.006 | $B_{100}$, C-II, E | Liver, intestine | Transport of endogenously produced TAG |
| Intermediate density lipoprotein (IDL) | | 1.006-1.019 | B, C, E | VLDL catabolism, liver | |
| Low density lipoprotein (LDL) | 20% Protein<br>10% TAG<br>48% Cholesterol<br>22% Phospholipid | 1.019-1.063 | $B_{100}$ | VLDL catabolism, liver | Transport of cholesterol to peripheral tissues |
| High density lipoprotein (HDL) | 50% Protein<br>8% TAG<br>20% Cholesterol<br>22% Phospholipid | 1.063-1.210 | A-I, A-II | Intestine, liver | Reverse transport of cholesterol from peripheral tissues to liver |

Table 12. Classification of lipoproteins. TAG – triacylglycerol.

3.1.1(e) Peripheral Tissue Accretion

On reaching the peripheral tissues, the triacylglycerol components of the chylomicrons cannot cross the cell membrane: they must first be degraded to release non-esterified (free) fatty acids and glycerol. This is mediated by the action of lipoprotein lipase (LPL) on the surface of tissue cells, which is activated in the post-prandial state by insulin. The expression and activity of LPL is tissue-specific and related to nutritional state, accounting at least in part for the differences in metabolism between tissues (Fielding & Frayn 1998, Karpe *et al* 1998).

The transport of fatty acids across the cell membrane is most probably mediated by more than one mechanism (McArthur *et al* 1999), the relative contributions of which may vary according to cell type (Abumrad, Harmon & Ibrahimi 1998). The passive diffusion ("flip-flop") of fatty acids across the membrane is dependent on fatty acid concentration and ionization (Hamilton 1999). Facilitated transport may be mediated by fatty acid binding proteins (such as plasma membrane fatty acid binding protein/$FABP_{pm}$) alone and/or in combination with fatty acid transport protein(s) (FATP) (Abumrad *et al* 1998). Several putative FABP and FATP have been identified, including leptin (Campbell *et al* 1998b). In addition, once inside the cell, fatty acids may be subject to intracellular transport by diffusion and/or cytoplasmic proteins (McArthur *et al* 1999). The combination of such specific transportation into and within cells may enable cells to direct fatty acid movement and so facilitate the positioning of fatty acids in specific functional sites within membranes, or within cell organelles for storage/oxidation.

Such facilitated transport may be of particular relevance to LCPUFA. The non-esterified fatty acids and glycerol released by LPL action on dietary triacylglycerol can be re-esterified within the cell to re-form triacylglycerols, a reaction dependent on the activation of fatty acids by fatty acyl CoA synthetase. Indeed, the conversion of fatty acids to fatty acyl CoA by fatty acyl CoA synthetase is required not only for re-assembly of dietary triacylglycerols, but also for *de novo* triacylglycerol synthesis and fatty acid oxidation. Long-chain fatty acyl CoA synthetase is a cell membrane protein (Gargiulo, Stuhlsatz-Krouper & Schaffer 1999) and although its role in the regulation of fatty acid metabolism has not been fully elucidated, it may function to ensure a concentration gradient across the cell membrane which facilitates efficient cellular uptake of non-esterified fatty acids, particularly LCPUFA. In addition, transport of fatty acids across the cell membrane by FATP, followed by rapid esterification by long-chain fatty acyl CoA synthetase could enhance intracellular LCPUFA transport by specific cytosolic FABP or acyl-CoA binding proteins (Gargiulo *et al* 1999).

Once absorbed, fatty acids are available to undertake various roles, including storage and/or oxidation, elongation and/or desaturation, eicosanoid formation and membrane synthesis. The fate of each fatty acid is largely determined by its nature, specifically

chain length and degree of saturation (Jones *et al* 1999). Short- and medium-chain saturates are either oxidized or stored for energy. Dietary linoleic (18:2n-6) and linolenic (18:3n-3) acids are incorporated into either triacylglycerols for adipose tissue storage, or into phospholipids for cell membrane structure. Alternatively, when required, these essential fatty acids (EFA) can be elongated and desaturated to their LCPUFA derivatives. LCPUFA are spared from oxidation and incorporated into phospholipids, with preformed dietary LCPUFA incorporated in preference to those derived from dietary EFA (FAO & WHO 1980).

### 3.1.2 Infant Fat Digestion and Absorption

The newborn infant must adapt from dependence on glucose as its principal source of energy *in utero,* to the use of fat as its major energy substrate after birth. Saturated fatty acids are the main source of energy for the neonate, while long-chain polyunsaturated fatty acids (LCPUFA) are used for membrane synthesis (Ling & Weaver 1997). However, complete fat digestion by the neonate is limited by low pancreatic lipase activity and low bile salt production, relative to adults (BNF 1992, Hamosh *et al* 1992, Hernell & Bläckberg 1992). Neonates can, in part, compensate for this through initial gastric hydrolysis of lipids, which is proportionally more important to neonates than to adults (Tso & Weidman 1987).

#### 3.1.2(a) Gastric Digestion

Lingual lipase is activated by sucking and in response to ingested fat. Both lingual and gastric lipase are active in the absence of bile salts and within a pH range of 2.3-6.5 (Hamosh *et al* 1992, Manson & Weaver 1997) (Table 10). Thus, they can function in the near neutral environment of the neonate's stomach, as well as in the more acidic environment of the adult's stomach. Indeed, the homology of both physical and functional characteristics between lingual and gastric lipase has led to debate regarding their distinction and relative contributions, and to their collective classification as "preduodenal lipase" (Manson & Weaver 1997).

The hydrophobic nature of preduodenal lipase and its inability to hydrolyze phospholipids and cholesteryl esters allows the enzyme to become embedded in the phospholipid surface of the milk fat globule (Hamosh *et al* 1992). Without disrupting

the integrity of the membrane, preduodenal lipase can catalyze the hydrolysis of fatty acids from the *Sn*-3 position of the triacylglycerol to produce 1,2-diacylglycerol. Only very limited hydrolysis by preduodenal lipase occurs at positions *Sn*-1 and *Sn*-2 (Hamosh *et al* 1992). Studies show that gastric lipolysis by infants preferentially releases medium chain fatty acids (MCFA), monounsaturated fatty acids (MUFA) and long chain polyunsaturated fatty (LCPUFA) acids from the triacylglycerol. Gastric lipolysis of breast milk fat is more efficient that that of formula milk fat; this may be attributable to the relative ease with which lipase can access the triacylglycerol within the breast milk globule compared to the formula fat globule (Armand *et al* 1996).

3.1.2(b) Intestinal Digestion

On passage of chyme into the duodenum, preduodenal lipase is inactivated by pancreatic trypsin. Although pancreatic lipase is secreted from 30 weeks gestation, its activity in neonates remains low (Hamosh *et al* 1992, Manson & Weaver 1997). Its dependency on colipase and bile salts further limits the efficiency of pancreatic lipase in neonatal fat digestion. The binding of pancreatic lipase to intact fat globules is, however, stabilized by any LCPUFA released on gastric hydrolysis (Hernell & Bläckberg 1992). Thus, it can still hydrolyze triacylglycerols at the *Sn*-1 and *Sn*-3 positions, producing NEFA and 2-monoacylglycerol (Hamosh *et al* 1992).

Human milk confers a unique advantage to breast-fed infants, compared to formula-fed infants, with regard to fat digestion. This is because breast milk contains a lipase which is activated in the duodenum by primary bile salts (Hamosh *et al* 1992, Hernell & Bläckberg 1992, Manson & Weaver 1997), hence it is called bile salt stimulated lipase (BSSL) (Table 10). Secreted throughout lactation, the concentration of BSSL varies between lactating mothers' milks (Manson & Weaver 1997). The higher concentration of BSSL in the colostrum of mothers of preterm infants suggests a compensatory role in augmenting the immature preduodenal and pancreatic lipases of preterm infants. Moreover, the action of BSSL is non-specific and efficient hydrolysis occurs at the *Sn*-1, -2 and -3 positions of the triacylglycerol (Figure 8). The action of BSSL is therefore not dependent on the nature and/or position of the fatty acid. *In vitro* and model experiments suggest that BSSL can increase the efficiency of neonatal fat absorption even in the absence of bile salts. Thus BSSL can aid pancreatic lipase

by digesting any remaining intact triacylglycerol, as well as the mono- and diacylglycerols resulting from hydrolysis by preduodenal and pancreatic lipase action, to produce NEFA and glycerol. BSSL has been shown to play a role in the digestion of other classes of milk lipid (Nyberg *et al* 1998).

Despite their low concentrations, bile salts contribute significantly to neonatal fat digestion. By stabilizing the hydrolysis products of preduodenal lipase, bile salts create an emulsion in which the relative surface area of the lipid droplets is increased to aid hydrolysis by pancreatic lipase and BSSL. An insufficient bile salt pool may be a limiting factor to complete fat digestion in the preterm infant.

3.1.2(c) Jejunal Absorption and Plasma Circulation

As in the adult, the products of fat digestion (non-esterified fatty acids, glycerol, monoacylglycerol) diffuse into the brush border of the neonatal intestinal mucosa. Absorption, binding to FABP, reconstitution of triacylglycerol and transport as chylomicrons occurs in infants as in adults. The selective incorporation into and release from chylomicrons of LCPUFA may contribute to their possible transport from the liver to neural tissues (Hernell 1990).

Human milk contains both medium-chain saturated (MCSFA) and long-chain polyunsaturated fatty acids (LCPUFA). The absorption of MCSFA without the aid of bile salts provides the neonate with a ready source of energy (BNF 1992). Of the absorbed products, short- and medium-chain fatty acids are oxidized either immediately, or are stored in adipose tissue where they remain available for future oxidation. Endogenous desaturation and/or elongation of LCPUFA may occur before they are used in the synthesis of membranes or eicosanoids.

3.1.2(d) Colonic Metabolism

An appreciable amount of ingested fat remains unabsorbed and passes into the colon (Ling & Weaver 1997), particularly in the preterm infant. Colonic bacteria can metabolize fatty acids via lipolysis, hydrolysis and desaturation. Conversion of fatty acids to toxic hydroxy-fatty acids may stimulate colonocyte proliferation, and may contribute to the genesis of colon cancer (Ling & Weaver 1997). This seems to be

increased when the intake of saturated fat is high, and decreased when LCPUFA intake is high.

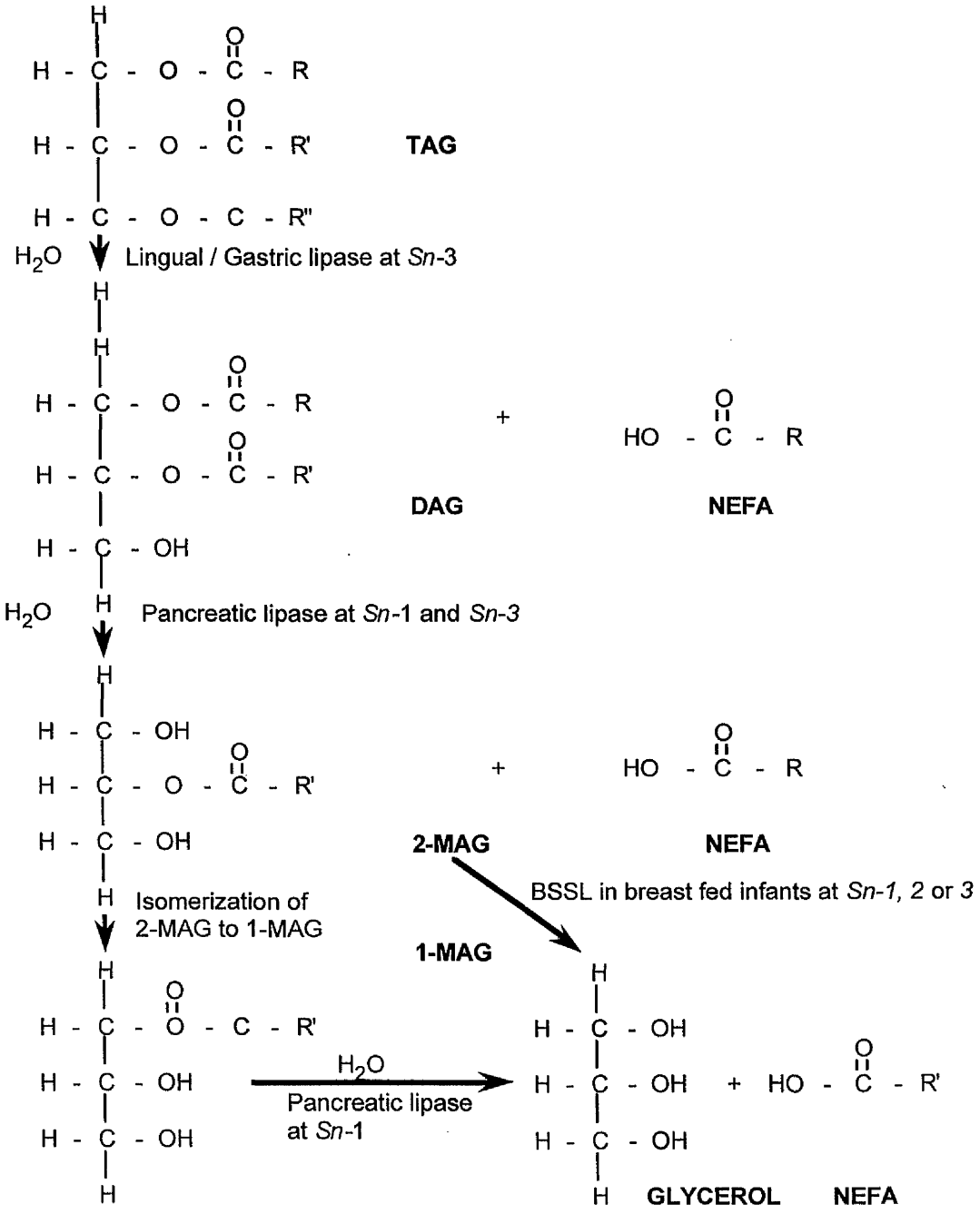



Figure 8. Hydrolysis of a triacylglycerol by gastrointestinal lipases. TAG - triacylglycerol, DAG - diacylglycerol, 2MAG - 2-monoacylglycerol, 1MAG - 1-monoacylglycerol, NEFA - non-esterified fatty acid, BSSL - bile salt stimulated lipase.

### 3.1.3 Fatty Acid Synthesis

A healthy diet normally meets all fatty acid requirements. If, however, dietary intake is inadequate, it may not provide the type or amount of all fatty acids required. The body must therefore be able to synthesize complete fatty acids, or elongate and/or desaturate fatty acids that have been ingested. In times of fasting, the body can synthesize fatty acids *de novo* from glucose and amino acids (see Hellerstein 1999 for review *of de novo* lipogenesis).

All tissues possess the enzymatic activities required for fatty acid synthesis, although not equally so (Bézard *et al* 1994). The main sites of fatty acid synthesis are the liver and lactating mammary glands (BNF 1992, Hamosh *et al* 1992, Salway 1994), with less occurring in adipose and renal tissue. Much of lactating mammary gland function is devoted to *de novo* fatty acid synthesis. To ensure an adequate supply of substrates, prolactin "upregulates" LPL, thus increasing the uptake of non-esterified fatty acids for elongation/desaturation. A maternal diet low in fat leads to a higher concentration of MCSFA in human milk as compared to a high fat maternal diet. Octanoic (8:0) and decanoic (10:0) acids are produced exclusively by the mammary glands and are therefore markers of endogenous maternal fat synthesis (Gurr 1993).

The synthesis of fatty acids involves the successive addition of 2C units to the carboxyl end of the C-chain. The 2C units are obtained from acetyl coenzyme A (acetyl CoA). While the main reactions of fatty acid synthesis occur in the endoplasmic reticulum of the cytosol (Bender 1997, Stryer 1988), its substrate acetyl CoA is first produced in the mitochondria by the oxidation of pyruvate (from glycolysis) or by the degradation of parent fatty acids, ketone bodies or amino acids. However, acetyl CoA cannot cross the mitochondrial membrane. To overcome this, citrate synthase catalyses the condensation of acetyl CoA with oxaloacetate (OAA) to form citrate and CoA. Transportation of citrate across the mitochondrial membrane and into the cytosol allows it to be cleaved by citrate lyase into cytosolic acetyl CoA and OAA.

Acetyl CoA carboxylase carboxylates acetyl CoA to form malonyl CoA. This biotin-dependent enzyme (Salway 1994) is "activated" when the diet is rich in carbohydrate and low in fat, and "deactivated" when the diet is high in fat or when fasting, thereby

regulating fatty acid synthesis. The next steps rely on the multienzyme fatty acid synthase for catalysis; one of the seven monomers of this complex is acyl carrier protein (ACP). ACP, in a two-stage process, reacts with individual acetyl CoA and malonyl CoA units to form acetyl-ACP and malonyl-ACP, respectively. The reaction of acetyl-ACP and malonyl-ACP decarboxylates the malonyl group, producing acetoacetyl-ACP. Acetoacetyl-ACP is then successively reduced, dehydrated and reduced again to form the 4C unit of butyrl-ACP.

Butyrl-ACP then cycles through the sequential addition of six more malonyl CoA units, by the same processes as above, until a saturated 16C molecule is produced. This product is palmitate, which mitochondrial elongase enzymes can elongate by adding more 2C units. Mitochondrial desaturase enzymes can insert double bonds into saturated fatty acids to produce unsaturated derivatives (Figure 9). For example, palmitate can be desaturated to palmitoleate (16:1n-7).

Desaturase enzymes (Table 13) act at specific positions on the acyl chain of the fatty acid, and are named according to the position at which they introduce double bonds (Sprecher 1992) relative to the carboxyl end (denoted Δ). As discussed previously (see Chapter 1, Section 1.1.2 Fatty Acids), fatty acids are metabolically related if the first double bond relative to the methyl end (n) occurs in the same position (n-3, n-6 *etc.*). Since desaturase enzymes cannot catalyze the insertion of double bonds other than at their specific site, the conversion of the fatty acids from one metabolic family to another is precluded (Figure 9).

| Enzyme | Position | Substrate | Activity in Humans |
|---|---|---|---|
| Δ4-desaturase | ΔC4-5 | C22 | + |
| Δ5-desaturase | ΔC5-6 | C20 | + |
| Δ6-desaturase | ΔC6-7 | C18 | + |
| Δ9-desaturase | ΔC9-10 | C16-18 | - |

Table 13. Desaturase enzymes.

Humans lack the desaturase enzymes which catalyze the insertion of double bonds beyond ΔC9 and C10 (or n-9 in an 18C chain), *i.e.* at ΔC12-13 and ΔC15-16 (Gurr 1993). Thus, humans cannot insert double bonds at n-3 or n-6 positions. Linoleic acid

(cis-9,12-octadecadienoic, double bonds at ΔC9-10/n-9 and ΔC12-13/n-6) and α-linolenic acid (cis-9,12,15-octadecatrienoic, double bonds at ΔC9-10/n-9, ΔC12-13/n-6 and ΔC15-16/n-3) are therefore essential constituents of the diet, *i.e.* essential fatty acids (EFA).

The conversion of EFA to their longer and more unsaturated derivatives occurs in adults consuming an *ad lib* diet (Salem *et al* 1999). In the fetus and neonate, levels of elongase and desaturase enzymes may be lower (Rodriguez *et al* 1998), although they are physiologically active during gestation (Rodriguez *et al* 1998) and within the first week of life (Salem Jr. *et al* 1996), even in preterm neonates (Carnielli *et al* 1996, Sauerwald *et al* 1997b). Whether preterm infants convert dietary EFA to a lesser extent than term infants is debatable (Innis *et al* 1999). Derivatives of linoleic and α-linolenic acid - arachidonic acid (AA), and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) respectively - are synthesized in only limited amounts, and may not adequately meet requirements (Carnielli *et al* 1996, Salem Jr. *et al* 1996). These LCPUFA may thus be conditionally essential to the fetus and neonate (BNF 1992).

The ability of the desaturase enzymes to act on various unsaturated fatty acids (UFA) leads to competition between the substrates for the enzymes. Δ6-desaturase has a preferred affinity for α-linolenic (n-3) > linoleic (n-6) > oleic (n-9) acids. Thus, when sufficient α-linolenic and linoleic acids are present in the diet, the conversion of oleic acid to its derivatives is negligible. An indicator of essential fatty acid deficiency is therefore the ratio of 20:3n-9 (Mead acid) to 20:4n-6 (AA) (triene/tetraene ratio), because the n-9 pathway will predominate only when α-linolenic and linoleic acids are deficient in the diet. The metabolism of fatty acids, and hence the fatty acid composition of membrane phospholipids, is therefore subject to dietary control. The levels of dietary linoleic and α-linolenic acids appear to determine their conversion to their LCPUFA derivatives, in both adults and neonates (Innis *et al* 1999, Li *et al* 1999, Sauerwald *et al* 1996). In both malnutrition and obesity, elongase and desaturase activities are modified, compromising EFA conversion and metabolism (Decsi, Molnár & Koletzko 1998, Holman *et al* 1981). Perturbations of the crucial Δ6-desaturase have

been implicated in eczema and diabetes (Horrobin 1993). Other factors regulating the activity of elongase and desaturase enzymes have been described (Bézard *et al* 1994), and may include age and gender (Babin *et al* 1999, Bolton-Smith, Woodward & Tavendale 1997), but have not been fully elucidated.

There is some evidence that fatty acid synthesis does not occur exclusively in the endoplasmic reticulum, (Sprecher & Chen 1999). The action of the putative Δ4-desaturase may instead be accounted for by a multi-step process which elongates 22:4n-6 and 22:5n-3 to their 24C derivatives, which are then further desaturated (Sauerwald *et al* 1997a). These fatty acids may then enter the peroxisomes and undergo retroconversion, a process of partial degradation similar to β-oxidation (removal of a 2C unit) (Sauerwald *et al* 1997b). This would generate 22:5n-6 and 22:6n-3, which are then able to return to the endoplasmic reticulum for metabolism (Carlson 1997, Innis *et al* 1999). The mechanisms controlling the preferential movement of 24C fatty acids to the peroxisomes, and the subsequent selectivity with which 22:5n-6 and 22:6n-3 are returned to the endoplasmic reticulum for incorporation into cell membranes have not been elucidated (Sprecher, Chen & Yin 1999). There is evidence that this alternative pathway for LCPUFA synthesis is active in infants, including those born prematurely (Sauerwald *et al* 1997b), although the contribution of the "classical" and "alternative" pathways has yet to be quantified (Sauerwald *et al* 1997a). The retroconversion of C24 to C22 fatty acids is implicated in fatty acid synthesis, while that of C22 to C20 fatty acids may be a prerequisite for β-oxidation (see Section 3.1.5 Fatty Acid Oxidation).

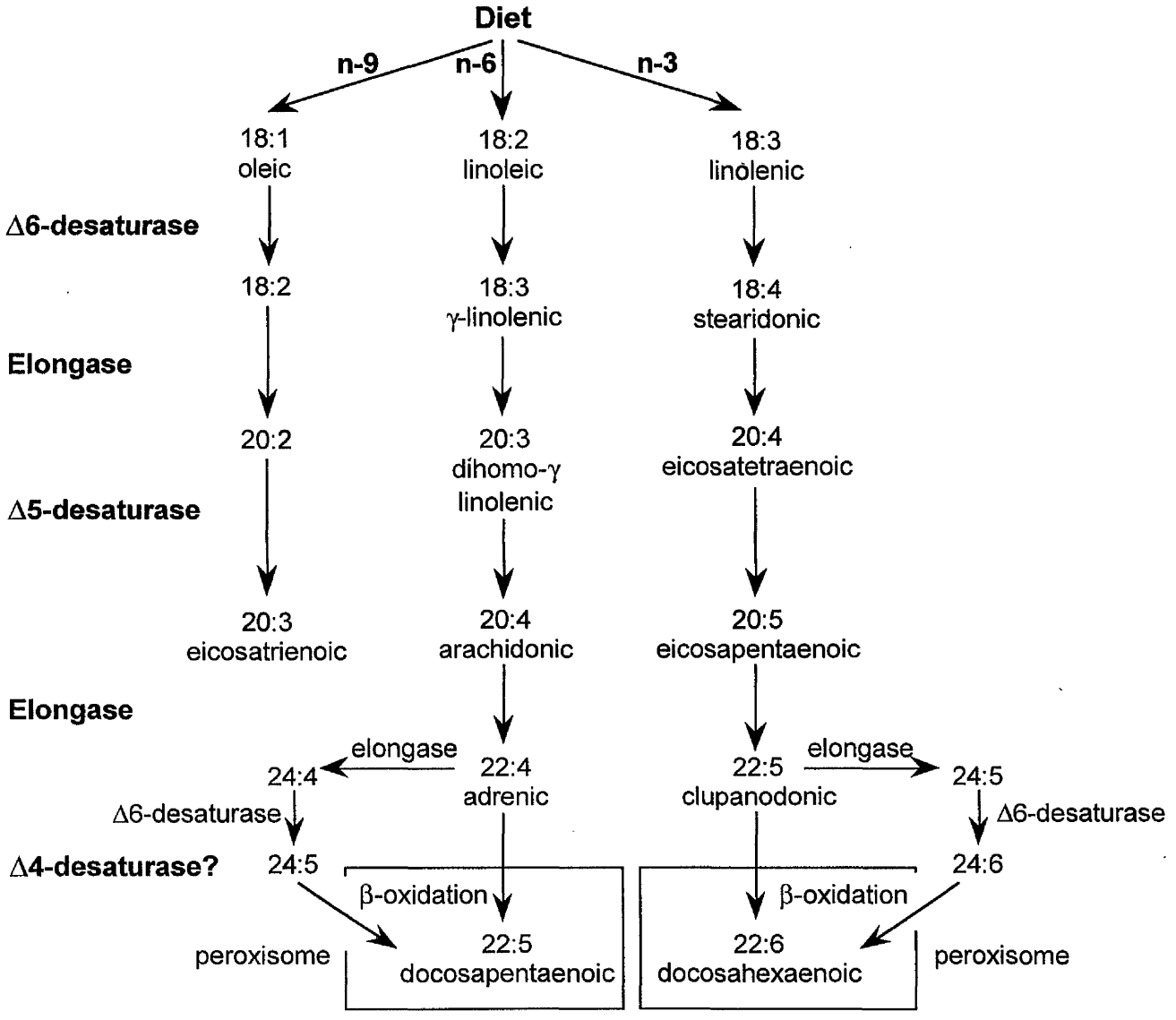



Figure 9. Elongation and desaturation of fatty acids

### 3.1.4 Triacylglycerol Synthesis

In addition to being the main form in which lipid is found in the diet, triacylglycerols are also the major form in which fatty acids are stored and transported. Short and medium chain fatty acids, obtained either from the diet or from *de novo* synthesis, are reconstituted into triacylglycerols before storage within adipocytes. Similarly, non-esterified fatty acids must be in the form of triacylglycerols before secretion into breast milk.

The formation of fatty acyl CoA from fatty acids and CoA, catalyzed by fatty acyl CoA synthetase, converts the fatty acids into an "activated" form. The fatty acid moieties of two separate fatty acyl CoA complexes esterify to glycerol phosphate at the *Sn*-1 and *Sn*-2 positions, forming diacylglycerol phosphate. The glycerol used is obtained from glucose in the liver and adipose tissue when the concentrations of insulin and glucose are high (Salway 1994). Dephosphorylation of diacylglycerol phosphate produces diacylglycerol, to which a third fatty acid from another fatty acyl CoA is added. This completes the triacylglycerol molecule (Figure 10).

The fatty acids on each of the three *Sn*- positions of the triacylglycerol molecule may differ, although their distribution is not entirely random. Their *Sn*- position, to an extent, reflects the tissue in which the triacylglycerol molecule has been synthesized (Table 14). The triacylglycerols made for storage purposes in adipose tissue show a preference at *Sn*-1 for a saturated fatty acid (SFA) and at *Sn*-2 for an unsaturated fatty acid (UFA), with *Sn*-3 having no preference regarding saturation (BNF 1992). In comparison, the triacylglycerols contained in breast milk have preferential binding at *Sn*-1 of UFA, at *Sn*-2 of SFA and random assignment at *Sn*-3.

| | Human milk fats | Human storage fats | Cows' milk fats |
|---|---|---|---|
| Sn-1 | UFA | SFA | Random |
| Sn-2 | SFA | UFA | Random |
| Sn-3 | Random | Random | Short-chain fatty acid |

Table 14. Assignment of fatty acids at *Sn*-positions of triacylglycerol molecules.

Triacylglycerols synthesized in the liver are exported as very low density lipoproteins (VLDL) (Table 12) and destined for tissues where oxidation occurs. Those synthesized

in adipose tissue are stored as a fuel reserve. Triacylglycerols stored in white adipose tissue provide the main energy reserve for the other tissues of the body and undergo slow turnover, rather than remain inert in storage (Gurr 1993). Triacylglycerols stored in brown adipose tissue, which is predominant in babies, contribute to thermogenesis, which is particularly important in neonates in whom shivering does not occur.

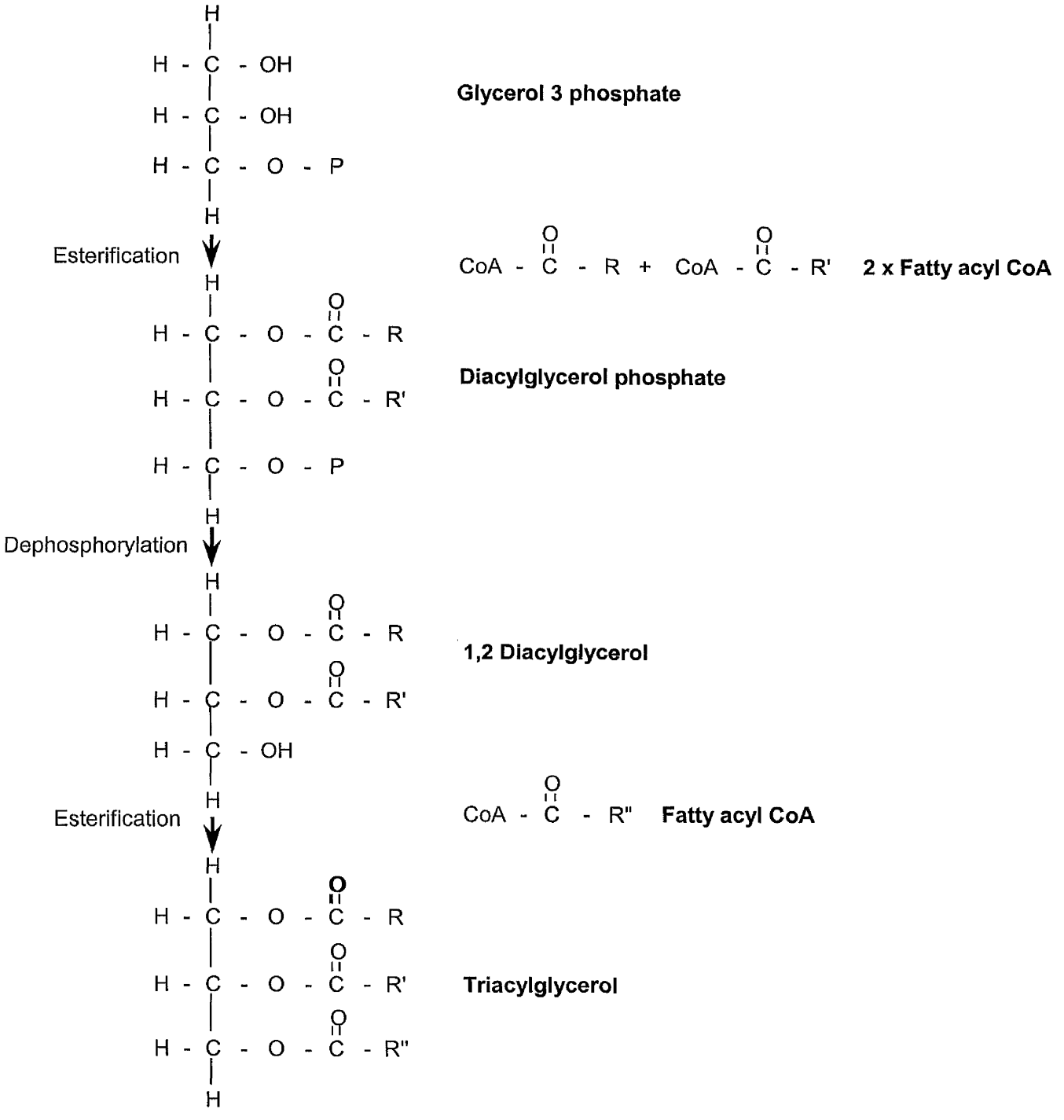



Figure 10. Synthesis of a triacylglycerol molecule.

### 3.1.5 Fatty Acid Oxidation

To utilize fatty acids for energy, cells must obtain them from stored triacylglycerols within adipose tissue (in the fasting state), or from circulating triacylglycerols in chylomicrons (in the fed state).

During periods of fasting or energy-restriction, circulating insulin levels are low and glucagon levels are high. Glucagon binds to the surface of adipose cells and stimulates the second messenger cyclic adenosine monophosphate (cAMP). This, in turn, activates a cAMP-dependent protein kinase which phosphorylates and activates hormone-sensitive lipase (HSL). HSL catalyses the hydrolysis of *Sn*-1 and *Sn*-3 fatty acids in the stored triacylglycerol, producing glycerol and free fatty acids. Transportation of glycerol to the liver allows its conversion to dihydroxy-acetone phosphate (DHAP), which enters the pathway of gluconeogenesis, producing glucose for oxidation in erythrocytes and cerebral tissue (Salway 1994). The NEFA are secreted from the adipocytes and transported, bound to albumin, to other tissues.

Conversely, in the post-prandial state, the antagonistic action of insulin prevents activation of HSL, instead activating lipoprotein lipase (LPL). Circulating chylomicrons are taken up by the tissue cells where LPL hydrolyses the triacylglycerols to free fatty acids and glycerol. The glycerol is transported to the liver and used as DHAP in glycolysis.

In either case, the resulting non-esterified fatty acids within tissue cells are oxidized to release adenosine triphosphate (ATP) in a process called β-oxidation. This is the oxidation of the β-carbon of each fatty acid chain, with the sequential removal of 2C units as acetyl CoA. In order to enter the mitochondrial matrix where β-oxidation occurs, the fatty acids are converted to fatty acyl CoA in the cytosol, by esterification with coenzyme A as catalyzed by fatty acyl CoA synthetase.

Fatty acyl CoA then crosses the outer mitochondrial membrane. When it reaches its inner face, carnitine acyltransferase I transfers the fatty acid to carnitine, forming acylcarnitine and releasing CoA (Borum 1987). Acylcarnitine is transported across the inner mitochondrial membrane by carnitine acylcarnitine translocase. The fatty acid is

transferred to another CoA, by carnitine acyltransferase II, to reform carnitine and fatty acyl CoA. Free carnitine cycles back to the inner mitochondrial membrane surface, where it can cross back over to the outer mitochondrial membrane. This "carnitine shuffle" (Figure 11) is a point of regulation in fatty acid oxidation (Rasmussen & Wolfe 1999), as it will not proceed when malonyl CoA is present (*i.e.* during fatty acid synthesis) or when the CoA is acylated (*i.e.* the process is saturated and sufficient fatty acids are present). The role of carnitine and its particular relevance to neonatal metabolism has been reviewed (Stanley 1998b).

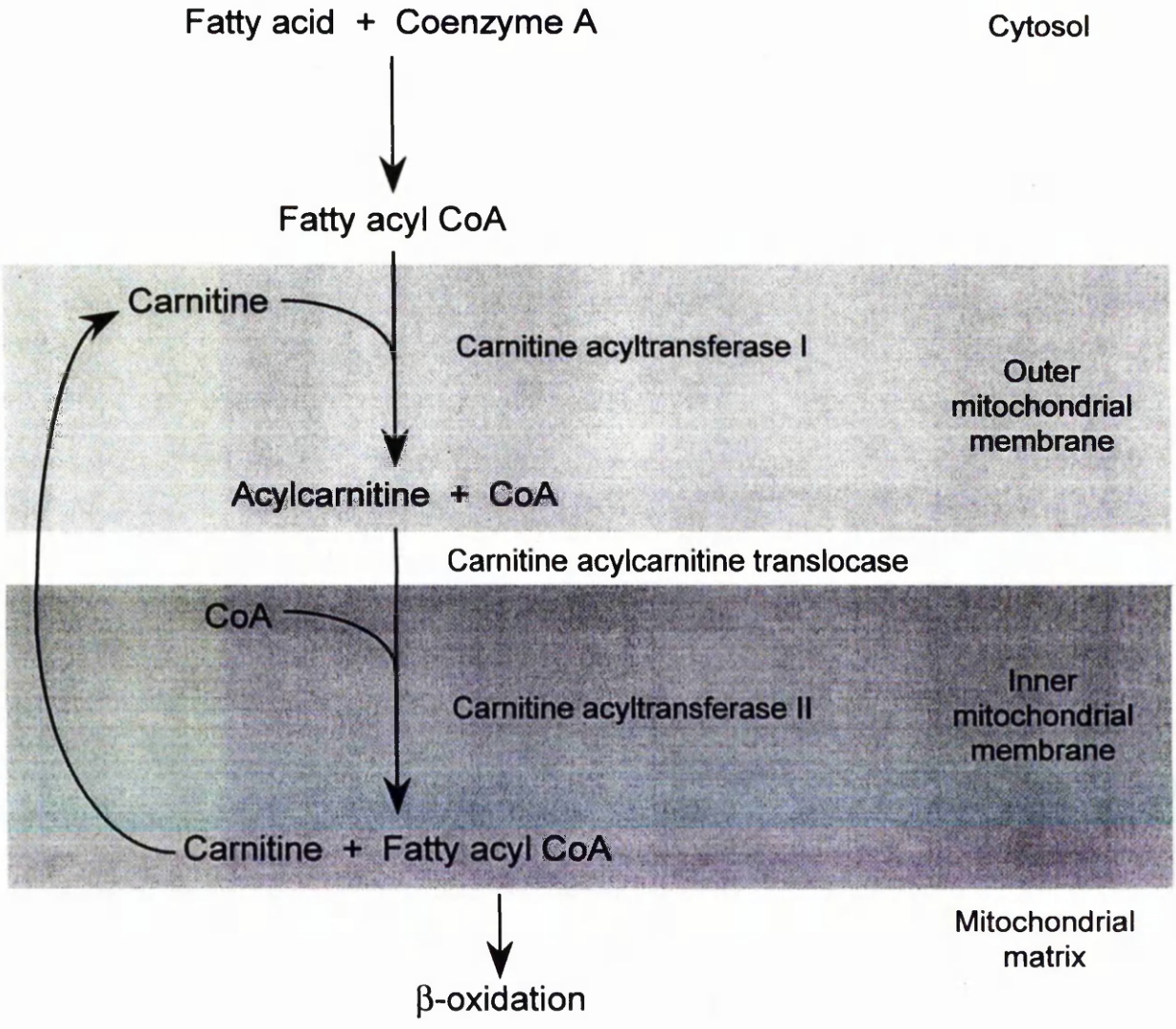



Figure 11. The "carnitine shuffle". Transport of fatty acids as fatty acyl CoA across the mitochondrial membranes before entering β-oxidation in the mitochondrial matrix.

The fatty acyl CoA enters the mitochondrial matrix, where the four step β-oxidation process occurs (Figure 12). The initial step is oxidation by acyl-CoA dehydrogenase, an enzyme specific for short, medium or long chain fatty acids. This removes two hydrogen atoms from fatty acyl CoA, with concomitant release of flavin adenine dinucleotide ($FADH_2$). This is followed by a hydration reaction, and then another oxidation, this time producing nicotinamide adenine dinucleotide (NADH). Thiolysis by CoA produces acetyl CoA and a fatty acyl CoA. The fatty acyl CoA recycles through these four steps, until the last 2C group becomes acetyl CoA (Tso & Weidman 1987). (Most fatty acids undergoing oxidation have an even number of C atoms. If, however, the original fatty acid had an odd number of C atoms, the last 3C group enters the tricarboxylic acid (TCA) cycle.)

On the rare occasion that long chain polyunsaturated fatty acids (LCPUFA) are used for oxidative energy production, they undergo a slightly different initiation into the oxidative process – retroconversion to shorter or more saturated fatty acids. Primarily, they enter carnitine-independent peroxisomal oxidation, which shortens the fatty acids, thus C22 fatty acids are converted to C20 species. The shorter derivatives form fatty acyl CoA complexes, which are transported out of the peroxisome and into the mitochondria via a carnitine-dependent pathway, and hence enter mitochondrial β-oxidation (BNF 1992, Borum 1987).

The acetyl CoA, $FADH_2$ and NAHD that are generated during β-oxidation enter the TCA cycle, each generating 12, 2 and 3 ATP respectively. Fewer $FADH_2$ and NADH molecules are produced on oxidation of UFA, thereby producing less energy (Champe & Harvey 1994), which is the reason that LCPUFA are a poor substrate for energy production.

To enter the TCA cycle, acetyl CoA must react with oxaloacetate (OAA). Under conditions of insufficient OAA, as in low carbohydrate states such as fasting and diabetes, or when excess acetyl CoA is produced, the liver can convert acetyl CoA into ketone bodies. This is the collective term for acetoacetate, 3-hydroxybutyrate and acetone. These then circulate to peripheral tissues and can be used by skeletal muscle,

renal cortex, cerebral and cardiac muscle tissues for oxidation via the TCA cycle to produce ATP.

The oxidation of EFA may serve more than one purpose. The production of ketones from EFA apparently stimulates the recycling of their hydrocarbon chains following β-oxidation into the cholesterol and fatty acids generated by *de novo* lipogenesis in brain tissue. EFA oxidation may thus actually induce the synthesis of essential brain lipids (Cunnane *et al* 1999).

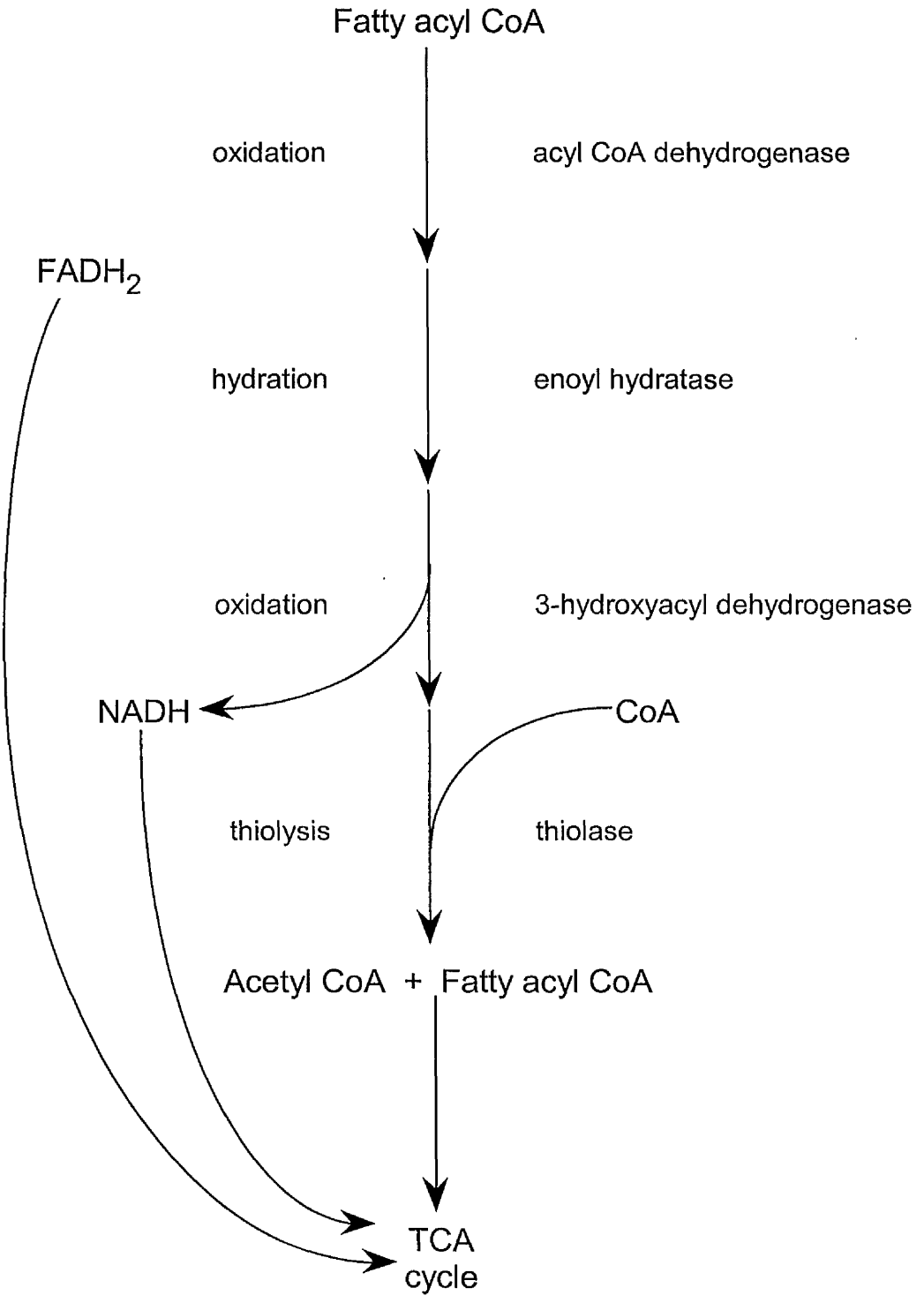



Figure 12. β-oxidation of fatty acids prior to energy production via the TCA cycle.

## 3.2 LIPID METABOLISM DURING PREGNANCY AND FETAL PUFA SUPPLY

### 3.2.1 Introduction

Pregnancy is a unique challenge to metabolism, requiring interaction and balance "between three distinct yet interdependent entities – mother, placenta, and fetus" (Coleman 1989). The physiology of pregnancy, its associated metabolism and complications have been extensively reviewed (Jansson & Powell 2000, King 2000, Knopp, Bonet & Zhu 1998). The adaptations which facilitate maternal/placental/fetal lipid metabolism are discussed, with emphasis on LCPUFA transport.

### 3.2.2 Overview of Physiology

Changes in maternal metabolism facilitate the transfer of vital nutrients, including lipids, across the placenta. The structure of placenta is unique with its outer layer of bipolar membranes of differing orientation and nature. The microvillous membranes are directed towards the maternal circulation, with the basal membranes facing the fetal circulation. Differences in the nature of the microvillous and basal membranes are implicated in the differential transport of nutrients between mother and fetus.

Accumulation of LCPUFA by the fetus elevates the fetal level of these fatty acids above that observed in the mother; indeed, the levels of LCPUFA increase progressively between maternal liver, placenta, fetal liver and fetal brain (Crawford, Hassam & Williams 1976). Such preferential accumulation or biomagnification (Crawford *et al* 1976) of LCPUFA by the fetus was considered to have arisen as a result of selective fatty acid uptake, oxidation and synthesis by the placenta in combination with fetal liver synthesis, but the contribution of maternal metabolism and LCPUFA mobilization with preferential placental transfer to the fetus is now recognised (Campbell, Gordon & Dutta-Roy 1996, Dutta-Roy *et al* 1996).

Since the placenta has no Δ5- or Δ6-desaturase activity, it cannot synthesize all classes of fatty acids; the fetus must therefore derive its LCPUFA from the maternal circulation or from its own elongation/desaturation reactions. Several factors have been shown to be involved in the control of lipid transfer from mother to fetus, although the relative importance of each has yet to be determined. Such contributing

factors include the transplacental gradient of NEFA and TAG (determined at least in part by maternal and/or fetal concentrations), fetal albumin concentration, placental metabolism of lipids, and fetal utilization of lipids (Kimura 1998). Moreover, the contribution of placental lipid transfer to fetal fatty acid status is undetermined, with conflicting evidence that it accounts for 20-100% of fetal requirements, in a species-specific manner (Kimura 1998).

### 3.2.3 Maternal Adaptations and Metabolism

Maternal weight gain during pregnancy varies between countries and between the developing and developed worlds (BNF 1994). The average overall gain during pregnancy in British women is 12.5kg, of which 3.5kg fat is estimated to be stored (Hytten & Leitch 1971). These fat depots accumulate in early to mid pregnancy due to enhanced maternal lipogenesis in adipose tissue. Such anabolism declines in the last trimester, when the mother enters a more catabolic state as a result of enhanced enzymatic lipolysis (Herrera & Munilla 1997). The NEFA and glycerol subsequently released are transported to the liver for TAG synthesis, accounting for the hypertriglyceridemia (particularly in VLDL form) observed during the last trimester.

### 3.2.4 Placental Transport and Metabolism

Of the lipids in the maternal circulation, the placenta is impervious to phospholipids and triacylglycerol and is capable of transferring NEFA only. In order to liberate NEFA from maternal TAG, the placenta possesses lipoprotein lipase (LPL) activity. Found exclusively on the microvillous membrane (Bonet *et al* 1992), placenta LPL hydrolyses maternal TAG-rich lipoproteins, including VLDL. The selectivity of placenta LPL for TAG packaged in post-hepatic lipoproteins may be a protective mechanism in defence of the fetus (Dutta-Roy *et al* 1996).

The available NEFA then require transport across the placenta. As mentioned previously, fatty acid binding proteins (FABP) are involved in the facilitated transport of NEFA across cell membranes and plasma membrane FABP ($FABP_{pm}$) has been identified in many tissues. The placenta contains a unique placenta $FABP_{pm}$ (p-$FABP_{pm}$), distinct from $FABP_{pm}$ (Campbell *et al* 1997), which is found exclusively on the microvillous membrane (Campbell & Dutta-Roy 1995). This protein has been

shown to preferentially bind LCPUFA (Campbell *et al* 1997, Campbell, Gordon & Dutta-Roy 1998), and therefore may account for the specific binding sites on and preferential uptake by the placenta membrane for LCPUFA (Campbell *et al* 1996) in a saturable process (Crabtree *et al* 1998). Such selectivity is not observed in liver cells (Dutta-Roy 2000).

Following uptake, fatty acids are subject to metabolism within the placenta cells, and may be utilized for placental lipid synthesis or lipolysis requirements, or transported to the fetus. The mechanisms controlling these processes have not yet been elucidated. Similarly, it is not known whether fatty acids are transferred to the fetus directly or whether intermediate metabolism is required (Coleman 1989). The form and transfer mechanisms by which fatty acids are transported from the placenta to the fetus also remain unknown (Dutta-Roy 1997).

However, fatty acid translocase (FAT) and fatty acid transport protein (FATP) have been found in both placental membranes (Campbell *et al* 1998a), as have several isomers of cytoplasmic FABP. These intracellular proteins may help "direct" NEFA; DHA and AA have been found to be differentially incorporated into the lipids classes, with DHA preferentially packaged within TAG as opposed to PL (Crabtree *et al* 1998). Moreover, another TAG hydrolase, distinct from LPL, has been found to be active in placental cells, and has been implicated in releasing NEFA from TAG stored in the placenta (Waterman, Emmison & Dutta-Roy 1998).

### 3.2.5 Fetal Lipid Accretion

The contribution of placental transfer and fetal synthesis to fetal LCPUFA accumulation is not known (Clandinin 1999). It has been estimated that the minimum levels of fatty acids required during the last trimester for the *de novo* synthesis of tissues are 3.6g/week (522mg/d) for n-6 and 469mg/week (67mg/d) for n-3 fatty acids (Clandinin *et al* 1981). For the accumulation of adipose tissue, approximately 2.8g of n-6 and 390mg of n-3 fatty acids must accrue each week *in utero* (Clandinin 1999).

Intra-uterine accretion of EFA and PUFA occurs in the liver, where they may be metabolized to LCPUFA. The rate of intra-uterine fatty acid accretion by the liver is

thought to be 13.5mg/week and 3.8mg/week for the n-6 and n-3 families respectively, most of which is accounted for by LCPUFA (Clandinin, Chappell & Van Aerde 1989, Clandinin *et al* 1981). The relative contributions of placental transport and/or liver synthesis to hepatic fatty acid accumulation have not been quantified (Clandinin *et al* 1989). From animal studies, it is evident that despite transfer of both αLA and DHA between mother and fetus, fetal tissues incorporate more preformed DHA (*i.e.* obtained from mother) than DHA derived *de novo* from αLA (Greiner *et al* 1997).

Indeed, the capacity of the fetal liver to synthesize LCPUFA is not known. Both Δ5- and Δ6-desaturases are expressed in the fetal liver during the second and third trimesters; the activity of each fluctuates during the second trimester and then remains level during the third trimester (Rodriguez *et al* 1998).

The accumulated fatty acids are mobilized from the liver and accrue in the structural lipids of other fetal tissues, particularly those of the nervous system, during the last trimester (Clandinin *et al* 1989). More of the LCPUFA derivatives than parent EFA are found in the phospholipids of these tissues (Clandinin *et al* 1989, Svennerholm 1968), in humans and other animals (Crawford *et al* 1992). The ubiquitous presence of LCPUFA, particularly AA and DHA, in cell membrane phospholipids suggests they are vital structural components capable of influencing membrane fluidity and excitability (BNF 1992, Hernell 1990). DHA is concentrated in the brain and retina, accounting for 20 to 60% of total phospholipid fatty acids in these tissues, a level observed in the brain and retina of many mammalian species (Crawford *et al* 1999, Hernell 1990).

The form in which DHA is transported to the brain and retina, and the cellular uptake mechanisms used by these tissues to obtain DHA have not been elucidated (Bazan 1992). In addition, the cellular interactions required within the tissues to ensure DHA supply to the neurons and photoreceptors are not fully understood (Delton-Vandenbroucke, Grammas & Anderson 1999), although the retina at least appears to have specific mechanisms for DHA uptake and incorporation (Rotstein, Aveldaño & Politi 1999). DHA may be transported from the liver to the brain and retina within lipoproteins (Hernell 1990); the photoreceptor cells of the retina have indeed been

found to possess a receptor for LDL (Bazan 1992). The presence of FABP and acyl-CoA synthetase specific for DHA may contribute to the preferential accumulation in these tissues (BNF 1992).

In addition to immature fetal liver metabolism, neither the fetal retina nor brain initially synthesize DHA, and their capacity to do so is a function of gestational age. Thus, placental transfer is crucial (Clandinin 1999), particularly during the earlier gestational period when growth is slower and the fetus able to store LCPUFA obtained via the placenta. Premature infants are compromised as a result of both being denied LCPUFA via the placenta and their immature metabolism; the potential for deficiency in the preterm infant is thus a matter of much concern to neonatologists (Crawford *et al* 1993, Crawford 2000), and the relevance of appropriate pre- and early ante-natal maternal nutrition is clear (Crawford 1993, Crawford *et al* 1993).

For n-3, n-6 and n-9 PUFA families, accretion in brain tissue increases with gestation, until maximum rates at term (Koletzko 1992, Martinez 1992). The amounts of n-6 and n-9 PUFA accumulating in the brain are approximately twice that of n-3 PUFA (Martinez 1992, Svennerholm 1968).

Estimation of the brain's accumulation of fatty acids in the last trimester varies from 33mg/week (Clandinin *et al* 1980) to 41mg/week (Clandinin *et al* 1981) for n-6 PUFA, and 15mg/week (Clandinin *et al* 1980) to 22mg/week (Clandinin *et al* 1981) for n-3 PUFA. In the last trimester of 26 to 40 weeks gestation, 80% of the DHA present in the fetal brain is assimilated (Clandinin *et al* 1980), *i.e.* coincidental with the brain growth spurt (Hernell 1990). Animal studies indicate that in other primates, as in the human fetus, DHA accumulation in brain tissue occurs mainly in late gestation, when developmental and functional changes are in process (Green *et al* 1999).

The retina also accumulates high levels of PUFA, with most active assimilation occurring after 24 weeks gestation (Koletzko 1992). DHA occurs mainly in the outer segment of rod photoreceptor cells. Its presence in the phospholipids of these cells, particularly phosphatidylethanolamine (Craig-Schmidt, Stieh & Lien 1996), implicates it in the membrane fluidity of these signal-transducing cells (Koletzko 1992). DHA is

synthesized by the retinal pigment epithelium (Anderson *et al* 1994, Innis *et al* 1999) which can transport DHA and other nutrients to the retina. On release from the rod photoreceptors during membrane biogenesis, DHA is preferentially incorporated into TAG, and AA into PL (Anderson *et al* 1994), a pattern similar to that of the preferential incorporation of these two LCPUFA by placental cells (Crabtree *et al* 1998, see above). The retinal pigment epithelium stores the DHA and transports it back to the photoreceptor cells, where it is re-integrated into new membranes, providing the retina with an efficient process to conserve DHA (Innis *et al* 1999). The role of DHA in the aetiology of retinal degenerations is controversial (Anderson *et al* 1999).

## 3.3 POSTNATAL FAT ACCUMULATION

### 3.3.1 Introduction

Fat accounts, on average, for 71% of the total weight gained by the human fetus (Ziegler *et al* 1976). The term human fetus of 3500g is 16% fat and by four months postpartum, the infant (6.5kg) is 25% fat. The human infant gains 25g/d when breast feeding, and ingests around 37g of lactose and 21g of fat each day (Weaver & Stephenson 1998). 35% of weight gain during the first six months of postnatal life is fat. 90% of all energy retained by growing tissues of the term baby is comprised of lipid (Weaver & Stephenson 1998). The fat accumulated is deposited mostly as white adipose tissue and in the membranes that make up the brain and nervous tissue (Weaver & Stephenson 1998).

The postnatal period is as critical as gestation to the accumulation of LCPUFA (Connor 2000). It has not been possible to quantify the proportion of fatty acids obtained via the placenta, endogenous synthesis and/or breast milk to the accretion of LCPUFA by the fetus and neonate (Innis, Hrboticky & Foote 1989).

Levels of LCPUFA do not increase for the first month postnatally, but do so between the first and third months (Clandinin *et al* 1989). Lower LCPUFA status has also been observed in postnatal plasma lipids, compared to cord plasma (Decsi & Koletzko 1994). If the rate of neonatal hepatic synthesis is either limited, or slower than the mobilization of LCPUFA from the liver to the tissues, the lack of immediate postnatal

accretion could be accounted for (Clandinin *et al* 1989). Evidence from studies on preterm, formula-fed infants suggests that it is the immaturity of the elongation/desaturation metabolism which causes the delay in extra-uterine accretion (Hernell 1990).

DHA accretion can be accomplished in breast-fed infants by utilization of the preformed DHA present in breast milk, or by either enzymatic conversion of α-linolenic acid (C18:3n-3), mobilization of subcutaneous tissues or hepatic fatty acid residues (Weaver & Stephenson 1998). The formula-fed infant, however, is not subject to a dietary source of DHA and therefore relies on the latter mechanisms to obtain adequate DHA.

### 3.3.2 Adipose Tissue

Storage lipid accumulates in adipose tissue, a form of connective tissue consisting of fat cells (adipocytes) which contain fat globules. There may be single (unilocular fat) or multiple (multilocular fat) fat globules within the cell. Unilocular fat, or "white" adipose tissue, is found in subcutaneous tissue and along the course of small blood vessels. Multilocular fat, or "brown" adipose tissue, accumulates in the thoracic and scapular regions and along the course of the large blood vessels. The fat content of adipocytes is mainly in the form of triacylglycerol.

By term, 80% of total fat is subcutaneous and 20% is in deep body sites (Widdowson 1985). Brown fat accounts for 7% (Weaver & Stephenson 1998) or 35-40g of total fat at term (Sparks 1984). There is an increase in the size, number and fat content of adipose cells after birth (Weaver & Stephenson 1998). During the first postnatal months, when fat is being deposited rapidly, the composition of plasma lipids, subcutaneous adipose tissue and brain reflect the composition of milk ingested (Weaver & Stephenson 1998, Widdowson *et al* 1975).

### 3.3.3 Brain Tissue

The brain is 60% structural lipid, 85% of which is found in the cerebrum. The brain increases in size by 750g during infancy, from 350g (10% of body weight at birth) to 1100g (still 10% of body weight at 12 months), attributable mainly to nerve cell growth

and proliferation and myelination (Weaver & Stephenson 1998). For this, the brain requires 60% of the total energy intake during the first year postpartum, obtaining the energy from dietary fat (Cockburn 1994).

Extrapolation from published data has led to estimation of the increase in brain DHA content in the first 6 months postpartum as 905mg in breast-fed and 450mg in formula-fed infants (Cunnane *et al* 2000a & b). The rate of total body DHA accumulation in breast-fed infants has been estimated as 10mg/d in the first six months postpartum (Cunnane 1999, Cunnane *et al* 2000b), with 5mg/d required for the brain alone (Cunnane, Francescutti & Brenna 1999, Cunnane *et al* 2000b). Considering "obligatory losses" of an assumed 50-60% of absorbed DHA, the authors postulate a dietary intake of 20mg/d would be required to meet this, a level provided by DHA in breast milk at 0.2% fatty acids (Cunnane 1999, Cunnane *et al* 1999, Cunnane *et al* 2000a & b). The authors stress that this amount is not based on association with function, but is intended to approximate the rate of DHA accumulation in breast-fed infants (Cunnane *et al* 2000b). For the infant fed formula containing αLA but devoid of supplementary DHA, the conversion of ingested αLA to *de novo* DHA would be required to proceed at a rate of 5%, a rate much greater than predicted to occur and therefore, the authors argue, evidence for DHA supplementation of infant formulae (Cunnane 1999, Cunnane *et al* 1999, Cunnane *et al* 2000a & b).

Infant formula containing αLA at less than 1% fat may be similar to breast milk in its composition of αLA, but may be inadequate to meet the elevated LCPUFA requirements of infants deprived of preformed DHA (Bjerve *et al* 1992, Innis *et al* 1989). The conversion (or bioequivalence) rate of αLA to DHA in the brain of infant baboons was estimated at 0.23% (Su *et al* 1999).

Chapter 4

# Biomarkers of Fatty Acid Status

## 4.1 INTRODUCTION

The contribution of animal studies to the understanding of fatty acid metabolism and status has been considerable, although their possible limitations must be appreciated when extrapolating to humans (Innis 2000). Several human biochemical indices of fatty acid status have been studied, each with its own validity and merit depending on the rationale for its use.

Generally, human fluids and tissues contain more than one class of lipid, which may be analysed separately or without distinction. Thus, each lipid fraction (triacylglycerols/TAG, phospholipids/PL, cholesterol esters/CE, non-esterified fatty acids/NEFA) or total lipids (total fatty acids/TFA) may be analysed. Moreover, fatty acid quantification may be relative or absolute. Relative amounts quantify each fatty acid as a proportion or percentage of all the fatty acids present in the lipid fraction analysed (e.g. % DHA in TAG fatty acids); absolute quantification expresses the concentration (mole) or actual weight (g) of each fatty acid in a standard amount of sample (e.g. mmole or mg DHA per ml or mg sample). The need for clarity regarding the lipid fraction analysed and the expression of results is highlighted by the variation in composition between fractions, and the difference in observed effects depending on the expression of results (Lopes *et al* 1991). The methods of quantification and their application are discussed further in Chapter 8 Materials and Methods.

## 4.2 CIRCULATING LEVELS OF FATTY ACIDS

Cells and tissues, including those of the nervous system, can obtain LCPUFA from preformed LCPUFA (from the diet by adults, via placental transfer from mother by the fetus, and from breast milk by the neonate), mobilization of adipose reserves, endogenous conversion of dietary EFA in the specific tissue, and/or EFA conversion in peripheral tissues (*e.g.* the liver, especially in the fetus and neonate). The relative contribution of these sources is not known (Innis 1991), however, in all cases, the transport of LCPUFA or their parent EFA is mediated by plasma. Plasma levels may not reflect tissue accretion, and by inference requirement, but do indicate the potential amount available to the tissues. Thus, although the fatty acid composition of plasma is not a specific proxy for that of tissues, it is a valid measure of fatty acid status. Moreover, since certain tissue samples are not as readily obtainable (see Section 4.3 Tissue Levels of Fatty Acids), it may be more useful to correlate diet and outcome

with circulating levels in larger studies than to perform smaller studies on tissue samples.

Plasma fatty acid composition has been shown to be a reliable biomarker (Ma *et al* 1995a), reflecting short-term dietary PUFA intake (Bjerve *et al* 1993, Dougherty *et al* 1987, Pauletto *et al* 1996). Serum lipids have been shown to accurately reflect the differential dietary intake of distinct populations (Torres *et al* 2000), with serum triacylglycerols indicative of recent diet (days) due to their rapid turnover, and serum phospholipids and cholesterol indicating dietary intake over the preceding weeks and months (Zeleniuch-Jacquotte *et al* 2000).

Devoid of a nucleus, the main site of lipids in the red blood cell (RBC) is the cell membrane, thus phospholipids are the predominant erythrocyte lipids. RBC do not synthesize fatty acids or phospholipids and so incorporate fatty acids/phospholipids only after they have been metabolized by other cells. RBC fatty acid composition does not therefore reflect dietary intake immediately, but does indicate previous intake. Total RBC lipid analysis is considered a suitable index of dietary intake (Dougherty *et al* 1987).

Correlation between fish consumption, dietary n-3 PUFA and plasma fatty acid status has been shown (Ma *et al* 1995b). Fish intake as infrequent as once per week influences plasma fatty acid concentrations (Bønaa, Bjerve & Nordøy 1992). In particular, dietary EPA and DHA correlate well with their corresponding levels in both plasma (Anttolainen *et al* 1996, Bønaa *et al* 1992, Ma *et al* 1995b) and RBC (Romon *et al* 1995). Supplementation of non-pregnant women with fish oils revealed that DHA increased in plasma and RBC in a dose-dependent manner (Otto, Houwelingen & Hornstra 2000). Fish oil supplementation, even in cases of malnourishment, enhances RBC n-3 status relative to pre-supplementation and to non-supplemented controls (Smit *et al* 2000a). There is some variability in the observation that circulating levels of AA are negatively correlated with intake of fish (Romon *et al* 1995) or fish oils. Plasma αLA and EPA have also been shown to be responsive to dietary intakes (Li, Mann & Sinclair 1999).

Circulating levels of fatty acids in relation to dietary intakes have been studied in great detail in neonates. This is partly attributable to the interest in postnatal diet and development, and also because neonates provide a unique opportunity for observation and intervention due to their exclusive diet of milk, whether breast or formula, until weaning. There is a wealth of data on the effect of neonatal dietary intake on fatty acid status, although the studies differ in the age of the infants, the diet (breast milk, standard or supplemented formula), the age at which the samples are taken, and the lipid fraction analysed (Table 15). Some studies have been observational, comparing breast-fed to standard commercial formula-fed infants, who were not randomized but whose respective diets were pre-assigned by parental choice. Other studies have involved some intervention whereby those infants whose mothers elected to formula feed were randomised to either a standard formula, or to a supplemented formula with a modified fatty acid composition often closer to that of breast milk.

Infant LCPUFA status, particularly DHA, is correlated with breast milk composition, even when maternal milk is low in EFA and LCPUFA (Smit *et al* 2000b). Differences have been noted in the levels of circulating fatty acids between infants receiving high or low intakes of breast milk, with those receiving greater amounts exhibiting higher total LCPUFA levels, in particular DHA and AA (Leaf *et al* 1996). Moreover, neonatal plasma and RBC DHA is correlated with breast milk DHA in a dose-dependent but saturable manner (Gibson, Neumann & Makrides 1997).

On comparison of those fed breast milk versus standard formula, breast fed infants have been repeatedly shown to have overall higher LCPUFA levels, and circulating levels reflect the composition of breast milk; the status of standard formula-fed infants reflects their diet which typically provides some EFA, but no LCPUFA. These findings have been noted in both term (Bakker, van Houwelingen & Hornstra 1999, Birch *et al* 1993, Granot, Golan & Berry 2000, Jørgensen *et al* 1996, Makrides *et al* 1993 & 1994) and preterm (Foreman-van Drongelen *et al* 1995b, Birch *et al* 1993) infants.

Such findings have led to the experimental use of formulae supplemented with EFA and/or LCPUFA, and comparisons of infants receiving standard or supplemented formula milks have been undertaken. Supplemented formulae have been shown to

result in higher PUFA status than standard formulae (Carslon, Werkman & Tolley 1996), in an apparent dose-related response (Jensen *et al* 1997). In addition, supplemented formulae appear to be as effective as breast milk in determining LCPUFA status (Bondía-Martínez *et al* 1998). Generally, infants fed breast milk or supplemented formula (EFA or LCPUFA) achieve higher status than those fed standard formula, with differential effects of breast milk, supplemented formula and standard formula reported (Agostoni *et al* 1995, Auestad *et al* 1997, Birch *et al* 1992, Birch *et al* 1998, Carlson *et al* 1996, Faldella *et al* 1996, Jørgensen *et al* 1998, Makrides *et al* 2000a, Uauy *et al* 1990). With regards to the supplement added, variations and combinations of EFA and LCPUFA have been used, the type and amount of supplementary fatty acids exerting influence over circulating levels. Perhaps most notably, differential results have been demonstrated between formulae supplemented with DHA alone or in addition to AA (Auestad *et al* 1997, Birch *et al* 1998).

| Study | Infants | Infant Feeding Regimens | Status |
|---|---|---|---|
| Billeaud *et al* 1997 | Preterm | High αLA formula, Low αLA formula | Plasma TAG/PL/CE, RBC PE |
| Carlson *et al* 1991 | Preterm | Standard formula, Low marine oil supplemented formula, High marine oil supplemented formula | Plasma TAG/PE/PC, RBC PE/PC |
| Clandinin *et al* 1999 | Preterm | Breast milk, Standard formula, Formulas with range of AA and DHA | Lipoproteins |
| Clark *et al* 1992 | Term | Breast milk, Standard formula, High αLA supplemented formula, Low αLA supplemented formula | Plasma total lipids, RBC total lipids |
| De-Lucchi *et al* 1988 | Term | Breast milk, Standard formula | RBC PE/PC/PS/SM/Cholesterol |
| Decsi *et al* 2000 | Term | Breast milk, Standard formula | Plasma TAG/PL/CE |
| Decsi & Koletzko 1995 | Term | Standard formula, LCPUFA supplemented formula | Plasma TAG/PL/Sterol Esters |
| Decsi, Thiel & Koletzko 1995 | Term | Breast milk, Standard formula | Plasma TAG/PL/Sterol esters |
| Hayes *et al* 1992 | Term | Breast fed, Coconut oil/soybean formula, Corn oil/soybean formula | Plasma TAG/PL/cholesterol/lipoproteins, RBC total lipids |
| Jensen *et al* 1996 | Term | Formulas (4) differing in αLA | Plasma PL, RBC PL |
| Kohn *et al* 1994 | Term | Breast milk, Standard formula, LCPUFA supplemented formula | Plasma total lipids, RBC total lipids |
| Koletzko, Decsi & Demmelmair 1996 | Term | Breast milk, Standard formula, LCPUFA supplemented formula | Plasma PL |
| Koletzko *et al* 1995 | Preterm | Breast milk, Standard formula, LCPUFA supplemented formula | Plasma PL |
| Koletzko *et al* 1989 | Preterm | Breast milk, Standard formula, LCPUFA supplemented formula | Plasma TAG/PL/Sterol Esters |
| Liu *et al* 1987 | Preterm | Low, intermediate and high EPA & DHA supplemented formulas | Plasma TAG/PE/PC |
| Makrides *et al* 1995a | Term | Breast milk, Standard formula, LCPUFA supplemented formula | RBC total lipids |
| Ponder *et al* 1992 | Term | Breast fed, Coconut oil/soybean formula, Corn oil/soybean formula | Plasma PL, RBC PE/PC |
| Vanderhoof *et al* 1999 | Preterm | Breast milk, Standard formula, LCPUFA supplemented formula | Plasma total lipids |
| Woltil *et al* 1999a | LBW | Breast milk, Low LCPUFA supplemented formula, High LCPUFA supplemented formula | RBC total lipids |
| Woltil *et al* 1999b | LBW | Breast milk, Low LCPUFA supplemented formula, High LCPUFA supplemented formula | Plasma CE, RBC total lipids, Platelet total lipids |

Table 15. Selected studies demonstrating relationship between dietary and circulating levels of fatty acids in neonates. αLA – alpha-linolenic acid; AA – arachidonic acid; DHA – docosahexaenoic acid; LCPUFA – long-chain polyunsaturated fatty acids; EPA – eicosapentaenoic acid; TAG – triacylglycerol; PL – phospholipids; CE – cholesterol esters; PE – phosphatidylethanolamine; PC – phosphatidylcholine; PS – phosphatidylserine; SM – sphingomyelin.

In adults, age and anthropometric indices, such as body mass index (BMI), have not been found to be associated with circulating levels of FA (Berry *et al* 2001, Romon *et al* 1995, Theret *et al* 1993). There have been conflicting reports of the effect of cigarette smoking on circulating FA status, with the most consistent observation being of lower levels of linoleic acid (18:2n-6) in the plasma (Ma *et al* 1995b, Pawlosky *et al* 1999) and RBC (Theret *et al* 1993) of smokers. Other studies have found either no association (Romon *et al* 1995), a positive correlation with MUFA (Berry *et al* 2001, Simon *et al* 1996) and n-9 PUFA (Simon *et al* 1996), and a negative association with AA and DHA (Simon *et al* 1996).

The effect of alcohol has been similarly unresolved, ranging from no effect observed (Berry *et al* 2001, Hjartaker, Lund & Bjerve 1997) to varying effects on SFA (Ma *et al* 1995b, Simon *et al* 1996). Most consistently, a positive association has been noted with palmitoleic acid (16:1n-9) in RBC (Romon *et al* 1995, Theret *et al* 1993) and serum (Simon *et al* 1996), while a negative association with 18:2n-6 has been observed in RBC (Romon *et al* 1995, Theret *et al* 1993), plasma (Ma *et al* 1995b) and serum (Simon *et al* 1996). One possible explanation for these variations may lie in the observation that alcohol intake had no effects on fatty acid status when moderate, but did so when high (60g/d or more) (Simonetti *et al* 1993).

Circulating levels of fatty acids are thus reliable indices of dietary intake in humans of varying age. Alcohol and cigarette smoking should, however, be taken into consideration when studying dietary and circulating fatty acids (Simon *et al* 1996).

## 4.3 TISSUE LEVELS OF FATTY ACIDS

### 4.3.1 Relation to Growth and Development

Whether circulating levels reflect tissue accretion is a matter of debate. A correlation between RBC and cerebral cortex DHA was observed in neonates (Makrides *et al* 1994). There is conflicting evidence from animal and human studies as to whether circulating (plasma and/or RBC) levels of DHA correlate with brain DHA (Cunnane 1999). It has been argued that circulating levels cannot indicate brain levels since plasma levels do not reflect requirement, merely intake and transport (Cunnane *et al* 2000b). This has led to the consideration of human tissue data as necessary to establish the relationship between dietary, circulating and tissue levels, and to provide

evidence for the need to supplement infant formulae (Cunnane 1999, Cunnane & Francescutti 1999).

However, sampling of certain tissues, such as brain and retinal tissue, can only be achieved at autopsy. The use of these tissues assumes that tissue composition reflects requirement, but the circumstances under which such specimens are available preclude tissue composition from becoming related to developmental outcomes, such as visual function, developmental score *etc.*

Much work has been concerned with the fatty aid status of neonatal tissue, again largely in response to the putative role of fatty acids in development. The fatty acid composition of brain tissue has been shown to reflect that of the diet in an age or dose-dependent manner. Breast-fed infants had higher levels of DHA in their cerebral cortex than formula fed infants (Farquharson *et al* 1992, Makrides *et al* 1994). Cerebral DHA varied with age and duration of breast feeding (Farquharson *et al* 1995a, Makrides *et al* 1994). For formula-fed infants, DHA was correlated with the level of αLA in the formula (Farquharson *et al* 1995a). The lower DHA in the formula fed groups was compensated for by increased incorporation of the structurally similar DPA (22:5n-6), in a manner negatively correlated with dietary DHA and αLA.

In cerebral white matter, DHA was again dependent on diet and age, declining with age more evidently in the formula-fed group. DPA also declined with age but did so more rapidly in the breast-fed group (Farquharson *et al* 1996). Similarly in cerebellar gray and white matter, DHA was higher in breast–fed than formula-fed infants; of those receiving formula, DHA increased with the αLA content of the formula. In cerebellar gray matter, DPA was preferentially incorporated in response to low DHA (Jamieson *et al* 1999). The levels of LA, αLA, AA and DHA all appear to be age and dietary related in both neonatal adipose (Farquharson *et al* 1993) and hepatic tissue (Farquharson *et al* 1995b).

Levels of LCPUFA in brain, adipose and hepatic tissue thus reflect the LCPUFA content of infant feeds. No such dietary related differences have been observed

between breast- and formula-fed infants in the DHA content of the retina (Makrides *et al* 1994).

Breast milk is also a reliable index of FA status, and the variation in its fatty acid composition in response to numerous factors is discussed in more detail in a later section (see Chapter 5, Section 5.2 Fatty Acid Status of Breast Milk). Problems arise when the analysis of breast milk is used to extrapolate the requirements of all infants. Breast milk of mothers delivered at term may indicate dietary fatty acid levels adequate for thriving neonates consuming sufficient volumes of milk. However, the needs of premature infants may be greater, due to their immature development and lower adipose stores (Innis 2000). Term breast milk should thus not be assumed as appropriate for preterms; milk from mothers delivered prematurely may be more suitable.

There is therefore great difficulty in determining the amounts of fatty acids required for appropriate growth and development of the fetus and neonate since no single index is universally adequate. The value of the various biochemical indices of FA composition in the study of growth and development is discussed in detail elsewhere (Innis 1991, Innis 1992b).

### 4.3.2 Relation to Storage and Potential Status

Adipose tissue may be sampled with minimum discomfort (Beynen & Katan 1985, Berry *et al* 1986) and is "ethically appropriate" (Cunnane & Francescutti 1999). Due to the slow turnover of its constituent lipids, adipose tissue may indicate long-term dietary habits (BNF 1992) and may overcome some of the limitations incurred with dietary analysis, particularly when considering fish and n-3 PUFA intake (Marckmann 1999). However, for intervention studies, prolonged time periods are required to detect an effect of dietary manipulation or supplementation on the composition of adipose tissue, and to relate adipose fatty acids to those of the circulation and other tissues (Mantizoris *et al* 1995).

## 4.4 CONCLUDING REMARKS

It has been suggested that since several factors (diet, synthesis, turnover) determine the fatty acid composition of each sample type (RBC, plasma, adipose, breast milk, brain

tissue) more than one index of status should be used to reflect dietary intake (Beysen & Fielding 1999). Each sample type may thus be considered a valid "snapshot" of fatty acid status.

Chapter 5

# Maternal and Fetal Fatty Acid Status

## 5.1 MATERNAL AND FETAL FATTY ACID STATUS

### 5.1.1 Introduction

There is a correlation between the dietary intakes of fish and n-3 fatty acids and RBC fatty acid composition of pregnant women (Olsen *et al* 1995a). In particular, the RBC ratio of n-3 EPA, DPA and DHA to n-6 AA can be used to rank intakes, even when the average intake is relatively low (Olsen *et al* 1995a). Plasma PL composition also reflects dietary intake during pregnancy (Al *et al* 1995b). Biomarkers of dietary fatty acids are thus reliable even in pregnancy. Certain biomarkers are specific to pregnancy and the fetus, such as blood and tissue from the umbilical cord and the status of the placenta itself. Moreover there are several other factors which influence the status of both mother and fetus. The assessment of fatty acid status during pregnancy, and the determinants of and relationship between maternal and fetal status are discussed.

### 5.1.2 Maternal Fatty Acid Status

5.1.2(a) Relative to Non-pregnant Female Status

In comparison to non-pregnant women, pregnant mothers have similar n-6 PUFA but increased EPA and DHA levels when quantified in absolute concentrations in plasma total lipids at term (Wang, Kay & Killam 1991). Plasma PL of pregnant women are lower in the relative levels of all n-6 and n-3 PUFA, but not Mead acid (20:3n-9), in comparison to non-pregnant women. The maternal fatty acid profile remains deviated 6 weeks postpartum, regardless of lactation, although it begins to return to the non-pregnant "norm" (Holman, Johnson & Ogburn 1991).

5.1.2(b) Changes during Pregnancy and with Parturition

In a study of early pregnancy (up to 10 weeks gestation), the absolute amounts of plasma PL fatty acids significantly increased compared to pre-pregnancy levels, with a 46% increase in plasma DHA (Otto *et al* 2001). Significant increases were not observed in RBC PL; RBC DHA was elevated but not significantly. With regards to their relative levels in both plasma and RBC PL, total SFA remained unchanged, LA decreased, and n-6 and n-3 LCPUFA increased. These changes could not be explained by dietary intakes, which remained constant, and indeed, the increase in % DHA was such that the correlation between dietary intake and RBC or plasma DHA was no longer evident. It has been suggested that these findings indicate that pregnancy-

associated hyperlipidemia starts early in gestation and that there are metabolic adaptations (increased synthesis and/or incorporation) that ensure adequate DHA for fetal requirements (Otto *et al* 2001).

In perhaps the most comprehensive study to date on the pregnancy-associated changes in maternal fatty acid status, plasma PL were obtained throughout pregnancy from 10 weeks gestation until delivery and then at 6 months postpartum (Al *et al* 1995c). Expression of the results in both absolute (mg/L) and relative (% TFA) terms has enabled the differential patterns of change to be appreciated. Absolute amounts of total fatty acids, total n-6, total n-3, total SFA, total MUFA, LA, AA and DHA all increased between the first trimester to term in maternal plasma PL (Al *et al* 1995c). Postpartum declines were noticed in all of these, although the level of total fatty acids, total n-6, total SFA, total MUFA, LA and AA decreased to first trimester levels, while total n-3 and DHA were lower postpartum than during early pregnancy (Al *et al* 1995c).

These patterns were not however reflected in the relative amounts of the fatty acids in maternal plasma PL. Total n-6, total n-3 and AA all decreased during pregnancy while LA remained constant and total SFA and MUFA increased. DHA, however, accounted for the largest increment but only until 18 weeks gestation when its relative amount fell. By 6 months postpartum, total n-6, MUFA, LA and AA were all similar to their mid-gestation levels, but maternal total n-3 and DHA were lower than in early pregnancy. The transient increase in DHA followed by its decline suggested that there was maternal mobilization of DHA followed by preferential transfer to the fetus (Al *et al* 1995c).

Indeed, elevated maternal PUFA in the NEFA fraction of plasma and RBC have been observed, particularly in the second trimester when RBC DHA and AA were maximal (Ashby, Robinette & Kay 1997). Moreover, several studies have noted decreases in maternal RBC and/or plasma % DHA levels between time-points within the last trimester and subsequent term delivery following healthy pregnancies (Al *et al* 1995a). In addition, the last trimester decline in plasma PL % DHA has been observed in pregnancies with gestational diabetes mellitus (Wijendran *et al* 1999).

Inverse associations between LA and AA, LA and DHA, αLA and AA and αLA and DHA have been observed in the individual PL of both maternal plasma and RBC during the last trimester (Ghebremeskel *et al* 2000). In addition, AA was positively correlated with DHA in plasma and RBC PL. Perhaps, however, the most important aspect of these findings is that they were observed in both British and Korean mothers, despite their dietary differences, and in their babies (see Section 5.1.3 Fetal Fatty Acid Status) in each population. Since these relationships were particularly evident for RBC PL, the authors suggest that the AA and DHA contents of membranes are regulated in both mother and fetus, and by maternal status and supply to fetus (Ghebremeskel *et al* 2000). However, the fatty aids were quantified based on peak area and thus the results were presumably obtained on analysis of the relative amounts. Given that as the proportion of one fatty acid increases or decreases, there must be a reciprocal change in another, it is possible that the relationships observed are dependent on the manner in which the individual fatty acids are quantified.

The post-partum decline in PUFA status is particularly evident in plasma PL, where DHA decreases following parturition and does so to a greater extent in lactating mothers, such that their DHA status is lower than that of non-lactating mothers and non-pregnant women (Otto *et al* 1999).

There is apparently no effect of labour on plasma or RBC fatty acid composition (Al *et al* 1990, Holman *et al* 1991). No significant differences in maternal fatty acid status have been noted following either vaginal or caesarean section delivery (van der Schouw *et al* 1991)

5.1.2(c) Factors Affecting Maternal Status

*(i) Parity*

There is conflicting evidence as to whether circulating maternal DHA status (relative and/or absolute) is inversely related to parity (Al, van Houwelingen & Hornstra 1997, van den Ham, van Houwelingen & Hornstra 2001, van Houwelingen, Ham & Hornstra 1999). The relative (% TFA) and absolute (mg/L) levels of plasma PL DHA during pregnancy and at parturition have been shown to be lower in multigravida compared to primigravida mothers (Al *et al* 1997). Indices of DHA sufficiency and deficiency did not vary between primigravida and multigravida mothers. Maternal plasma PL DHA

as a % TFA was negatively correlated with the number of pregnancies. However, a subsequent study failed to detect a difference between parous and nulliparous non-pregnant women in absolute (mg/L) RBC or plasma PL levels of total fatty acids, and in the % DHA in plasma PL (van den Ham *et al* 2001). RBC PL % DHA was significantly lower in parous women, but there was no difference in the DHA sufficiency index (ratio of DHA to DPA/22:5n-6) between parous and non-parous women. In addition, no relation between RBC or plasma DHA and the number of pregnancies was detected and no effect of time between successive pregnancies was observed (van den Ham *et al* 2001). While it appears that circulating DHA returns to non-pregnant levels in both primiparous and multiparous women within one year postpartum, it remains to be seen whether the same is true for tissue DHA (van Houwelingen *et al* 1999). This could explain why parity affects the DHA status of pregnant and not non-pregnant parous women; while circulating DHA status of non-pregnant parous and non-parous women would remain similar, a putative depletion of DHA stores following pregnancy would explain the lower DHA status of multigravida mothers during pregnancy. Parity may therefore not directly effect maternal status in the non-pregnant state, but rather may limit mobilization of maternal stores during subsequent pregnancies.

*(ii) Maternal Diet*

Maternal PUFA status at term has been shown to vary with fish and/or n-3 consumption, either throughout or for defined periods during pregnancy. Mothers consuming an above average amount of oily fish ingested greater quantities of n-3 PUFA, which became manifest in a higher % EPA and DHA, and lower AA in RBC PL, compared to mothers consuming less oily fish than average (Sanjurjo *et al* 1995). The increased % EPA and DHA, and ratio of n-3 LCPUFA to AA, in RBC total lipids of Faroese mothers compared to Danish mothers reflects the large marine and n-3 intake of the Faroe Islands (Olsen *et al* 1991).

Mothers supplemented with n-3 PUFA between weeks 26 and 35 of gestation increased their % DHA in both RBC and plasma total lipids over the period of supplementation, while the DHA status of unsupplemented mothers remained unchanged. As a result, by the end of supplementation, those mothers receiving supplements were significantly higher in RBC and plasma DHA than unsupplemented

mothers. Moreover, the DHA of supplemented mothers at delivery remained elevated at a level significantly higher than their unsupplemented comparisons (Connor *et al* 1996).

Supplementation of mothers with fish oil for 10 weeks until term significantly changed maternal plasma PL PUFA status relative to that of unsupplemented mothers (van Houwelingen *et al* 1995). Higher % n-3 PUFA and lower n-6 PUFA, including DPA (n-6), as well as lower Mead acid were observed in supplemented mothers. The % of n-3 PUFA in maternal plasma PL at 37 weeks gestation correlated with maternal levels at delivery (van Houwelingen *et al* 1995).

Both vegetarian and diabetic (type I, insulin dependent diabetes mellitus/IDDM) mothers display fatty acid patterns different from non-diabetic omnivorous mothers, but similar to each other, in their RBC total lipids at term. Relative levels of n-6 and n-3 PUFA, including DHA, are lower in vegetarian and diabetic compared to omnivorous mothers. These differences can be explained by the lower PUFA intakes of vegetarians, but not so in the diabetic mothers whose dietary composition resembled that of healthy omnivores, suggesting that metabolic differences exist in IDDM (Lakin *et al* 1998).

*(iii) Maternal Lifestyle/Habits*

Differences in fatty acid composition between smokers and non-smokers have not been detected in maternal plasma PL (Al *et al* 1995c). An elevated % of 18:0 (stearic acid) was noted in the plasma total lipids of non-smokers who had a healthy pregnancy delivered at term (Matorras *et al* 1994). The EFA and LCPUFA status of RBC membranes remains unchanged following smoking, alcohol consumption, or smoking with alcohol consumption during pregnancy, although the possibility exists that the apparent lack of effect is due to metabolic adaptations to ensure adequate mobilization of fatty acids (Smuts *et al* 1999).

*(iv) Gestation*

Mothers who delivered prematurely at 34 weeks had higher RBC % levels of LA, AA, DPA (n-6), DHA, but lower levels of 16:0, 18:0, 18:1 and αLA than control mothers at 34 weeks who went on to deliver at term (Reece *et al* 1997). Maternal RBC LA, AA,

DPA (n-6) and DHA were all higher at preterm delivery than term delivery. Similarly in plasma, the % contribution of LA, AA and EPA were each higher at preterm than term deliveries.

*(v) Health of Mother and Fetus*

Gestational diabetes mellitus (GDM) has been associated with varying patterns of maternal fatty acid status (Wijendran *et al* 1999). During the last trimester, plasma PL from mothers with GDM had lower relative and absolute n-6 LCPUFA, αLA and DPA (n-3), but higher DHA. However, % DHA was related to both dietary intake and pre-pregnancy BMI, such that when these were considered as covariates, the differences in % and concentration of DHA between GDM and normal maternal plasma were not significant (Wijendran *et al* 1999).

Mothers suffering from pregnancy-induced hypertension (PIH) had lower % LA in serum PL and CE, as well as higher DPA (n-3), DHA and total n-3 in serum PL, than mothers without this condition (van der Schouw *et al* 1991). Further analyses and the observation that the fetal status was not markedly different from normal, have suggested that these differences arose as a result of maternal metabolic adaptations occurring, at least in part, in PIH mothers to ensure an adequate fetal PUFA status (van der Schouw *et al* 1991).

These findings were largely repeated in a study of maternal plasma analysis during pregnancy and at term. Differences in the absolute amounts of fatty acids in plasma PL between mothers with and without PIH were not apparent during gestation, but a marked decline in LA, αLA and total n-6 in PIH mothers by term led to a significantly reduced αLA level compared to control mothers (Al *et al* 1995a). Relative fatty acid levels were also similar throughout pregnancy but differed at delivery in PIH and normal pregnancies, with lower LA and αLA in hypertensive mothers (Al *et al* 1995a). The % DHA in plasma PL from the PIH mothers increased between 32 weeks gestation and delivery, in direct contrast to the decline in % DHA seen over this time in the normal pregnancy group. The authors argue that this elevation in DHA occurred after the onset of PIH and is therefore unlikely to be causal, but may have occurred either to ensure adequate mobilization/transfer of DHA, or in response to the stress of

PIH (Al *et al* 1995a). Severity of hypertension did not further affect maternal fatty acid status (Al *et al* 1995a).

Maternal fatty acid status is modulated in pre-eclamptic toxaemia (PET). The absolute amounts of LA, αLA and EPA were lower in maternal plasma total lipids at term following pregnancy with PE compared to normal pregnancy (Wang *et al* 1991). Higher absolute amounts of palmitic (16:0), oleic (18:1n-9) and LA in serum NEFA have also been observed in PET (Lorentzen *et al* 1995). The relative amount of LA was, however, decreased in serum PL and TAG in mothers with PET. These differences were observed both during early pregnancy in a prospective cohort who later developed PE and in late pregnancy in a retrospective cohort diagnosed with PET. The authors thus suggest that the altered fatty acid status is not a consequence of but a contributing factor to PET (Lorentzen *et al* 1995).

Mothers who had an intrauterine growth retardation (IUGR) pregnancy had a higher % EPA but no differences in the absolute amounts of n-6 or n-3 PUFA in their plasma lipids when compared to controls (Matorras *et al* 1994). The reasons for this are unknown, indicating that further studies are required to elucidate the changes in maternal status in relation to IUGR (Matorras *et al* 1994).

### 5.1.3 Fetal Fatty Acid Status

Blood obtained from the umbilical cord following delivery is indicative of fetal/neonatal fatty acid status. RBC and plasma from the umbilical arteries do not differ significantly from that obtained from the umbilical vein (Al *et al* 1990).

The relative amounts of both LA and αLA have been inversely correlated with AA and DHA in cord plasma and RBC PL, while cord blood AA has been positively correlated with DHA. Moreover, these relationships were observed in both term and preterm infants, suggesting that mechanisms exist to ensure appropriate fetal AA and DHA levels (Ghebremeskel *et al* 2000).

5.1.3(a) Factors Affecting Fetal Status

*(i) Parity*

DHA, measured in absolute (mg/L) or relative (% TFA) terms, in umbilical cord plasma PL did not differ between neonates of primigravida and multigravida mothers (Al *et al* 1997). The % DHA in relation to DPA (22:5n-6) (DHA sufficiency index) was lower in neonates of multigravida mothers and negatively correlated with the number of pregnancies; conversely, the DHA deficiency index (% 22:5n-6/22:4n-6) was higher in cord plasma PL from multigravida mothers and was positively correlated with number of pregnancies.

*(ii) Maternal Diet*

Fetal fatty acid status is greatly influenced by maternal diet. Cord RBC PL following pregnancy in which mothers consumed oily fish more frequently were characterised by a higher % EPA and lower AA than was observed when maternal fish consumption was lower than average (Sanjurjo *et al* 1995).

Cord RBC and plasma total lipids obtained following pregnancy in which mothers were supplemented with n-3 PUFA from weeks 26 to 35 had significantly higher % DHA than in cord blood from an unsupplemented pregnancy (Connor *et al* 1996). Maternal fish oil supplementation from 30 weeks gestation until term significantly increased the percentage of all n-3 PUFA and decreased all n-6 LCPUFA in umbilical cord plasma PL relative to unsupplemented pregnancies (van Houwelingen *et al* 1995).

The % DHA is reduced and that of DPA (n-6) is elevated in cord plasma PL of neonates born to vegetarian mothers, who have high LA and low EPA/DHA intakes (Reddy *et al* 1994). Given that vegetarian intakes of AA, EPA and DHA may be nil (Sanders 1999), the presence of these fatty acids in cord blood suggests that endogenous synthesis is occurring. However, given that the dietary LA/αLA ratio is high in vegetarians, excessively high maternal LA consumption should be avoided to prevent inhibition of DHA synthesis (Sanders 1999).

High maternal intake of LA, either from habitual diet or supplementation, increases both the absolute and relative levels of individual and total n-6 PUFA, while decreasing n-3 levels in umbilical plasma PL (Al *et al* 1995b). However, maternal

plasma PL LA and n-3 status at 20 weeks gestation is correlated with corresponding umbilical plasma and arterial wall status, such that maternal supplementation with LA from the 20th week of gestation does not increase neonatal LA status. This suggests that the timing of maternal intake an important consideration, particularly when supplementation is undertaken (Al *et al* 1995b).

*(iii) Genetics*

There are suggestions that genetic influences contribute to neonatal fatty acid status (Hornstra *et al* 1992). The cord plasma PL of Canadian Inuit neonates exhibit higher relative levels of SFA and MUFA, with lower n-3 and n-6 PUFA, compared to Dutch infants. In particular, the Inuit neonates have lower % DHA and AA, but higher levels of the precursors LA and DHGLA (20:3n-6), as well as Mead acid (20:3n-9), despite the substantial marine component of the maternal diet. Moreover, cord plasma fatty acid content varies with maternal diet within the Inuit population, with higher n-3 PUFA found in communities where maternal fish consumption is greater. Thus, while maternal diet is a contributing factor, it may not be the only determinant of neonatal FA status, as it is possible that genetic differences, in particular low Δ5-desaturase activity, have evolved to ensure that n-3 levels do not become excessive when maternal supply is plentiful (Hornstra *et al* 1992).

Neonates with a family history of atopy have been shown to differ in their umbilical plasma and RBC fatty acid composition from neonates free of atopic risk, but the precise pattern of these differences has varied between studies (Beck, Zelczak & Lentze 2000).

*(iv) Maternal Lifestyle/Habits*

No differences in cord plasma PL fatty acids have been detected between neonates born to mothers who smoked or not (Al *et al* 1995c). Cord plasma total lipids were, however, lower in % DHA following a pregnancy in which mothers did not smoke (Matorras *et al* 1994).

Maternal smoking has been associated with elevated Mead acid levels in cord RBC membranes of preterm neonates; maternal alcohol consumption was coincident with a non-significantly lower cord DHA status. In combination, however, maternal alcohol

consumption and smoking were associated with significantly reduced cord RBC DHA which continued to decline further postnatally (Smuts *et al* 1999). Several reasons have been suggested to explain these observations, including increased transfer and/or uptake of DHA in compensation for the effects of alcohol and tobacco. However, there remains the possibility that the circulating RBC are not indicative of other tissues, whose fatty acid composition may have been compromised (Smuts *et al* 1999).

*(v) Gestation*

Umbilical plasma PL % LA has been found to be negatively correlated, while AA, DPA (n-3) and DHA were positively correlated with gestational age (Al *et al* 1995a). Interestingly, both the relative and absolute amounts of total n-3 and DHA in cord plasma PL were positively correlated with age (Al *et al* 1995c). Indeed, from analysis of both prenatal and umbilical samples, it has been observed that both the absolute and relative amounts of DHA in plasma PL increase with gestation (van Houwelingen *et al* 1993).

Increasing gestation also corresponds with increasing n-3 LCPUFA in cord plasma TAG (Berghaus, Demmelmair & Koletzko 2000). Gestational-age dependent relationships have been noted for % n-3 and n-6 EFA and LCPUFA in umbilical cord plasma TAG and CE; DHA in the TAG fraction, however, was constant and did not increase with gestation (Hoving *et al* 1994).

*(vi) Health of Mother and Fetus*

Neonates born following an IUGR pregnancy showed no differences in the fatty acid composition (absolute and relative amounts) of their plasma total lipids compared to neonates of an appropriate WGA (Matorras *et al* 1994). Similarly, the relative fatty acid compositions of cord serum PL, TAG and CE were not altered following pregnancy involving PIH (van der Schouw *et al* 1991), possibly because there is maternal adaptation to ensure an efficient maternal-fetal gradient. In cord plasma PL, the absolute amounts of fatty acids did not differ following normal or PIH pregnancy, although the % DHA was higher in PIH neonates (Al *et al* 1995a).

### 5.1.4 Relationship between Maternal and Fetal Blood Status

5.1.4(a) Comparison of Mother and Offspring

Several authors have noted the depletion of EFA and enrichment of LCPUFA in the neonate compared to mother (Koletzko, Demmelmair & Socha 1998). Generally, levels of LA and αLA are higher while AA and DHA are lower in maternal compared to cord plasma and RBC. Higher fetal levels of Mead acid (20:3n-9) have also been observed (van der Schouw *et al* 1991).

Lower EFA and higher LPCUFA in plasma TAG (Berghaus *et al* 2000) and plasma PL (Al *et al* 1990) have been observed in umbilical cord compared to maternal plasma. Umbilical plasma TAG and CE have higher % n-3 and n-6 LCPUFA and Mead acid, but lower % EFA than the corresponding lipid fractions in maternal plasma. Moreover, these observations were made for neonates born from 23 weeks until term (Hoving *et al* 1994).

While absolute amounts of total fatty acids, SFA, MUFA, n6, n3, LA, AA and DHA were lower in umbilical plasma PL compared to maternal plasma at delivery, neonatal levels did not differ from maternal levels at 6 months postpartum, which were assumed to be indicative of maternal pre-conceptional levels (Al *et al* 1995c). Moreover, umbilical plasma PL relative amounts of SFA, n-3, AA and DHA were all higher, while n-6 and LA were lower, than maternal levels throughout and following pregnancy. Thus although neonates may have lower absolute amounts of fatty acids, the relative enrichment of LCPUFA and depletion of EFA suggests preferential transfer from mother to fetus.

The findings in plasma have been largely replicated in RBC. Several differences between maternal and cord RBC total lipids at term delivery have been noted, including lower maternal relative amounts of LA, AA, DPA (22:5n-6) and DHA, and higher αLA and EPA (Reece *et al* 1997). However, the only significant differences were a lower % AA and higher % EPA in mothers than neonates.

Furthermore, elevated fetal % AA and DHA has been observed in all serum lipid fractions in both normal and PIH pregnancies (van der Schouw *et al* 1991). However, these patterns were slightly different in the case of neonates delivered prematurely.

Mothers delivering at preterm had significantly higher % LA and AA and lower DHA in their plasma PL than did cord plasma. Maternal RBC total lipids were, however, higher in % LA and lower in AA, EPA and DHA than cord RBC (Reece *et al* 1997).

5.1.4(b) Correlation of Fatty Acid Status of Mother and Offspring

Correlations have also been detected at delivery between maternal and cord plasma PL in the % of SFA, MUFA, n-6, n-3, LA, DPA (n-6), AA and DHA (Al *et al* 1990, Al *et al* 1995c, van Houwelingen *et al* 1995). Maternal plasma TAG % LA was positively correlated with that of cord plasma when delivery occurred after 34 weeks gestation; LA in maternal and cord plasma CE fractions were similarly related when delivery occurred at term (Hoving *et al* 1994). The % DHA in cord RBC and plasma has been correlated not only with maternal blood DHA, but also with maternal dietary n-3 (Connor *et al* 1996). In neonates born appropriate WGA, correlations between maternal and cord plasma total lipids were observed for % 14:0 (myristic acid) and EPA. A similar maternal-fetal relationship for % EPA was also observed in IUGR pregnancies, in which maternal and fetal oleic (% and absolute) and palmitoleic (%) acids were also correlated (Matorras *et al* 1994).

### 5.1.5 Fatty Acid Status of Placental Tissue

Placental tissue resembles the umbilical cord vessels in its fatty aid composition (Al *et al* 1990). It can be argued that since synthesis and transfer by the placental is central to fetal accretion of fatty acids, analysis of placental tissue provides an opportunity to "track" fatty acid patterns from mother to placenta to fetus.

Whether placental tissue is subject to the same differential compositions between populations as noted above for blood samples should indicate whether the composition of the maternal circulation is modified by the placenta before reaching the fetus. The relative level of 18:0 is higher in preterm than term placental tissue (Reece *et al* 1997).

Placental tissue from vegetarian and diabetic mothers is characterised by higher % EFA and lower LCPUFA (particularly from the n-3 family) in total lipids than that of non-diabetic omnivorous mothers. This may be attributable to lower dietary intakes of the vegetarian mothers, and metabolic differences of the diabetic mothers (Lakin *et al* 1998).

### 5.1.6 Fatty Acid Status of Umbilical Cord Tissue

Umbilical cord vessels have been analysed on the basis that their continuing growth and lack of a vasa vasorum, coupled with the inability of tissue to synthesize fatty acids *de novo*, allows the vessels to reflect the fatty acid status of fetal blood over a prolonged period of time (Crawford *et al* 1989, Foreman-van Drongelen *et al* 1995a, Hornstra *et al* 1989). In the umbilical cord, the vein is the afferent (supplying) vessel, conducting blood from the placenta to the fetus, while the arteries are the efferent (draining) vessels, returning blood from the fetus back to the placenta. This has lead to the suggestion that umbilical venous wall lipids provide an indication of longer term fatty acid supply to the fetus, while umbilical arterial wall lipids reflect the fatty acid status of the blood returning from the fetus to the mother, and hence the fetal fatty acid status (Foreman-van Drongelen *et al* 1995a, Hornstra *et al* 1992).

The fatty acid composition of the umbilical artery compared to vein was found to be lower in % n-6 and n-3 PUFA, but higher in n-7 and n-9 fatty acids as well as 20:3n-9 (Mead acid) and 22:5n-6 (DPA), the indicators of n-3 and n-6 deficiency, and DHA deficiency respectively (Al *et al* 1990, Hornstra *et al* 1989). In addition, all umbilical vessels have less LA and n-6 PUFA but more Mead acid than do adult colonic arteries and veins (Hornstra *et al* 1989). These observations have led investigators to suggest that neonatal EFA status is marginal and may be inadequate for fetal requirements. Although the actual reasons for the apparent fatty acid deficiency are not known, it has been speculated that maternal diet and its influence on maternal-fetal transfer is important in ensuring that a poor fetal status does not prevail (Al *et al* 1990).

Umbilical cord tissue has also been analysed whole, without dissection and separation of vessels. The differential patterns of fatty acid composition observed between omnivorous, vegetarian and diabetic mothers in the total lipids of whole cord tissue (Lakin *et al* 1998) suggests that analysis of the entire tissue is capable of reflecting maternal dietary and metabolic differences.

5.1.6(a) Factors Affecting Umbilical Vessel Status

*(i) Maternal Status*

The relative amounts of PL n-3 in umbilical arterial and venous walls have been correlated with the levels in maternal plasma PL (van Houwelingen *et al* 1995).

*(ii) Parity*

DHA, as % TFA, was lower in PL of cord arterial and venous vessel walls from multigravida compared to primigravida mothers (Al *et al* 1997). The DHA deficiency index (% 22:5n-6/22:4n-6) and the DHA sufficiency index (% 22:6n-3/22:5n-6) were also more favourable in the cord vessels of primigravida mothers. DHA relative levels (% TFA) and sufficiency indices were negatively correlated with the number of pregnancies.

*(iii) Maternal Diet*

Phospholipids from both umbilical arterial and venous vessel walls were enriched in EPA and DHA, and reduced in DPA (n-6), following maternal fish oil supplementation during the last trimester (van Houwelingen *et al* 1995). In addition, maternal fish oil supplementation does not differentially affect the fatty acid composition of umbilical arterial tissue PL relative to that of the umbilical vein (van Houwelingen *et al* 1995).

As a consequence of high maternal LA intake, the concentration and relative amount of n-6 PUFA are higher, while that of n-3 PUFA are lower, in umbilical vessels (Al et 1995b). A maternal vegetarian diet results in cord artery PL with lower % SFA and DHA, and higher n-6 LCPUFA and Mead acid, a fatty acid pattern which closely resembles that of the maternal diet (Reddy *et al* 1994).

*(iv) Genetics*

Despite a higher maternal consumption of marine foods, Canadian Inuit neonates have lower % n-3 and n-6 PUFA, except for higher LA and DHGLA, in the PL of their cord vessels compared to Dutch neonates. In combination with similar findings in cord plasma PL (see above), it has been suggested that genetic determinants also contribute to fetal fatty acid status (Hornstra *et al* 1992).

*(v) Gestation*

Gestational age has been positively correlated with % DHA and negatively with % Mead acid (20:3n-9) in the umbilical arterial wall PL (van Houwelingen *et al* 1995). Furthermore, in umbilical arterial wall PL, % DPA (n-6) was negatively and % DHGLA, adrenic acid, AA and DHA were all positively correlated with gestational age (Al *et al* 1995a). The relative amount of LA in umbilical venous wall PL has been negatively correlated with gestational age, while that of both AA and DHA has been positively correlated (Al *et al* 1995a).

The relative and absolute amounts of n-6 and n-3 PUFA are reduced in the cord tissue PL of preterm compared to term infants, and more markedly so in the umbilical arteries compared to vein. In addition, indices of EFA deficiency, including Mead acid (20:3n-9) and DPA (22:5n-6), are elevated in preterm cord vessels (Foreman-van Drongelen *et al* 1995a). These findings may, in part, be explained by the positive correlation between n-3 or n-6 PUFA (arterial and venous) and gestational age in premature infants.

*(vi) Singleton and Multiple Pregnancies*

Multiple and singleton pregnancies do not differ in the absolute amounts of fatty acids present in umbilical vessel PL; there are, however, differences in their relative fatty acid levels (Foreman-van Drongelen *et al* 1996). Arterial and venous cord tissues have lower n-6 and n-3 PUFA, but higher n-9 PUFA following multiple pregnancies. As a result, the EFA status and EFA deficiency indices are less favourable in the cord tissue from multiple compared to singleton pregnancies. Although there is no differentiation between twins and triplets, the cord fatty acid composition of quintuplets is even poorer. These observations may be further indications that fetal requirements strain, and may even exceed, maternal supply and that maternal status is a limiting factor in fetal status (Foreman-van Drongelen *et al* 1996).

*(vii) Health of Mother and Fetus*

Neither absolute nor relative fatty acid levels in umbilical arterial and venous tissues differed following PIH compared to normal pregnancies (Al *et al* 1995a). PET has been shown to be associated with abnormal cord vessels. The relative levels of n-3

and n-6 PUFA are lower in arterial and venous tissues following pregnancy with PE (Velzing-Aarts *et al* 1999).

## 5.2 FATTY ACID STATUS OF BREAST MILK

### 5.2.1 Introduction

In addition to its macronutrient content, breast milk contains a multitude of non-nutritive components including hormones, enzymes and immunoglobulins. The nature (Table 16) and composition (Table 17) of breast milk changes during lactation.

| Time postpartum | Milk | Appearance | Maximal concentration of |
|---|---|---|---|
| Birth-5d | Colostrum | Thick, creamy colour | Proteins, immunoglobulins |
| 6d-15d | Transitional | Thin, white colour | Lactose, water-soluble vitamins |
| 16d-Weaning | Mature | Thinner, blue/white colour | Fat, calories |

Table 16. Changes in breast milk with time postpartum.

| Nutrient | per 100g | Colostrum | Transitional | Mature |
|---|---|---|---|---|
| Energy | kJ | 236 | 281 | 289 |
| | kcal | 56 | 67 | 69 |
| Water | g | 88.2 | 87.4 | 87.1 |
| Protein | g | 2.0 | 1.5 | 1.3 |
| Carbohydrate | g | 6.6 | 6.9 | 7.2 |
| Starch | g | 0 | 0 | 0 |
| Total sugars | g | 6.6 | 6.9 | 7.2 |
| Fibre | g | 0 | 0 | 0 |
| Fat | g | 2.6 | 3.7 | 4.1 |
| Saturated | g | 1.1 | 1.5 | 1.8 |
| Monounsaturated | g | 1.1 | 1.5 | 1.6 |
| Polyunsaturated | g | 0.3 | 0.5 | 0.5 |
| Cholesterol | mg | 31 | 24 | 16 |
| Nitrogen | g | 0.31 | 0.23 | 0.2 |
| Sodium | mg | 47 | 30 | 15 |
| Potassium | mg | 70 | 57 | 58 |
| Calcium | mg | 28 | 25 | 34 |
| Magnesium | mg | 3 | 3 | 3 |
| Phosphorous | mg | 14 | 16 | 15 |
| Iron | mg | 0.07 | 0.07 | 0.07 |
| Copper | mg | 0.05 | 0.04 | 0.04 |
| Zinc | mg | 0.6 | | 0.3 |
| Chloride | mg | N | 86 | 42 |
| Manganese | mg | Tr | Tr | Tr |
| Selenium | ug | N | | 1 |
| Iodine | ug | N | N | 7 |
| Retinol | ug | 155 | 85 | 58 |
| Carotene | ug | | | |
| Vitamin D | ug | N | N | 0.04 |
| Vitamin E | mg | 1.30 | 0.48 | 0.34 |
| Thiamin | mg | Tr | 0.01 | 0.02 |
| Riboflavin | mg | 0.03 | 0.03 | 0.03 |
| Niacin | mg | 0.1 | 0.1 | 0.2 |
| Tryptophan | mg | 0.7 | 0.5 | 0.5 |
| Vitamin B6 | mg | Tr | Tr | 0.01 |
| Vitamin B12 | ug | 0.1 | Tr | Tr |
| Folate | ug | 2 | 3 | 5 |
| Pantothenate | mg | 0.12 | 0.20 | 0.25 |
| Biotin | ug | Tr | 0.2 | 0.7 |
| Vitamin C | mg | 7 | 6 | 4 |

Table 17. Nutrient composition of human breast milk (data from Holland *et al* 1991).

The properties of breast milk and the impact of feeding on both mother and infant have been the subject of much discussion (Emmett & Rogers 1997, Heinig & Dewey 1996 & 1997, Rogers, Emmett & Golding 1997, Whitehead & Paul 2000). The energy content of breast milk increases with lactation, and is estimated to be 530kcal/l at 6 weeks lactation, rising to 580kcal/l at 3 months lactation (Goedhart & Bindels 1994). The metabolizable energy intake decreases from 98kcal/kg/d at 5 weeks to 89kcal/kg/d at 11 weeks (Lucas *et al* 1988). However, the increase with age in infant body weight and volume of milk consumed, ensures a net increase in total metabolizable energy obtained. Total carbohydrate content of breast milk provides 40-50% of the energy in milk (Lawrence 1994).

Fat is present in human milk at concentrations of 2-4g/100ml (Table 17). Human milk fat accounts for 45-50% of total milk energy. It has been calculated that the average amount of total fat ingested by infants of 1-6 months age, based on a breast milk volume of 670-900ml/day, is 29-38g/day (Dewey & Lönnerdal 1983).

The fat content of breast milk is important as an energy source; its fatty acid composition is important with regard to the provision of EFA and LCPUFA. The lipid content of breast milk has been reviewed extensively (Jensen 1989a & b, Jensen 1999), including those factors identified as influencing composition (World Health Organization/WHO 1985). Breast milk contains EFA and LCPUFA whereas infant formulae only contain EFA; breast milk thus provides LCPUFA during early postnatal life when accumulation of LCPUFA is high, and is the first potential source of LCPUFA in early postnatal life.

### 5.2.2 Total Lipids

There are various classes of lipids in milk, contained within the milk fat globule which is synthesized in the mammary gland (Mepham 1987). Triacylglycerols, contained in the core of the milk fat globule, account for 98% of the fat in breast milk (Hachey *et al* 1987, Jensen 1989a, Hamosh & Bitman 1992, Hamosh *et al* 1992, Innis 1992a, Goedhart & Bindels 1994). Small amounts of diacylglycerols, monoacylglycerols and non-esterified fatty acids may be present in breast milk (Jensen 1989a). Phospholipids account for 0.5-1% of total milk lipids (Hachey *et al* 1987, Jensen 1989a) and are present in concentrations of 150-200mg/l (Hamosh *et al* 1984). Phospholipids are

associated with the milk fat globule membrane; they are derived from *de novo* synthesis within the mammary gland, inserted into the membranes of the milk secreting cells and their intracellular Golgi apparatus, which envelop the milk fat globules on their extrusion from the mammary cells (Hamosh *et al* 1984, Mepham 1987).

Cholesterol is the main sterol present in breast milk, and is found at relatively high concentrations of 100-150mg/l (Hamosh *et al* 1984, Goedhart & Bindels 1994). This has raised many questions on lipid metabolism and the putative "early origins" hypothesis of later disease (Goedhart & Bindels 1994, Huisman *et al* 1996). Cholesterol is obtained from the circulation and from *de novo* synthesis by the mammary gland and, as with phospholipids, is found in the milk fat globule membrane derived from the apical cell and Golgi membranes (Hamosh *et al* 1984, Mepham 1987).

### 5.2.3 Fatty Acids

5.2.3(a) Within Triacylglycerols

The fatty acids present in milk triacylglycerols are arranged in a specific positional distribution (Innis 1992a, Goedhart & Bindels 1994). Thus, each fatty acid has a preferred site (*Sn*-1, 2 or 3) at which to be esterified on the triacylglycerol. The most abundant triacylglycerols in breast milk are those with the combinations of fatty acids 16:0/18:1/18:1 or 16:0/18:1/18:2 at the *Sn*-1, 2 and 3 positions respectively (Winter *et al* 1993). These are suitable substrates for preduodenal lipase, which acts at the *Sn*-3 position to preferentially release medium chain and long chain unsaturated fatty acids, rather than saturated fatty acids. The long chain fatty acid released helps to stabilise fat globules in the duodenum, allowing pancreatic lipase to act at the *Sn*-1 and -3 positions. Pancreatic lipase releases C18 essential fatty acids (EFA) in preference to their longer, more unsaturated derivatives. This, and the fact that the unsaturated fatty acids (UFA) present in milk have a high coefficient of absorption (ESPGAN 1991), suggest that breast milk and the infant digestive system are suitably adapted to each other (Weaver 1992 & 1993).

5.2.3(b) Sources

The fatty acids present in the triacylglycerols of milk are derived from maternal diet, maternal adipose stores and *de novo* synthesis within the mammary gland. Stable isotope tracer studies have allowed quantification of the contribution made by these sources. Medium chain saturated fatty acids (MCSFA, C<16) synthesized in the mammary gland account for 10-12% of total fatty acids, while LCPUFA derived from the diet comprise 29% of total fatty acids. The remaining 59% of fatty acids are derived from both tissue synthesis and adipose tissue (Hachey *et al* 1987).

The incorporation of dietary LA into milk accounts for approximately 30% of milk LA, a rate which remains stable throughout lactation. The conversion of the dietary n-6 EFA to its LCPUFA metabolites also remains stable with time (Demmelmair *et al* 1998). There is a delay of 6-10 hours before dietary fats are incorporated into and appear in breast milk (Emken *et al* 1989, Fidler *et al* 2000b). The relationship between weight gain during pregnancy, and hence maternal fat deposition, and fat content of milk is unclear (Michaelsen *et al* 1994). The amounts (%) of both EFA in colostrum and mature milk are correlated with their respective levels in maternal white adipose tissue (Pugo-Gunsam *et al* 1999). There is some evidence to suggest that as *de novo* synthesis of MCSFA increases, the proportions of n-6 and n-3 EFA and LCPUFA are "protected" by preferential incorporation into milk TAG at the expense of stearic (18:0) and oleic (18:1n-9) acids (Schmeits *et al* 1999b).

5.2.3(c) Contributions

The fatty acid content of human milk is affected by many factors (WHO 1985, Jensen 1989a); its variability and the reporting of the relative (% fatty acid) fatty acid composition makes it difficult to define concentrations. However, percentage contributions of individual fatty acids to total fatty acid content have been assessed in many studies on mature breast milk from Europe, Africa, Asia and America. The relative contributions of the fatty acid classes can be summarized as follows (ESPGAN 1991, Goedhart & Bindels 1994, Innis 1992a, Koletzko Thiel & Abiodun 1992):

- Saturated fatty acids (SFA) account for 45-53% of total fatty acids, including palmitic acid at 20-24% of total fatty acids
- Monounsaturated fatty acids (MUFA), particularly oleic acid (18:1n-9), account for 30-38% of total fatty acids
- Total n-3 and n-6 polyunsaturated fatty acids (PUFA) account for 13-16% of total fatty acids including:
    - linoleic acid (18:2n-6) as 11-16% of total fatty acids
    - α-linolenic acid (18:3n-3) as 1% of total fatty acids
    - arachidonic acid (20:4n-6) as 0.5-0.7% of total fatty acids
    - docosahexaenoic acid (22:6n-3) as 0.3-0.6% of total fatty acids.

As with non-processed foods in the adult diet, the quantities of *trans* fatty acids in breast milk are low. Obtained from maternal dietary sources, the relative amounts of *trans* fatty acids are negatively correlated with EFA levels in breast milk (Innis & King 1999).

### 5.2.4 Factors Influencing Total Lipid and Fatty Acid Composition

5.2.4(a) Suckling

The amount of total milk lipid increases during a feed from foremilk to hindmilk (Jensen 1989a, Drury & Crawford 1990, Lawrence 1994) from approximately 2mg/100ml to 4mg/100ml. The second breast suckled at a feed produces foremilk with more lipid than that suckled first (Hall 1979). It has been suggested that the rising fat content of milk acts as a "satiety signal", but whether these variations are involved with appetite and thirst control in breast-fed babies is debatable (Hall 1975). The fatty acid composition remains constant throughout a feed (Gibson & Kneebone 1980) and does not differ between breasts (Hall 1979), thus a sample taken at any time during a feed is indicative of the fatty acid composition of the whole feed.

5.2.4(b) Diurnal Rhythm

Total milk lipid content increases from morning to afternoon (Hall 1979, Jensen 1989a). However, the time at which fat content peaks varies with the geographical location of the population studied (Harzer *et al* 1983). Whether this is a function of

dietary intake is a matter of debate (Hall 1979, Harzer *et al* 1983). Diurnal rhythm does not influence fatty acid composition (Hall 1979, Harzer *et al* 1983).

5.2.4(c) Stage of Lactation

The amount of total lipid increases from colostrum to mature milk, then stabilizes. Most studies are in agreement with the levels of fat in colostrum, transitional and mature milk, as shown in Table 17 (Bitman *et al* 1983, Harzer *et al* 1983). Prolonged lactation with supplementary foods (weaning) is associated with a decline in the volume of milk but not its fat content (Dewey, Finley & Lönnerdal 1984).

Of the lipid classes, only triacylglycerols remain constant in concentration (98-99% of total fat), with cholesterol and phospholipids decreasing as lactation continues (Bitman *et al* 1983). This may however be due to an increase in fat globule size (volume/surface diameter), with a concomitant decrease in the surface thickness of the fat globule. Since the surface area of the fat globule is related to the membrane surrounding it, thinning of the membrane could account for the relatively lower amount of cholesterol and phospholipids seen (Rüegg & Blanc 1981, Simonin, Rüegg & Sidiropoulos 1984).

With regards to fatty acid composition, the total contributions of saturated, monounsaturated and polyunsaturated fatty acids do not change over time (Makrides *et al* 1995b). However, within each subclass of fatty acid type, variations exist. The percentage contribution of medium chain saturated fatty acids (MCSFA) synthesized *de novo* increases with lactation (Boersma *et al* 1991, Finley *et al* 1985, Gibson & Harzer *et al* 1983, Kneebone 1981). Whether this is due to the higher concentrations of essential fatty acids (EFA) and polyunsaturated fatty acids (PUFA) in phospholipids and cholesterol than in triacylglycerols (Drury & Crawford 1990, Hamosh *et al* 1992), or to maturation of mammary gland fatty acid synthetase activity, is not known. The relative amount of monounsaturated fatty acids (MUFA) decreases with ongoing lactation (Gibson & Kneebone 1981).

Of the polyunsaturates, both the essential LA and αLA increase during lactation, although the opposite is true of their long chain metabolites (Gibson & Kneebone 1981). Thus, the percentage contributions of LCPUFA, particularly DHGLA (20:3n-

6), AA (20:4n-6), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3), are all higher in colostrum than mature milk (Boersma *et al* 1991, Makrides *et al* 1995b, Rønnenberg & Skåra 1992), and within the first month compared to later (Xiang *et al* 1999). It has therefore been suggested that as colostrum provides a source of "ready made" LCPUFA while the infant's metabolism is immature, mature milk provides sufficient parent EFA for the infant to synthesize a new pool of long chain metabolites.

The absolute amounts of EFA increase during the first month of lactation, in parallel with the increase in total fat, and stabilize for the remainder of lactation. Although the % AA and DHA decline in the first month postpartum, their concentrations remain relatively stable (Marangoni *et al* 2000). Since the LCPUFA are more evident in PL than the other lipid classes, the decline in relative PL levels may account for this observation (Agostoni *et al* 1999). Moreover, these findings suggest differential patterns in relative and absolute amounts of milk fatty acids, indicating that both measurements are relevant and pertinent (Marangoni *et al* 2000).

In most studies, the ratio of n-6:n-3 PUFA is reported to increase in mature milk (Rønnenberg & Skåra 1992). This appears to be due to a constant level of the n-6 family and a decreased level of the n-3 family (Rønnenberg & Skåra 1992, Serra *et al* 1997).

5.2.4(d) Geography

Many studies have been undertaken in several countries. Results that differ significantly between countries can apparently be explained by dietary differences (Al-Othman *et al* 1996, Borschel *et al* 1986, Innis & Kuhnlein 1988, Laryea *et al* 1995, Pugo-Gunsam *et al* 1999, Serra *et al* 1997, Xiang *et al* 1999). The main differences seen are in the relative contributions of saturated and unsaturated fatty acids, with increased saturated fatty acids due to enhanced synthesis by mammary tissue when fat content of diet is low (Drury & Crawford 1990). As mentioned above, there may be an association between geography or location and diurnal rhythm (Jensen 1989a) which further occludes any potential variation with geography. However, although regional differences within a country have been observed, the similar LA/αLA ratio of all regions (Fidler, Salobir & Stibilj 2000a) suggests that geographical location is not the overriding determinant of breast milk composition.

5.2.4(e) Maternal Diet

The total fat content of breast milk is independent of maternal fat intake (Vuori *et al* 1982). Studies of undernourished mothers reveal that the total amount of fat in milk is not affected by maternal dietary restriction (Jensen 1989a). However, there are changes within the fatty acid composition with an increased proportion (but not absolute mg/l) of saturated fatty acids (SFA) and a decreased proportion of EFA in the milk of undernourished mothers (Drury & Crawford 1990, Schmeits *et al* 1999a). This suggests that except in extreme undernourishment, the amount of fat remains relatively constant, with increased endogenous synthesis of SFA by the mammary gland to compensate. If, however, the volume of milk produced by a malnourished mother is low, then the increase in medium chain saturated fatty acids (MCSFA) will not be effective in augmenting the infant's intake (Borschel *et al* 1986). Supplementation of Gambian mothers with low energy intake failed to significantly increase either the volume of breast milk produced, or its fat concentration (Prentice *et al* 1980, Prentice *et al* 1983).

Lactating mothers in The Gambia traditionally consume a low fat diet (16% of total energy intake) which contains a high proportion of oleic acid from groundnuts. Analysis of their breast milk showed a concomitantly high predominance of oleic acid, followed by palmitic and linoleic acids (Prentice *et al* 1989). Despite the low contribution of fat to the maternal intake and the dietary restrictions imposed during the rainy season, long chain fatty acid profile of the breast milk resembled that associated with a maternal medium fat diet, while the proportion of saturated fatty acids synthesized *de novo* by the mammary gland were lower than expected on such a low fat diet. Thus, mobilization of maternal body fat to ensure an adequate supply of EFA and their LCPUFA derivatives in breast milk was probably occurring. These findings agree with the theory that fatty acid composition is indicative of maternal diet when energy consumption is adequate, and of maternal adipose tissue when energy consumption is low (Hambraeus 1990).

A study on the effect of vegetarian and vegan diets on breast milk composition (Sanders & Reddy 1992) concurred with previous reports that vegetarian and vegan diets are slightly lower in fat than omnivorous diets, obtaining 30-35% total energy

from fat. The intake of α-linolenic acid was variable but was usually higher than in omnivorous diets. There was a higher proportion of C10-14 saturated fatty acids but lower proportion of C16-18 saturated fatty acids in breast milk from vegans than omnivores. This may perhaps reflect increased *de novo* synthesis of C10-14 saturated fatty acids which tend to be lower in maternal vegan intake. Breast milk from vegan mothers had a higher proportion of linoleic and α-linolenic acids than that of omnivorous mothers. This is in agreement with an earlier study in which linoleic acid, derived mainly from vegetable fat, was higher in the breast milk of vegetarian than omnivorous mothers (Finley *et al* 1985). The ratios of both linoleic:α-linolenic and total n-6:n-3 were higher in vegan breast milk (Sanders & Reddy 1992). The n-6 PUFA, particularly DHGLA and AA were similar in vegans, vegetarians and omnivores, although vegans had lower DHA *i.e.* n-3. This suggests that since vegans have no dietary sources of DHA and AA, adequate amounts are made from their EFA precursors and secreted into milk (Makrides, Neumann & Gibson 1996a).

Comparison of breast milk from Inuit women consuming a diet rich in fish oil sources (marine mammal flesh) with Canadian women from Vancouver consuming a "typical" North American diet, revealed that EPA (20:5n-3) and DHA (22:6n-3) were elevated in breast milk from Inuit women (Innis & Kuhnlein 1988). N-6 fatty acids were not altered. This led the authors to suggest that there is no competition between n-3 and n-6 fatty acids for inclusion in breast milk (Innis & Kuhnlein 1988). Breast milk from mothers who ate fish at least occasionally has been shown to have higher levels of DHA than that from mothers who abstain from fish (Finley *et al* 1985).

Several studies have shown that supplementation of lactating mothers with fish oil supplements increased the proportion of EPA (20:5n-3) and DHA (22:6n-3) in breast milk (Jensen *et al* 1992). The effect of fish oil supplementation depends on the dose consumed, until stabilisation or saturation of milk levels, and may persist following cessation (Harris, Connor & Lindsey 1984). Fish oil supplements providing all n-3 PUFA (EFA and LCPUFA) subsequently elevated the % levels of total and individual n-3, while decreasing total n-6 (Henderson *et al* 1992). Cod liver oil supplements, containing high proportions of EPA and DHA in particular, incurred a dose-related increase in the relative amounts of these fatty acids in breast milk, while the proportion

of AA remained unchanged (Helland *et al* 1998). Supplementation with DHA as the only PUFA (200mg/d from weeks 4-6 postpartum) caused a linear increase in both the relative and absolute amount of DHA in breast milk, without affecting the content of other fatty acids (Fidler *et al* 2000b). When a range of DHA doses (0.2-1.3mg/d), all without EPA, were given as supplements, breast milk % DHA increased in a dose dependent manner; breast milk % AA and total lipids were not affected (Makrides *et al* 1996a). The effects of supplementation on breast milk composition are discernible within a short period of time (hours-days) (Fidler *et al* 2000b, Helland *et al* 1998). Such maternal supplementation during lactation also modulates the fatty acid composition of maternal plasma and RBC, and of infant RBC in a manner correlated to intake and breast milk levels (Helland *et al* 1998, Henderson *et al* 1992, Makrides *et al* 1996a). Reassuringly, supplementation of lactating mothers with n-3 oils does not affect levels of the anti-oxidant vitamins A and/or E in maternal or infant plasma or in breast milk (Helland *et al* 1998, Henderson *et al* 1992, Makrides *et al* 1996a).

5.2.4(f) Season

In studies of Gambian mothers, milk volume and total fat concentration are decreased by the end of the rainy season (Prentice, Prentice & Whitehead 1981, Prentice 1980). Although the rainy season is a time of low nutritional intake (Paul & Mûller 1980, Prentice 1980), seasonal variations in fat concentrations were correlated only with maternal subcutaneous fat stores, which were not themselves correlated to maternal energy intake. Since the rainy season involves increased energy expenditure, as well as decreased energy intake, perhaps subcutaneous fat, and hence fat concentration, are dependent more on energy output than intake (Whitehead 1979). Evidence for this can be seen in the nutritional supplementation of lactating Gambian mothers, which eliminated the intake deficits of the rainy season thus equalizing seasonal intakes (Prentice *et al* 1980): despite remaining at a higher weight than unsupplemented mothers, those supplemented still displayed the characteristic weight loss of the rainy season. Quite what contribution season would make on its own, when intake, expenditure and season are not correlated, (as in Western countries) is not clear. Moreover, correction for season and stage of lactation revealed that each individual mother produced milk with a relatively constant fat concentration.

5.2.4(g) Socio-economic Status

Total breast milk fat is not affected by socio-economic status. Fatty acid composition is again a reflection of the diet consumed according to the socio-economic group to which the mother belongs - for example, mothers of low socio-economic status consuming a high carbohydrate diet have increased C12-14 SFA (*i.e.* lauric and myristic acids) in their milk, reflecting increased *de novo* synthesis in the breast (Brasil *et al* 1991). Diet and geography must also be considered with socio-economic status, as the dietary intake will reflect both economic level and cultural habits. For example, in some countries, lower socio-economic groups will have a high fat intake, whereas, in other countries a high carbohydrate intake will be more characteristic of low socio-economic status (Borschel *et al* 1986).

5.2.4(h) Parity

Parity has striking effects on the composition of the milk of lactating Gambian mothers (Prentice *et al* 1981, Prentice *et al* 1989). Maternal intake of energy and fat were not affected by parity. However, the proportion of endogenously produced saturated fatty acids in breast milk decreased with increasing parity. This effect could not be explained by differences in diet and nutritional status, suggesting that the ability to synthesize fatty acids *de novo* is lost with parity. There are some limitations in extrapolating effects of parity on breast milk from The Gambia to the West, as the timings of pregnancies in these societies differ (Jensen 1989a). Studies in Sudan (Laryea *et al* 1995) and Saudi (Al-Othman *et al* 1996) show increased saturated fatty acids with increasing parity. When controlling for other variables, Laryea *et al* (1995) showed no significant influence of parity on MUFA, but an increase in PUFA with parity. Al-Othman *et al* (1996) demonstrated a decline in total UFA and MUFA with parity, and an increase in PUFA with parity of eight or more, compared to parity of less than four.

5.2.4(i) Maternal Age

Although mammary glands are not sufficiently developed to sustain breast feeding until 18 months after the first menstrual period (Stout 1992), this does not seem to affect either total fat or FA composition (de Nóbrega 1992). When age is controlled for socio-economic level, there is no difference in the total fat or fatty acid composition of breast milk of adolescent as opposed to adult mothers (Brasil *et al*

1991). Adequate nutritional counselling may improve both the dietary intake and the success of lactation by adolescent mothers (Lipsman, Dewy & Lönnerdal 1985).

5.2.4(j) Length of Gestation

Breast milk from mothers who have delivered pre-term infants does not differ in total fat content in comparison to that from mothers who deliver at term, suggesting that preterm milk may provide equal calories, although preterm infants have higher energy requirements.

Bitman *et al* (1983, 1984) found that both term and preterm milks share the same pattern with regard to ongoing lactation: total lipids and MCSFA increase from colostrum to mature milk, while LCPUFA of C20-22, cholesterol and phospholipids all decrease with maturity. However, the initial contributions of MCSFA, LCPUFA, cholesterol and phospholipids were higher in early preterm milk (Bitman *et al* 1983 & 1984), but by day 21, both term and preterm milks had similar LCPUFA levels. Whether this is a disadvantage caused by immaturity of the mammary glands, or an advantage attributable to the evolutionary design of milk specifically for the preterm infant, is debatable (Jensen 1999).

In contrast, Rønnenberg and Skåra (1992) found that preterm colostrum had lower saturated fatty acids and n-3 PUFA, but a higher n-6:n-3 ratio, than term colostrum. Luukkainen, Salo & Nikkari (1994) found that SFA and MUFA remained stable in both tern and preterm milks throughout the first six months of lactation. Preterm and term milks had similar levels of LA and αLA, which increased with lactation. While LCPUFA of C20-22 decreased in both groups between the first week and the first month of lactation, there were no further changes in the preterm levels. The LCPUFA of the term milks, however, continued to decrease until six months postpartum, giving rise to differences in the LCPUFA levels between preterm and term milks at the end of the six months of lactation.

5.2.4(k) Maternal Health and Disease

*(i) Insulin Dependent Diabetes Mellitus*

Several studies of the milk of mothers with insulin dependent diabetes mellitus (IDDM) show that although there is no difference in total milk lipid, there are

differences in fatty acid compositions in the breast milk of women with IDDM (Jackson *et al* 1994). LCPUFA (in particular, DHGLA and AA) transiently increase in the breast milk of IDDM mothers within the first seven days of lactation, but then show a sustained decrease, to the extent that LCPUFA were lower in mature IDDM than control milk. The initial increase in LCPUFA can be explained by a delay in establishing lactation in IDDM mothers, but it is not clear whether the greater decrease in LCPUFA profile is caused by differences in maternal dietary essential fatty acid intake or altered fatty acid metabolism. These changes were not correlated with any parameters of IDDM such as severity, glycosylated haemoglobin, post-prandial glucose or insulin dosage. In contrast to the LCPUFA, the proportion of MCSFA (C10-14) was similar in both groups until 14 days postpartum, with an elevation thereafter in the breast milk of IDDM mothers. However by controlling for gestational age, it has been suggested that the rise in MCSFA was due to the earlier deliveries of IDDM mothers, since MCSFA are also higher in preterm milk (Hamosh & Bitman 1992).

*(ii) Cystic Fibrosis*

In a comparison of breast milk samples from mothers with cystic fibrosis with that of mothers without, total fat was slightly lower, compared to that from healthy controls, but within an acceptable range (Bitman *et al* 1987). The main differences noted were a decreased proportion of linoleic acid and AA, but an increased proportion of 16:2n-7, α-linolenic acid (18:3n-3), eicosadienoic acid (20:2n-6), adrenic acid (22:4n-6), DPA (22:5n-6) and clupanodonic acid (also DPA, 22:5n-3) in the breast milk of mothers with cystic fibrosis. It is not clear whether this is attributable to differences in maternal intake, maternal malabsorption or abnormal fatty acid metabolism (as higher levels of Δ6-desaturase enzymes are found in mothers with cystic fibrosis).

*(iii) Type 1 Hyperlipoproteinemia*

Lipoprotein lipase (LPL) deficiency could putatively prevent transfer of dietary LCPUFA from maternal blood to milk, thus accounting for the increased MCSFA, and decreased total fat, LA and LCPUFA in the breast milk of mothers with type 1 hyperlipoproteinemia (Berger *et al* 1983, Myher, Kuksis & Steiner 1984, Steiner, Myher & Kuksis 1985).

*(iv) Hypobetalipoproteinemia*

Breast milk from a mother with this condition contained decreased total fat, EFA and LCPUFA, but increased short and medium chain fatty acids (Wang & Illingworth 1987). Thus the lack of apolipoprotein B and hence chylomicrons and very low density lipoprotein (VLDL) that characterize hypobetalipoproteinemia, may prevent acquisition of LCPUFA from the circulation, resulting in a compensatory increase in mammary gland synthesis of saturated fatty acids.

*(v) Infection*

Systemic maternal infection does not affect the macro- or micronutrient content of breast milk (Zavaleta *et al* 1995). Localized breast infection may transiently decrease total fat content of milk (Ramadan, Salah & Eid 1972) , but volume and composition of milk from the unaffected breast is not compromised, with composition returning to normal once dysfunction has resolved (Prentice & Prentice 1984).

*(vi) Atopy*

Breast milk samples from mothers who do not have active atopic dermatitis but whose infants do, reflect previous studies of the plasma fatty acids of infants with atopic dermatitis (Businco *et al* 1993). Thus, SFA, MUFA, LA and αLA were all raised in the breast milk of mothers with atopic children. Conversely, the LCPUFA products (EPA, DPA, DHA for n-3, and DHGLA, AA, GLA for n-6) were all decreased. In addition, breast milk from mothers who themselves were atopic had lower relative amounts of n-6 fatty acids, particularly AA, when compared to non-atopic mothers (Thijs *et al* 2000). However, the ratio of LA to its LCPUFA was comparable regardless of mothers' atopic status, and supplementation with borage oil, a source of LA and GLA, rapidly increased breast milk GLA and DHGLA but not AA in the milk of atopic mothers. This suggests that the putative defect in EFA metabolism, which has been implicated in the aetiology of atopy due to the potential consequences for prostaglandin, interleukin and immunoglobulin E (IgE) regulation, may indeed exist (Thijs *et al* 2000).

*(vii) Exercise*

Aerobic exercise by lactating women does not affect either the volume or composition of breast milk (Dewey *et al* 1994).

## 5.3 RELATIONSHIP BETWEEN INFANT PUFA INTAKE AND DEVELOPMENT

There have been several attempts to define the relationship between dietary PUFA and development in the neonate. Such studies have compared breast with formula fed infants and/or supplemented with standard formulae. However, studies have differed according to whether they have involved preterm or term neonates, the age at which the neonates were studied, and the functional outcome measured. With regards to visual development, both acuity (Carlson *et al* 1993b, Hoffman *et al* 1993, Makrides *et al* 2000b) and attention (Carlson & Werkman 1996, Werkman & Carlson 1996) have been assessed. Other indices of neonatal development have included growth (Carlson *et al* 1992 & 1993a, Makrides *et al* 1999, Martinez *et al* 1999), developmental quotient (DQ) (Agostoni *et al* 1996 & 1997), Bayley scales of mental and/or psychomotor development (Birch *et al* 2000, Makrides *et al* 2000b, Scott *et al* 1998), vocabulary (Scott *et al* 1998), auditory and nerve physiology (Bouglé *et al* 1999), and problem solving (Willatts *et al* 1998a & b).

Few studies have detected differences in developmental outcome between infants fed breast milk, supplemented formulae or standard formulae. In those that have reported a difference in development, optimal outcomes were associated with breast milk and supplemented formulae, particularly in preterm neonates. However, these studies were often incomparable in terms of both the experimental formulae provided and the indices of development measured (Gibson & Makrides 1998 & 2000b, Makrides, Neumann & Gibson 1996b). This has led to much debate regarding the conflicting results (Gibson & Makrides 1999a, Heird, Prager & Anderson 1997, Jørgensen, Lauritzen & Fleischer-Michaelsen 1999). Indeed, this topic has been critically reviewed many times (Carlson 1999, Carlson & Neuringer 1999, Fernstorm 1999, Gibson & Makrides 1999b, Jacobson 1999, Lauritzen *et al* 2001, Neuringer 2000, SanGiovanni *et al* 2000a, Uauy *et al* 1992, 1994 & 1996, Wainwright 1992, Xiang & Zetterström 1999), underlining the uncertainty of the published findings.

Statistical considerations (Gore 1999), sample size (Morley 1998) and the differences in statistical power of such studies (Tolley and Carlson 2000) have led to the recognition of meta-analysis as the most appropriate method of elucidating a

relationship between diet and development. Both visual (SanGiovanni *et al* 2000b) and cognitive development (Anderson, Johnstone & Remley 1999) have been the subject of separate published meta-analyses. Systematic reviews and meta-analyses by the Cochrane Collaboration have been undertaken to assess the benefit of supplementing infant formulae to both preterm (Simmer 2001a) and term (Simmer 2001b) neonates. Both Cochrane reviews concluded that supplementation was safe; although supplementation may increase early visual maturation in preterm neonates (Simmer 2001a), no long term benefit to vision, growth or cognitive development were evident in either preterm (Simmer 2001a) or term (Simmer 2001b) neonates. Supplementation of infant formula does, however, remain at the forefront of nutritional debate (Heird 1999, Koletzko & Sinclair 1999) and has been comprehensively reviewed elsewhere (Forsyth 1998).

It should be noted that many studies of infant and child development compare the developmental indices of dietary groups without including any biochemical indices of fatty acid status. In addition, previous studies have considered the relationship between postnatal diet and development; development in relation to fetal nutrition and has not been assessed. The inter-dependency of maternal and fetal nutrition, and hence the effect of maternal supplementation during pregnancy, has therefore not been addressed when considering infant development, although there is evidence to suggest that maternal ante-natal nutrition influences later visual development (Williams *et al* 2001).

## 5.4 PERSPECTIVE AND CONCLUDING REMARKS

The contribution of maternal diet to maternal status and the subsequent impact of maternal status on that of the fetus is unquestionable. That adequate accumulation of EFA and LCPUFA *in utero* is necessary for the development of fetal tissues is obvious. Postnatal changes in neonatal circulating PUFA levels are determined by status at birth and by postnatal diet in both term (Guesnet *et al* 1999) and preterm (Foreman-van Drongelen *et al* 1995c) infants. The relationship between breast milk PUFA composition and brain growth (Xiang *et al* 2000), and the inadequacy of weaning foods as sources of LCPUFA (Jackson & Gibson 1989) highlight the importance of breast milk as the postnatal source of LCPUFA.

The inter-dependency of maternal and fetal nutrition is thus cause to examine the possibility that manipulation of maternal diet will enhance both maternal and infant PUFA status and provide a means by which to optimise infant development.

Chapter 6

# Study Design

## 6.1. AIMS

The study was designed to provide information that would help to define recommended PUFA intakes and/or supplementation programmes aimed at optimising maternal and fetal DHA status.

## 6.2 HYPOTHESES

The study aimed to test the hypotheses that supplementation of pregnant mothers with DHA:

(1) increases maternal blood, placental tissue, umbilical cord tissue and breast milk DHA,

(2) increases fetal blood DHA,

(3) improves neonatal and infant visual development.

## 6.3 STUDY DESIGN

A randomized, double-blind, placebo-controlled trial was undertaken, in which 100 pregnant women, including 25% expected to undergo caesarean sections, were enrolled at 15 weeks gestation. At this time a (baseline) blood sample was obtained and the women randomized to receive either a fish oil supplement (rich in DHA) or a high oleic acid sunflower oil placebo (50 in each treatment group) (Table 18).

The mothers were asked to continue the supplements until delivery, and further blood samples were obtained from them at 28 weeks gestation and delivery. Samples of umbilical cord blood, cord tissue and placenta were also obtained at delivery. Prior to discharge home, breast milk samples were obtained from breast-feeding mothers.

It had been hoped to obtain maternal adipose tissue in the event of caesarean section. However, the adipose tissue samples obtained were too few in number and insufficient in size to allow satisfactory analysis.

The study thereafter monitored neonatal and infant visual development, using visual evoked potentials (VEP) and electroretinograms (ERG), at birth, two and six months. At subsequent post-partum visits at 2 and 6 months, additional samples of maternal blood, maternal breast milk, and maternal and infant buccal cells were obtained. The postnatal part of the study is not part of this dissertation.

| Age of fetus/infant | 15 wk | 28 wk | Birth† | 2 mo | 6 mo |
|---|---|---|---|---|---|
| Fatty acid Supplements | _____fish oil capsules_____ .....placebo capsules..... | | | | |
| Blood Samples | O | O | O□ | O | O |
| Placenta & Cord | | | ● | | |
| Breast Milk | | | O | O | O |
| Buccal Cells | | | O□ | O□ | O□ |
| VEP | | | □ | □ | □ |
| ERG | | | □ | □ | □ |

† within first week of postnatal life, O mother, □ infant, ● placenta & cord

Table 18. Summary of study protocol.

## 6.4 SAMPLE SIZE CALCULATION

The DHA status of non-pregnant and unsupplemented women within the Glasgow population was found to be generally low and with little variation. In 136 non-pregnant women, mean RBC DHA was 4.57% TFA (SD 0.82) (Berry *et al* 2001). Assuming the same range of values at 15 week gestation, we hypothesised that supplementation of mothers would increase maternal DHA status by 20% of baseline (approximately 1SD), from mean 4.57% TFA to 5.48% TFA.

To detect such a 1SD difference between the two study groups, with significance level (p) of 0.05 and power of 90%, the following calculation was performed (du V Florey 1993):

take $\alpha$ (significance level) as 0.05

take $\beta$ as 0.1 or 10% (power = 100-$\beta$=90%)

take s (standard deviation) as 0.82

take d (difference of interest, equal here to 1SD) as 0.82

If $n = 2(\text{multiplier of } \alpha + \text{multiplier of } \beta)^2 s^2/d$

$= 2(1.96 + 1.28)^2 0.82^2/0.82^2$

$= 2(10.4976)0.6724/0.6724$

$= \frac{20.9952 \times 0.6724}{0.6724}$

$= 20.9952$

Thus, to detect a 1SD difference in RBC DHA as % TFA, the number required in each group at the final end-point of the study (6 months postpartum) was 21.

Assuming a 20% 'drop-out' rate between the time points of 15 weeks, 28 weeks, term, 2 and 6 months (Shepherd 1998), the following calculation was used to determine the initial number required:

| | | |
|---|---|---|
| 15 weeks | 100 - 20% = | 80 |
| 28 weeks | 80 - 20% = | 64 |
| Birth | 64 - 20% = | 51 |
| 2 months | 51 - 20% = | 41 |
| 6 months | 41 ÷ 2 = | 20.5 |

Thus, by recruiting a total of 100 participants at 15 weeks, in each group by 6 months n≡21.

## 6.5 RANDOMIZATION

Randomization of participants to receive either fish oil or placebo capsules was achieved by using a table of random numbers (Table 5.4 from Pocock 1983). The table lists permuted blocks of numbers 0 to 19; numbers 10 to 19 were ignored, to allow randomization in blocks of ten, based on numbers 0 to 9. An arbitrary starting point within the table was identified, and random rows of numbers were used. The required sample size of 100 was divide into blocks of 10, and the random numbers were assigned to each block of ten. A member of departmental staff external to the study assigned treatments on the basis of the random odd or even numbers. Thus, an equal number in each treatment group were obtained per block of ten participants recruited, and treatments were assigned on the basis of random number rather than sequence of recruitment. The randomization code remained unknown throughout the study, until after the samples were collected and analysed.

## 6.6 SUPPLEMENTS

A previous study of non-pregnant women in the Glasgow area revealed that 30% did not consume any fish (Berry *et al* 2001). Of those who did consume fish, the usual intake would be one portion per week, with the most common types consumed being

haddock, cod or canned tuna, all of which contain only 0.1 or 0.2g DHA per 100g of food. Considering that an average portion (MAFF 1993) of cod or haddock is 120g, which would provide 0.24g DHA, and that the average 45-100g portion of canned tuna would provide approximately 0.1g DHA, current DHA intakes in our study population were expected to be low. The total n-3 PUFA from such dietary sources of fish would however approximate to 0.1g/day, equivalent to the average British adult intake of n-3 from fish. Adherence to the recommended intake of one portion of oily fish per week, or supplementation with an equivalent amount of DHA, would therefore at least double the amount of DHA consumed in the study population.

It appeared prudent that, rather than providing an unfeasibly high mega-dose of fish oil/DHA which could be obtained only from consumption of a large number of capsules, the dose provided should be similar to the recommended intake, which can normally be achieved through dietary sources alone without additional supplementation. Thus supplementation by an amount of DHA which could be obtained on consuming one portion of oily fish per week, or a portion of white fish or tuna several times per week, would allow examination of both biochemical and developmental responses to the recommended intake. Hence the rationale on which the dietary n-3 recommendations for the study population of expectant mothers and their infants are based could be considered.

The dose of DHA chosen for supplementation was 200mg per day, an amount previously used in supplementation studies (Makrides *et al* 1996a). Such an amount would at least double the average intake in the study population while providing an amount of DHA obtainable from consumption of fish, including white fish, and fulfilling the DoH recommendations for n-3 LCPUFA intake of 0.2g/day. The fish oil used to obtain this dose was a blended fish oil, Marinol D40, which typically provided 100mg DHA in 323mg of oil per capsule and contained 40% of its fatty acids as DHA (Table 19). Participants consumed two capsules per day. The amount of EPA per capsule (approximately 20mg) was not sufficient to have any pharmacological effect on bleeding time or platelet aggregation (DoH 1994).

The placebo could not contain DHA, EPA, αLA or any other n-3 PUFA which could be converted to DHA. It was also advisable to have low levels of n-6 fatty acids in the

placebo, in order to avoid inducing competition for metabolism between supplementary n-6 and dietary n-3 fatty acids in the placebo group. The placebo used therefore contained sunflower oil with high levels of oleic acid and no significant amounts of LCPUFA (high oleic sunflower oil, 323mg) (Table 19). Participants consumed two capsules per day, the same number of capsules as those receiving fish oil supplements.

Due to the teratogenic effects of high concentrations of vitamin A, the antioxidant used for capsule stability was vitamin E. The presence of gelatine in the capsule shell material was unavoidable, and may have precluded some vegetarian and ethnic mothers in the study population from participating.

Both fish oil and placebo capsules were donated by R.P. Scherer Limited (Swindon, U.K.). Capsule composition was certified by the manufacturer and also analysed by GC-MS using the same methodology as for sample analysis (see Chapter 8 Materials and Methods) (Table 19). The shells of the both the active and placebo capsules were composed of the same material and composition, and weighed approximately 159mg. The capsules were identical in appearance and could not be distinguished on the basis of odour, *etc.* Study participants reported no side effects, such as reflux or after-taste, after capsule consumption.

| | Marinol D40 Active Supplement | | HOSF Placebo | |
|---|---|---|---|---|
| **Fatty Acid (%TFA)** | **Manufacturer's Data** | **GC-MS** | **Manufacturer's Data** | **GC-MS** |
| C14:0 | 3.7 | 5.1 | | 0.0 |
| C16:0 | 6.7 | 10.3 | 4 | 5.2 |
| C16:1n-7 | 4.3 | 4.3 | | 0.2 |
| C18:0 | 2.4 | 3.0 | 5 | 4.7 |
| C18:1n-9 | 15.6 | 10.4 | 81 | 77.6 |
| C18:2n-6 | 1.2 | 1.6 | 7.2 | 11.2 |
| C18:3n-3 | 0.8 | 0.7 | | 0.5 |
| C18:4 | 1.4 | | | |
| C20:0 | 0.1 | 0.2 | | 0.3 |
| C20:1 | 2 | | | |
| C20:5n-3 | 7.2 | 0.0 | | |
| C22:1 | 2.7 | | | |
| C22:5n-6 | 4.1 | 0.6 | | 0.0 |
| C22:6n-3 | 40.4 | 56.9 | | 0.0 |
| Sum of Above | 92.6 | 93.3 | 97.2 | 99.8 |
| Others | 9.4 | 6.72 | 3 | 1.22 |
| Total | 102.0 | 100.0 | 100.2 | 101.0 |

Table 19. Composition of active supplement and placebo capsules, according to certification by the manufacturer and analysis by GC-MS. Marinol D40 – fish oil capsule; HOSF – high oleic sunflower oil.

## 6.7 INCLUSION AND EXCLUSION CRITERIA

Mothers who were expected to deliver their babies at term, including 25% by caesarean section, and planned to feed their babies on breast and/or formula milk were eligible to participate in the study. Mothers with diabetes, twin pregnancies, pre-eclampsic toxaemia (PET), a past history of abruption or postpartum haemorrhage (PPH), allergy to fish products, a thrombophilic tendency or who were receiving drugs that affect thrombocyte function (non-steroidal anti-inflammatory drugs/NSAID *e.g.* aspirin, ibuprofen) were excluded from the study. Samples collected at delivery from pregnancies that concluded prematurely before 36 weeks, or in which the neonate had an Apgar score of less than 7 at 10 minutes, weight below the 3$^{rd}$ centile for gestational age, visual, medical or developmental problems were not included in the final analysis of results.

## 6.8 ETHICAL APPROVAL

Prior to commencement of the study, approval was obtained from both Glasgow University Ethics Committee and Yorkhill Research Ethics Committee.

## 6.9 STUDY IDENTIFICATION

In order to make the study identifiable to both hospital staff and participants amongst the many concurrent studies within the hospital, the study was given a distinct identity. The study was known as the FOMI study (FOMI – Fish Oils for Mothers and Infants), and could be identified by its distinctive logo used on all letter headings, leaflets and posters.

## 6.10 MEDICAL AND NURSING STAFF

Hospital staff involved in the care of the study population, including consultant obstetricians, antenatal clinic midwives and administrative staff, labour suite and postnatal ward staff were advised of the study. It was particularly necessary to liase with staff to facilitate efficient enrolment with minimum disruption to the clinic routine, and to ensure that the required samples were obtained at the time of delivery.

## 6.11 STUDY PARTICIPATION

Leaflets (Appendix 1 Introductory Leaflet) introducing the study were distributed to all mothers enrolling (booking) at approximately 10 weeks gestation at the antenatal clinic of the Queen Mother's Maternity Hospital, Glasgow, Scotland. Posters advertising the study were also displayed in the clinic area. The next routine visit to the antenatal clinic was generally scheduled for 28 weeks gestation, but 75% of mothers attended at approximately 15 weeks gestation for an α-fetoprotein (AFP) screening test.

At their AFP appointment, suitable participants were identified from their medical case notes and were invited to participate in the study. At this time, the study was explained in full detail; if a mother indicated her desire to participate, informed consent was obtained (Appendix 2 Consent Form). The participant was provided with a bottle of capsules, randomized according to enrolment order, and a leaflet with advice on capsule consumption (Appendix 3 Advice Leaflet). The medical case notes of the participant were labelled and details of the study added (Appendix 4 Case Note Consent). A sample of blood was obtained when the participant underwent venepuncture for her AFP test.

After enrolment a letter was sent to the participant acknowledging her involvement. Both the hospital consultant and GP of the participant were notified of her participation. Prior to her 28 weeks clinic appointment, the participant was contacted by telephone. If wishing to withdraw from the study, the participant was sent a letter, her consultant and GP notified, and her medical case notes amended. If the participant was continuing in the study, it was arranged that she would be seen at either the hospital clinic or her GP surgery to assess her compliance and diet, and to provide her with a second bottle of capsules. The required blood sample was obtained at the time of routine venepuncture.

Before her estimated delivery date (EDD), the participant was telephoned to determine her compliance with capsule consumption and her wish to continue or withdraw from the study. Samples were obtained at delivery in labour suite. Mother and baby were subsequently visited in the postnatal wards prior to discharge home, and details regarding the baby obtained from medical notes and mother.

The participants received no financial incentives to participate or continue in the study, although gifts were given to each infant at 2 and 6 months and travelling expenses for the postpartum visits were provided.

### 6.12 DIET AND LIVING HABITS OF PARTICIPANTS

Details regarding the participant's dietary intake of fish, smoking and exercise patterns, and alcohol consumption were collected using a questionnaire (Appendix 5). Although the application of food frequency questionnaires in assessing long-term diet and overall eating patterns is complex (Hu *et al* 1999, Tseng 1999, Willett *et al* 1985, Willett *et al* 1988), they are useful in ranking individuals (Schaefer & Augustin 2000). The information required for this study *i.e.* type, amount and overall frequency of fish consumption, was easily and best attainable by this method (Hjartåker, Lund & Bjerve 1997). Quantification of the portion size of fish consumed was achieved using appropriate material from the Food Atlas (Nelson, Atkinson & Meyer 1997).

## 6.13 ACKNOWLEDGEMENT OF STUDY PARTICIPANTS AND HOSPITAL STAFF

Women taking part in the study did so of their own volition, and at a time of particular importance to them. It is important to acknowledge that for many women, pregnancy may not be regarded as an easy time to participate in clinical studies (Mohanna & Tunna 1999). It is therefore appropriate that the mothers who consented to consuming the study capsules and allowing sample collection are referred to not as subjects, but as participants, in the fullest sense of the word (Boynton 1998, Chalmers 1999, Jackson 1999).

The participants have understandably expressed their interest in the findings of the study, and will be informed of the conclusions as promptly as possible. The results of the study will also be disseminated to all the medical and nursing departments aware of and of assistance to the study.

Chapter 7

# Study Participants

## 7.1 ENROLMENT

100 women were invited to take part in the study when attending the ante-natal clinic for an elective AFP test at approximately 15 weeks gestation (mean 15+4 weeks/109 days, SD 3 days, range 14+6/104 days to 17+0/119 days, Table 20). This time point was chosen to allow supplementation during the second and third trimesters, and also because it was the most convenient of the mothers' clinic visits.

| | 15 weeks | | 28 weeks | | Delivery | |
|---|---|---|---|---|---|---|
| | Fish Oil Group | Placebo Group | Fish Oil Group | Placebo Group | Fish Oil Group | Placebo Group |
| n | 50 | 50 | 33 | 36 | 31 | 31 |
| Mean | 110 (15+5) | 109 (15+4) | 199 (28+3) | 198 (28+2) | 280 (40+0) | 281 (40+1) |
| SD | 4 | 2 | 5 | 4 | 10 (1+3) | 9 (1+2) |
| Min | 104 (14+6) | 105 (15+0) | 190 (27+1) | 183 (26+1) | 254 (36+2) | 256 (36+4) |
| Max | 119 (17+0) | 115 (16+2) | 217 (31+0) | 209 (29+6) | 295 (42+1) | 292 (41+5) |
| Median | 110 (15+5) | 109 (15+4) | 198 (28+2) | 198 (28+2) | 281 (40+1) | 281 (40+5) |
| Mode | 109 (15+4) | 109 (15+4) | 195 (27+6) | 200 (28+4) | 285 (40+5) | 287 (41+0) |

Table 20. Time of antenatal sampling and delivery in days (weeks + days) after conception for all those enrolled who continued to take part in the study.

## 7.2 WITHDRAWALS AND EXCLUSIONS

Of the 100 original participants, 29 withdrew from the study before their next routine ante-natal clinic appointment (Table 21) at approximately 28 weeks gestation (mean 28+2 weeks/198 days, SD 5 days, range 26+1/183 days to 31+0/217 days, Table 20). A further seven participants withdrew between 28 weeks gestation and birth, and two infants in the placebo group were excluded following delivery (Table 21): one was delivered prematurely (34+5 weeks/243 days) and one was below the 3rd centile for weight for gestational age (0.57th centile).

| | Number of Participants | | | Number Withdrawn | | | Exclusions | | |
|---|---|---|---|---|---|---|---|---|---|
| Gestation | Whole Study Sample | Fish Oil Group | Placebo Group | Whole Study Sample | Fish Oil Group | Placebo Group | Whole Study Sample | Fish Oil Group | Placebo Group |
| 15 weeks | 100 | 50 | 50 | | | | 0 | 0 | 0 |
| 28 weeks | 71 | 35 | 36 | 29 | 15 | 14 | 0 | 0 | 0 |
| Birth | 62 | 31 | 31 | 7 | 4 | 3 | 2 | 0 | 2 |

Table 21. Numbers and withdrawal rates in study sample and treatment groups.

## 7.3 MATERNAL CHARACTERISTICS

On enrolment, information on previous obstetric history was recorded, and parity defined as one or more pregnancies, regardless of outcome. 42 women were nulliparous; of the 58 parous women, the mean time-period between their last previous pregnancy and the current (study) pregnancy was 3.45 years (SD 3.1, range 0.5 to 11 years, Table 22). All pregnancies were singleton and 13 participants were expected to deliver by caesarean section. There were no significant differences between fish oil and placebo groups in terms of numbers of parous women (chi-square 1.478, $p>0.2$), mean number of years between previous and current pregnancies (t-test, $p=0.2$), or expected delivery mode (chi-square 0.796, $p>0.2$) (Table 23).

| | Whole Study Sample | Fish Oil Group | Placebo Group |
|---|---|---|---|
| Nulliparous | 42 | 18 | 24 |
| Parous | 58 | 32 | 26 |
| Interval between last and current pregnancies (Years, mean (SD)) | 3.5 (2.9) (n=50) | 3.9 (3.2) (n=27) | 2.9 (2.5) (n=23) |

Table 22. Obstetric history of whole study sample and both treatment groups. Parity was defined as one or more pregnancies, regardless of duration or outcome.

| | Whole Study Sample | Fish Oil Group | Placebo Group |
|---|---|---|---|
| Expected Delivery Mode (n=100) | | | |
| SVD | 87 (87%) | 45 (90%) | 42 (84%) |
| Caesarean section | 13 (13%) | 5 (5%) | 8 (16%) |
| Actual Labour (n=62, 31 in each group) | | | |
| Spontaneous | 49 (79%) | 24 (77%) | 25 (81%) |
| Induced | 13 (21%) | 7 (23%) | 6 (19%) |
| Actual Delivery Mode (n=62, 31 in each group) | | | |
| SVD | 35 (56%) | 19 (61%) | 16 (52%) |
| Assisted | 11 (18%) | 3 (10%) | 8 (26%) |
| Caesarean section | 16 (26%) | 9 (29%) | 7 (22%) |

Table 23. Expected and actual modes of delivery for the study participants. Absolute numbers and percentages are shown. SVD - spontaneous vaginal delivery. Assisted delivery describes the use of forceps (low- or mid-cavity, rotational) or ventouse extraction.

The height and weight of participants were recorded from their medical notes. The height and weight of all mothers attending the antenatal clinic were recorded at the time of initial clinic booking, at approximately 10 weeks gestation. Because data on participants' pre-pregnancy weight relied on their own self-report, the weight recorded by nursing staff at time of booking was recorded. There were no significant

differences in maternal height (t-test, p=0.36) or weight (t-test, p=0.98) between fish oil and placebo groups (Table 24).

| | Height (m) | | | Weight at Booking (kg) | | |
|---|---|---|---|---|---|---|
| | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** |
| Mean | 1.66 | 1.65 | 1.66 | 68.12 | 68.16 | 68.09 |
| SD | 0.07 | 0.08 | 0.06 | 11.64 | 12.55 | 10.79 |
| Min | 1.51 | 1.51 | 1.52 | 44.70 | 44.70 | 52.00 |
| Max | 1.85 | 1.85 | 1.77 | 103.40 | 103.40 | 99.50 |
| Median | 1.67 | 1.66 | 1.67 | 66.35 | 66.35 | 66.35 |
| Mode | 1.67 | 1.63 | 1.67 | 58.00 | 58.00 | 62.20 |

Table 24. Heights and weights of study participants, as recorded by nursing staff at time of booking (approx. 10 weeks gestation).

## 7.4 FISH INTAKE, ALCOHOL AND SMOKING

Participants completed a detailed questionnaire (Appendix 5) regarding diet, exercise, smoking and alcohol intake. Qualitative dietary information regarding types of fish, cooking oil and spreading fats was collected using a food frequency questionnaire, with quantitative data regarding portion sizes collected using appropriate material from the Food Atlas (Nelson *et al* 1997). Frequency and type of fish consumed was also assessed at 28 weeks gestation and delivery (Table 25). At each time point, participants were asked to report any fish consumed within the 24-36 hours prior to sample collection. There were no significant differences in frequency of fish intake at 15 weeks (chi-square 0.077, p>0.2), 28 weeks (chi-square 1.066, p>0.2) or delivery (chi-square 1.939, p>0.2) between fish oil and placebo groups.

| Gestation | 15 weeks | | | 28 weeks | | | Delivery | | |
|---|---|---|---|---|---|---|---|---|---|
| **Frequency of Fish Intake** | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** | **Whole Study Sample (n=71)** | **Fish Oil Group (n=35)** | **Placebo Group (n=36)** | **Whole Study Sample (n=56)** | **Fish Oil Group (n=29)** | **Placebo Group (n=27)** |
| Never | 5 (5%) | 2 (4%) | 3 (6%) | 1 (1%) | 0 (0%) | 1 (3%) | 1 (2%) | 0 (0%) | 1 (4%) |
| <Once per week | 43 (43%) | 22 (44%) | 21 (42%) | 25 (35%) | 14 (40%) | 11 (31%) | 18 (32%) | 12 (41%) | 6 (22%) |
| Once per week | 27 (27%) | 13 (26%) | 14 (28%) | 27 (38%) | 14 (40%) | 13 (36%) | 13 (23%) | 5 (17%) | 8 (30%) |
| Twice or more per week | 25 (25%) | 13 (26%) | 12 (24%) | 18 (25%) | 7 (20%) | 11 (31%) | 24 (43%) | 12 (41%) | 12 (44%) |

Table 25. Frequency of fish consumption, in the whole study sample and in the randomized treatment groups, during gestation and at time of delivery. Absolute numbers and percentages are shown.

Although quantitative information regarding exercise, alcohol and smoking was collected, for the purposes of statistical analysis, this information was categorised into Yes/No (Table 26). There were no significant differences in the numbers of alcohol consumers (chi-square 0.045, $p>0.2$), smokers (chi-square 0.065, $p>0.2$) or those undertaking exercise (chi-square 0.364, $p>0.2$) between fish oil and placebo groups.

| | Alcohol | | | Smoking | | | Exercise | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** | **Whole Study Sample (n=100)** | **Fish Oil Group (n=50)** | **Placebo Group (n=50)** |
| No | 67 | 34 | 33 | 81 | 40 | 41 | 45 | 21 | 24 |
| Yes | 33 | 16 | 17 | 19 | 10 | 9 | 55 | 29 | 26 |

Table 26. Lifestyle characteristics of study participants. Alcohol consumption during pregnancy was defined as 0 units per week - no, 0.5 or more units per week - yes; smoking during pregnancy was defined as 0 cigarettes per day - no, 1 or more cigarettes per day - yes; exercise was defined as 0 hours per week - no, 0.5 or more hours per week - yes.

Participants were also asked to record any other dietary supplements taken at any time during gestation (Table 27). Information regarding the type of supplement consumed was recorded. However, since quantitative data regarding frequency of supplement consumption relied on the participants' self-report, supplement consumption was

categorised depending on type, regardless of dosage and frequency. There were no significant differences between fish oil and placebo groups in the numbers of those consuming other supplements (chi-square 0.065, p=0.799).

| | Whole Study Sample (n=100) | Fish Oil Group (n=50) | Placebo Group (n=50) |
|---|---|---|---|
| None | 81 | 41 | 40 |
| Vitamin/ Herbal | 15 | 7 | 8 |
| Evening Primrose Oil | 2 | 2 | 0 |
| Fish Oil/ Cod Liver Oil | 5 | 2 | 3 |

Table 27. Numbers in study sample consuming supplements other than those given in study. NB. One woman in the placebo group took both vitamin and other fish oil supplements, one woman in the fish oil group took both evening primrose oil and herbal supplements, and one woman in the fish oil group took both evening primrose oil and cod liver oil supplements.

## 7.5 SOCIO-ECONOMIC STATUS

Socio-economic status was measured using the Carstairs score (Carstairs and Morris, 1990) (Table 28). This score is based on Scottish residential postcodes and categorises the deprivation of areas based on four standard census variables believed to represent material disadvantage – male unemployment, no car, overcrowded housing and low social class (class IV or V, semi- or unskilled occupation). A Z-score is available for each postcode sector area, and these values can be ranked into Deprivation Categories (DEPCAT) on a scale of 1 to 7, with 1 the most affluent and comparable to social class I, and 7 the most deprived, comparable to social class V.

The Carstairs score and deprivation categories used here are based on the 1991 census (Greater Glasgow Health Board). The distribution of DEPCAT scores within the study sample was not significantly different to that within Glasgow (chi-square 12.137, p=0.06), therefore the study sample can be considered as representative of the socio-economic status of the population of Glasgow. Fish oil and placebo groups were not significantly different (chi-square 1.796, p>0.2) when DEPCAT scores were considered in categories of affluent (DEPCAT 1 and 2), middle (DEPCAT 3 and 4) and deprived (DEPCAT 5, 6 and 7).

| DEPCAT Score | Glasgow Postcode Sectors with DEPCAT Score (n=151) | Whole Study Sample (n=100) | Fish Oil Group (n=50) | Placebo Group (n=50) |
|---|---|---|---|---|
| 1 | 14 (9%) | 14 (14%) | 6 (12%) | 8 (16%) |
| 2 | 14 (9%) | 18 (18%) | 7 (14%) | 11 (22%) |
| 3 | 13 (9%) | 8 (8%) | 3 (6%) | 5 (10%) |
| 4 | 21 (14%) | 20 (20%) | 13 (26%) | 7 (14%) |
| 5 | 11 (7%) | 4 (4%) | 3 (6%) | 1 (2%) |
| 6 | 35 (23%) | 21 (21%) | 10 (20%) | 11 (22%) |
| 7 | 43 (29%) | 15 (15%) | 8 (16%) | 7 (14%) |

Table 28. Socio-economic status of study sample. DEPCAT Score 1 - most affluent, 7 - least affluent.

## 7.6 COMPLIANCE WITH CAPSULE CONSUMPTION

At 15 and 28 weeks gestation, participants were given a bottle containing 200 capsules. Compliance in consumption of study capsules was assessed by asking participants to return these bottles at the following study time-point (28 weeks gestation and delivery, respectively). The number of capsules and time period over which they were consumed were determined, indicating the dosage obtained (Cleland *et al* 1992).

## 7.7 DELIVERY

Of the 62 women remaining in the study at the time of delivery, 13 (21%) underwent inducement of labour. In total, 35 (56%) delivered by SVD, 11 (18%) had an assisted delivery, and 16 (26%) had a caesarean section (5 elective, 11 emergency) (Table 23). Mean gestation was 40 weeks/280 days (SD 1+2 weeks/9 days, range 36+2 weeks/254 days to 42+1 weeks/295 days, Table 20). There were no significant differences in gestational length (t-test, $p=0.8$), type of labour (chi-square 0.097, $p>0.2$) or actual delivery mode (chi-square 2.78, $p>0.2$) between fish oil and placebo groups.

## 7.8 INFANT CHARACTERISTICS

All infants had an Apgar score of 7 or more at ten minutes after birth. Five infants (three in fish oil group, two in placebo group) required phototherapy for neonatal jaundice; four infants (two in each treatment group) required additional non-urgent medical attention (*e.g.* antibiotics).

There were no significant differences in the sexes of the infants (chi-square 1.042, $p>0.2$) or rate of breast-feeding between the fish oil and placebo groups (chi-square 0.295, $p>0.2$) (Table 29).

| | Whole Study Sample (n=62) | Fish Oil Group (n=31) | Placebo Group (n=31) |
|---|---|---|---|
| Gender | | | |
| Male | 28 | 16 | 12 |
| Female | 34 | 15 | 19 |
| Feeding | | | |
| Exclusive Breast Milk | 41 | 19 | 22 |
| Exclusive Formula Milk | 20 | 11 | 9 |
| Breast + Formula Milk | 1 | 1 | 0 |

Table 29. Characteristics of infants. Mode of feeding was recorded prior to discharge. N.B. The infant receiving both breast and formula milks received only 70ml of formula within the first 24 hours after delivery, then continued to be exclusively breast-fed. For the purposes of statistical analysis, this infant was considered as breast-fed.

## 7.9 INFANT ANTHROPOMETRY

Measurements of infant anthropometry (weight, length and occipital frontal circumference (OFC)), recorded within the first 24 hours following delivery, were obtained from medical case notes. For these indices, standard deviation scores (SDS) and centiles compared to British 1990 growth reference data were obtained using the British 1990 Growth Reference Programme (Child Growth Foundation, 1996). There were no significant differences in actual infant birth weight (t-test, $p=0.22$), length (t-test, $p=0.12$) or OFC (t-test, $p=0.74$) between fish oil and placebo groups (Table 30). There were no differences between groups in SDS for infant weight (t-test, $p=0.17$), length (t-test, $p=0.089$) or OFC (t-test, $p=0.74$) (Table 30).

| | | Weight (g) | | | Length (cm) | | | OFC (cm) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Whole Study Sample (n=62) | Fish Oil Group (n=31) | Placebo Group (n=31) | Whole Study Sample (n=57) | Fish Oil Group (n=27) | Placebo Group (n=30) | Whole Study Sample (n=61) | Fish Oil Group (n=30) | Placebo Group (n=31) |
| Measurement | | | | | | | | | | |
| | Mean | 3603 | 3529 | 3677 | 53 | 53 | 54 | 35 | 35 | 35 |
| | SD | 473 | 531 | 403 | 2 | 3 | 2 | 1 | 1.4 | 1.5 |
| | Min | 2800 | 2280 | 2760 | 47 | 47 | 49 | 31 | 31 | 31 |
| | Max | 4550 | 4550 | 4370 | 58 | 58 | 57 | 38 | 38 | 38 |
| | Median | 3600 | 3500 | 3700 | 53 | 53 | 54 | 35 | 35 | 35 |
| | Mode | 3840 | 2850 | 3840 | 55 | 54 | 55 | 35 | 35 | 35 |
| SDS | | | | | | | | | | |
| | Mean | 0.3 | 0.1 | 0.4 | 1.3 | 1.1 | 1.6 | 0.0 | -0.1 | 0.1 |
| | SD | 1.0 | 1.0 | 0.9 | 1.2 | 1.3 | 1.1 | 1.1 | 0.9 | 1.3 |
| | Min | -1.9 | -1.9 | -1.7 | -2.5 | -2.5 | -1.7 | -2.8 | -2.8 | -2.5 |
| | Max | 1.9 | 1.6 | 1.9 | 3.7 | 3.6 | 3.7 | 2.6 | 1.5 | 2.6 |
| | Median | 0.3 | 0.2 | 0.4 | 1.4 | 1.2 | 1.7 | 0.1 | 0.1 | 0.2 |
| | Mode | 1.2 | | | 2.8 | | 2.9 | 0.31 | | -0.4 |
| Centile | | | | | | | | | | |
| | Min | 3 | 3 | 4 | 0.6 | 0.6 | 5 | 0.3 | 0.3 | 0.6 |
| | Max | 97 | 95 | 97 | 100 | 100 | 100 | 100 | 93 | 100 |

Table 30. Infant anthropometry. OFC - occipital frontal circumference; SDS - standard deviation score.

## 7.10 BLOOD, TISSUE AND BREAST MILK SAMPLES

Antenatal maternal blood samples were collected at enrolment (n=97) and 28 weeks gestation (n=62) (Table 31). Maternal blood samples (n=59) were obtained within (mean) 20 hours of delivery (SD 31 hours, range 2 hours prior to delivery to 96 hours following delivery). 73% of maternal blood samples were obtained within 24 hours of delivery; 85% were obtained within 48 hours. Analytical problems occurred for three samples of maternal red blood cells (RBC), due to insufficient sample volume (less than 1ml RBC). These samples were obtained from separate individuals: (1) a mother in the fish oil group at 15 weeks gestation; (2) a mother in the fish oil group at delivery and (3) a mother in the placebo group at delivery. These RBC samples were excluded from the final statistical analyses; their corresponding plasma samples were of sufficient volume and were included in the analyses.

Placenta (n=53), umbilical cord tissue (n=53) and umbilical cord blood (n=56) were obtained within 2 hours of delivery. Breast milk samples (n=26) were obtained within 1 to 7 days (mean 3, SD 1) following delivery: 85% of breast milk samples were obtained within the first 4 days following delivery.

Those individuals from whom samples were obtained were representative of their treatment group and the whole study sample at each time point. There were no significant differences between fish oil and placebo groups in terms of time of maternal blood sample (t-test, p=0.35) or breast milk sample (t-test, p=0.14) following delivery.

| | | Number of Samples | | | % of Total Possible | | |
|---|---|---|---|---|---|---|---|
| **Gestation** | **Sample** | **Whole Study Sample** | **Fish Oil Group** | **Placebo Group** | **Whole Study Sample** | **Fish Oil Group** | **Placebo Group** |
| 15 weeks | Maternal RBC | 96/100 | 47/50 | 49/50 | 96 | 94 | 98 |
| | Maternal plasma | 97/100 | 48/50 | 49/50 | 97 | 96 | 98 |
| 28 weeks | Maternal RBC | 63/71 | 30/35 | 33/36 | 89 | 86 | 92 |
| | Maternal plasma | 63/71 | 30/35 | 33/36 | 89 | 86 | 92 |
| Birth | Maternal RBC | 57/62 | 29/31 | 28/31 | 92 | 94 | 90 |
| | Maternal plasma | 59/62 | 30/31 | 29/31 | 97 | 97 | 94 |
| | Umbilical cord RBC | 56/62 | 27/31 | 29/31 | 95 | 87 | 91 |
| | Umbilical cord plasma | 56/62 | 27/31 | 29/31 | 95 | 87 | 91 |
| | Placenta tissue | 53/62 | 26/31 | 27/31 | 86 | 87 | 85 |
| | Umbilical cord tissue | 53/62 | 26/31 | 27/31 | 84 | 84 | 85 |
| | Adipose tissue | 5/16 | 4/9 | 1/7 | 31 | 44 | 14 |
| | Breast milk | 26/42 | 12/20 | 14/22 | 63 | 60 | 65 |

Table 31. Numbers of samples obtained at each time point from the whole study sample and in each group. Expressed as absolute numbers, and as a percentage of the number possible in each group. RBC - red blood cells.

Chapter 8

# Materials and Methods

## 8.1 METHODS OF FATTY ACID ANALYSIS

### 8.1.1 Introduction

Some lipids are easily accessible for analysis, for example in samples of oils, which are by definition liquid at room temperature. In contrast, lipids in biological materials, such as human fluids and tissues, are constituents of heterogeneous samples, contained within cells and structures, amid proteins and organelles. Thus, it is necessary to separate and *extract* these integral lipids free from the non-lipid substances present in the tissue. The first stage in fatty acid analysis is therefore the extraction process, in which lipids are obtained with minimum contamination by other substances.

Unless the lipid is present in the tissue as a particular type (*e.g.* in breast milk and adipose tissue, lipid is present mainly in triacylglycerol form; in red blood cells/RBC phospholipids are the predominant lipid class), the extracted lipid will contain all types of lipid. Classes of lipid (triacylglycerols, non-esterified fatty acids, phospholipids) can be distinguished and separated from the mass of total lipids by various chromatographic methods, *e.g.* thin layer chromatography (TLC) or preparative high-performance liquid chromatography (HPLC).

Once the total or required class of lipids have been isolated, the fatty acids must be in a suitable form for analysis. Current methods of fatty acid analysis employ gas chromatography (GC) to separate, coupled with either a flame ionisation detector (FID) or a mass spectrometer (MS) to detect individual fatty acids. These methods require the fatty acids to be in the form of a volatile derivative, *e.g.* methyl esters (fatty acid methyl esters/FAME). Fatty acids must therefore undergo methylation to produce their respective FAME. Since FAME are *derived* from fatty acids, the conversion of fatty acids to FAME is termed the derivatization procedure.

This chapter reviews the processes of lipid extraction, FAME derivatization, and subsequent analysis by GC-MS. The validation and details of the actual methodology used are described.

### 8.1.2 Lipid Extraction

The use of chloroform and methanol in lipid extraction is widely documented, and the nature and dynamics of their interaction well understood (Bligh & Dyer 1959). Folch

*et al* (1951) originally developed a method of isolating lipids from brain tissue by homogenizing the tissue in a 2:1 chloroform:methanol (v/v) mixture, followed by purification of lipid matter free from non-lipid substances by water. Folch, Lees and Sloane Stanley (1957) later refined this technique with the addition of mineral salts to aid the removal of non-lipid contaminants without incurring loss of lipids. Fatty acids are of an amphipathic nature, having both a hydrophobic hydrocarbon chain and a hydrophilic carboxyl group (COOH) that dissociates at pH 4.5 ($COO^-$). In long chain fatty acids, the hydrophobic element predominates, causing the fatty acids to be insoluble in water, a polar solvent. Chloroform and methanol are solvents that dissolve ionic or polar solutes. Fatty acids, including those of long chain length, are soluble in chloroform, while other cellular components are precipitated by methanol. The addition of chloroform and methanol to a biological sample extracts the lipids by the formation of a biphasic solution, consisting of an upper aqueous/methanol layer, and a lower layer of chloroform containing lipids. Upon removal of the top layer, addition of a salt solution aids removal of residual non-lipid contaminants, methanol and water, and encourages the polar lipids into the non-polar solvent phase. The solution again separates into layers, and a lower layer of chloroform containing lipids can be retrieved.

The method of Folch *et al* (1957) has been widely adopted and modified (Bligh & Dyer 1959, Dodge & Phillips 1967). Indeed, "a modified Folch extraction is now the method of choice when total lipids are needed" (Jensen 1989a). A modified Folch extraction consists of mixing the sample with chloroform: methanol (2:1, v/v), followed by centrifugation to separate the phases, with removal of the top layer, and washing the bottom layer with dilute aqueous salt solution to remove non-lipid components. The use of potassium chloride (KCl) at an appropriate concentration has been established (Folch *et al* 1957, Dodge & Phillips 1967). The inclusion of antioxidants within the solvents prevents fatty acid autooxidation, and aids the reproducibility of analyses (Dodge & Phillips 1967).

Once the chloroform/lipid layer has been obtained, the amount of lipid must be quantified, to facilitate analysis of a defined weight of lipid. This requires evaporation of the chloroform solvent in order to concentrate the lipid mass. Methods for the removal of the chloroform include the use of heat under a stream of nitrogen

(containing less than 5mg/kg of oxygen) (International Union of Pure and Applied Chemistry 1977a), a vacuum centrifuge and a rotary vacuum evaporator (Dodge & Phillips 1967). The dry weight of the lipid can be calculated by performing the evaporation process in previously weighed glassware. The weight of lipid recovered is thus the difference between the initial weight of the empty vial, and the subsequent weight with the dried sample.

### 8.1.3 Derivatization

Polar groups, such as the COOH group of fatty acids, can only be applied directly to gas chromatography columns if they are volatile (C<14). In order to make long chain fatty acids volatile, the polar groups can be derivatized by means of methylation, producing FAME that can be resolved by GC.

Several agents have been used for the methylation of fatty acids, and their application in the derivatization of all lipid classes documented (Eder, Reichlmayr-Lais & Kirchgessner 1992). Most commonly, methylation is catalysed by boron trifluoride-methanol, methanolic sulphuric or hydrochloric acid, and methanolic sodium methoxide. Boron trifluoride-methanol may have some limitations in the derivatization of certain phospholipids (Eder *et al* 1992), and may incur loss of short and medium chain fatty acids, as well as the production of artefacts (Kohn *et al* 1996). Sodium methoxide is efficient even at ambient temperature (Alexander & Justice Jr. 1985, Eder *et al* 1992), but its use is limited when appreciable amounts of free fatty acids are present in the sample, such as in human breast milk (Kohn *et al* 1996).

Anhydrous methanolic hydrochloric acid (methanolic HCl, MeOH/HCl) is an established catalyst (International Union of Pure and Applied Chemistry 1977a) and has wide application in methylation catalysis, provided conditions are suitably anhydrous to ensure the solubilization and esterification of even non-polar lipids such as triacylglycerols. The presence of an additional solvent aids solubilization of non-polar lipids, facilitating esterification by methanolic HCl (Kohn *et al* 1996).

The relative levels of saturated, C18 monounsaturated, and long chain PUFA appear to be stable regardless of the derivatization agent, and results obtained using the various agents should be comparable (Kohn *et al* 1996). However, the use of methanolic HCl

when absolute quantification of fatty acids is required appears preferable (Kohn *et al* 1996). Although methanolic hydrochloric acid and methanolic sulphuric acid are of similar efficacy (Eder *et al* 1992), the use of methanolic HCl has already been established in this laboratory (Farquharson *et al* 1996).

To facilitate esterification, temperature and time conditions must be appropriate. Several studies have investigated the optimal conditions; only sodium methoxide will efficiently esterify at room temperature, while boron trifluoride and methanolic HCl require heating. Heating is traditionally performed using heating blocks, although alternatives such as microwave oven irradiation (Jeyashoke, Krisnangkura & Chen 1998) may now be used.

### 8.1.4 Gas Chromatography

Once the fatty acids are in a suitable form for analysis, they must be separated into their distinct molecular species in order to be identified and quantified. Chromatography is a separation technique dating back to the ancient times of Pliny the Elder, and was developed in the 1940s through the work of 1952 Nobel Prize (Chemistry) Laureates A.J.P. Martin and R.L.M. Synge. It separates the compounds or analytes (in this case, fatty acids) in a solute (mixture of compounds to be separated) based upon their partition or distribution between two physically distinguishable phases. These phases are a mobile phase and a stationary phase, which provide a driving force and retardation effects respectively. The ratio of the concentration of the solute in the mobile phase to that in the stationary phase is called the partition coefficient. The analytes separate because their relative distribution between the two phases differ. The development and application of chromatography is widely covered by major biochemistry textbooks (Fowlis 1995, Freifelder 1982, Wilson 1994).

The stationary phase or sorbent may be in the form of a solid, gel or immobilized liquid held stationary by attachment to a solid support or matrix. When the stationary phase is contained within a tubular system (*i.e.* column), the type of chromatography is called column chromatography. The mobile phase may be in a liquid or gaseous form. In column chromatography, a mixture of the mobile phase and solute passes through the column.

One form of column chromatography is gas chromatography. In gas chromatography, the stationary phase is either a solid or a liquid, and the mobile phase is a gas (carrier gas). In gas liquid chromatography (GLC), the column (usually glass) is packed with an inert support coated with a liquid phase (therefore it is also known as packed-column gas chromatography).

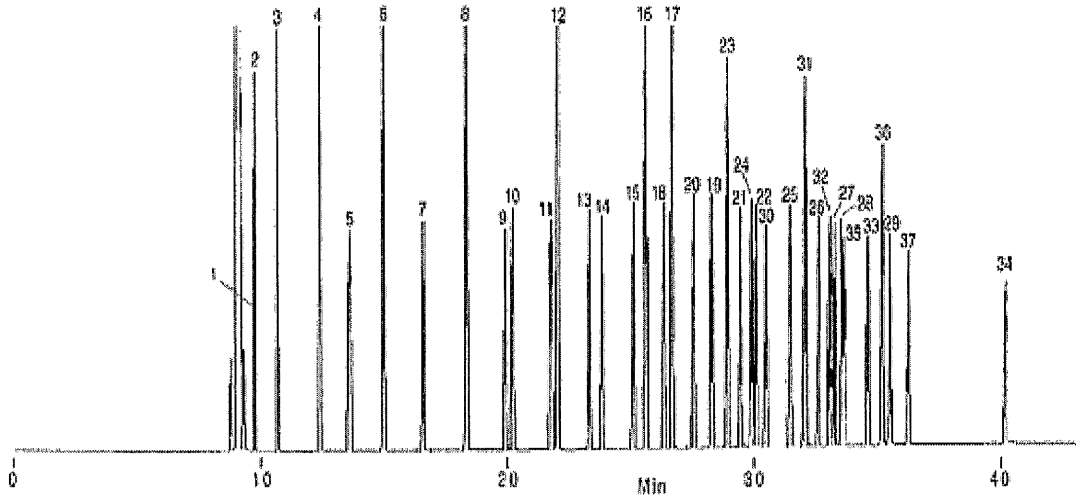
In capillary gas chromatography (GC), the column is usually made from fused silica, with the stationary phase bonded directly to the silica column (thus there is technically no support or liquid). The silica tube is thin (*i.e.* a capillary), with an external diameter of 0.2-0.8mm and an internal diameter (id) of 0.05-0.5mm (most commonly 0.25mm or 0.32mm id). The length of the capillary column ranges up to 100m, with 15-30m most typical. The outer surface of the column is protected with a coating material, usually polyimide. The stationary phase bonded to the inside of the capillary forms a film, the thickness of which (film thickness) affects the ratio of the stationary to mobile phases, and hence analyte separation. Film thickness varies from 0.1-1.0μm. Thermally stable polymers in liquid or gel form are used as the stationary phase; the most common materials are polysiloxanes (non-polar) and polyethylene glycols (polar). Phases of varying polarity are available, with a polar phase required for FAME chromatography.

In comparison to GLC, GC offers greater resolution and increased sensitivity, creating the advantage of a higher degree of precision, at a higher speed. In addition, the fused silica of GC columns is more robust than the glass of GLC columns.

In GC, the sample is injected as a solution of (volatile) analytes in (volatile) solvent. To allow the analytes to be present in the gas phase, they must be in a volatile state prior to injection, hence the requirement for long chain fatty acids to be in forms such as methyl esters (FAME). The carrier gas must be dry and free of oxygen, and is usually nitrogen, hydrogen or helium. On injection, the solute enters a chamber, which is heated independently from the column to ensure vaporisation, usually to 150-250°C, 20-30°C greater than the maximum oven temperature. The injection system found on a capillary GC operates in split/splitless mode. In split mode, only part of the vaporised mixture of carrier gas and solute enters the column, with the remainder

being diverted to waste via a valve (split valve). The ratio of the amount diverted to the amount entering the column is called the split ratio, and is typically 50:1 or 100:1. Thus, in split mode, a small but representative sample of the solute is chromatographed and analysed. In splitless mode, the split valve is closed to ensure that the entire sample enters the column; this achieves greater sensitivity.

Once the vaporised solute reaches the column, the analytes redistribute or partition between the gaseous mobile and the stationary phase according to the partition coefficient of their molecular species. The column is in a thermostatically controlled oven, as temperature influences analyte volatility. The initial oven temperature is usually below that of the injector, but rises at a pre-determined rate. The analytes all spend the same amount of time in the gaseous phase, but are retained for different lengths of time by the stationary phase. Each analyte is thus eluted at a different rate and has a characteristic retention time. As the analytes leave the column in order of their retention time, their presence is detected by a detector system which records each separate analyte as a peak on a chart or chromatogram (Figure 13).




Retention Time (Mins)

Analyte Data - Fatty acid methyl esters

1. C4:0 (Butryic)
2. C6:0 (Caproic)
3. C8:0 (Caprylic)
4. C10:0 (Capric)
5. C11:0 (Undecanoic)
6. C12:0 (Lauric)
7. C13:0 (Tridecanoic)
8. C14:0 (Myristic)
9. C14:1 (Myristoleic)
10. C15:0 (Pentadecanoic)
11. C15:1 (cis-10-Pentadecenoic)
12. C16:0 (Palmitic)
13. C16:1 (Palmitoleic)
14. C17:0 (Heptadecanoic)
15. C17:1 (cis-10-Heptadecenoic)
16. C18:0 (Stearic)
17. C18:1n9c (Oleic)
18. C18:1n9t (Elaidic)
19. C18:2n6c (Linoleic)
20. C18:2n6t (Linolelaidic)
21. C18:3n6 (g-Linolenic)
22. C18:3n3 (a-Linolenic)
23. C20:0 (Arachidic)
24. C20:1n9 (cis-11-Eicosenoic)
25. C20:2 (cis-11,14-Eicosadienoic)
26. C20:3n6 (cis-8,11,14-Eicosatrienoic)
27. C20:3n3 (cis-11;14,17-Eicosatrienoic)
28. C20:4n6 (Arachidonic)
29. C20:5n3 (cis-5,8,11,14,17-Eicosapentaenoic)
30. C21:0 (Henicosanoic)
31. C22:0 (Behenic)
32. C22:1n9 (Erucic)
33. C22:2 (cis-13,16-Docosadienoic)
34. C22:6n3 (cis-4,7,10,13,16,19-Docosahexaenoic)
35. C23:0 (Tricosanoic)
36. C24:0 (Lignoceric)
37. C24:1n9 (Nervonic)

Figure 13. Typical chromatogram of fatty acid methyl esters obtained by GC. Obtained from Supelco at www.sigma-aldrich.com/saws.nsf/SupProducts, showing chromatogram of Supleco 37 Component Fame Mix.

In order to detect each peak as a specific analyte, several methods have been used, including flame ionisation detectors (FID), with identification based on retention time. The most conclusive method of identification is the mass spectrometer. Ions can be separated based on their mass/charge (M/Z) ratio, and the deflection of ions of equal velocity in a magnetic or electrostatic field depends on their mass. Bombarding the

analytes with an electron beam (ionisation source), allows the mass spectrometer to generate ions which can be separated by an analyser such as a quadrupole mass filter and detected by an electron multiplier. The relative amount of each ion can be measured, generating a spectrum based on mass (mass spectrum), which is characteristic of that compound and can be compared to known spectra, allowing conclusive identification of the analytes. A mass spectrometer is more sensitive than a flame ionisation detector, and also allows definitive identification of compounds when used in conjunction with a library of the spectra of known compounds or standards.

When using MS, the carrier gas in the GC must be helium. The eluted sample from a GC system can be directly channelled into the ionisation source of a mass spectrometer via a heated tube (transfer line). This combination of systems to separate and identify compounds is known as gas chromatography mass spectrometry (GC-MS), and allows concurrent compound identification as the chromatograph is being developed.

Once the analytes have been identified, they must be quantified. To do this, the chromatographic and identification data are transferred to computerised data systems or integrators. The area of the peak of each analyte on the chromatogram (peak area) is proportional to the amount of the given analyte, but proportionality is not constant and varies with each analyte (hence the need for accurate identification). Quantification can be relative or absolute, and both methods rely on peak area.

The most common method of relative quantification is to integrate all the peaks in a chromatogram, and to express each peak as a percentage of the total peak area.

$$\% \text{ Analyte A} = \frac{\text{Peak area A} \times 100}{\Sigma(\text{Peak area A} + \text{Peak area B}.....)}$$

Such results are expressed as area percentages: each analyte is expressed as an area percentage of total analytes. For fatty acid analysis, each fatty acid is thus expressed as a percentage of total fatty acids (% TFA). Since the amount of each analyte is therefore dependent on and relative to all the analytes included, the results will vary when different analytes are measured. Unless the same analytes are consistently

analysed, it is difficult to compare relative results from different analyses or laboratories (Cabré *et al* 1992).

Absolute quantification requires initial calibration using varying and known amounts (concentrations) of an analyte, or mixture of analytes, in pure or reference form (standard) to produce several chromatograms with different peak sizes. A standard curve can then be constructed to relate amount/concentration to peak area. Such calibration data can be stored and used to determine the absolute amount from the peak area in the sample. The standard used for the calibration is physically separate from (*i.e.* an external standard) but similar to the sample, containing the types and concentrations of analytes believed to be in the sample. To ensure calibration best reflects day-to-day variation in the methodology, the external standard should be processed concurrently with the sample, thus an external standard is used to calibrate the quantification of each batch of samples analysed. The external standard should be analysed by GC-MS before the batch of samples, and then re-analysed after the samples to determine any changes in the system.

Peak area is, however, subject to variation in the preparation (in this case, extraction and derivatization) and chromatographic procedures. To overcome this, a known concentration of an analyte similar to, but of a different molecular species from, the analyte(s) in the sample is added to the sample, *i.e.* an internal standard. The internal standard is added either before the preparation process begins to ensure any loss of the standard is equal to loss of the analyte, or prior to analysis. The internal standard must also be present in the external standard, although not necessarily in the same concentration as it is added to the samples. This allows the ratio of the peak areas of the internal standard and/or analyte in the sample to be compared to the corresponding peaks in the calibration standard. In the external standard, the peak areas relate to known concentrations of the internal standard and each analyte. To account for differences in detector response, a calibration or response factor for each compound in the external standard is calculated as follows:

$$\text{Response factor for Analyte} = \frac{\text{Area}_{\text{Analyte in ES}}}{\text{Area}_{\text{IS in ES}}} \times \frac{\text{Concentration}_{\text{IS in ES}}}{\text{Concentration}_{\text{Analyte in ES}}}$$

(IS - internal standard, ES – external standard)

The response factor (RF) can then be used in the calculation of absolute amount or concentration of each analyte in the sample:

$$\text{Concentration of Analyte} = \frac{\text{Area}_{\text{Analyte in Sample}}}{\text{Area}_{\text{IS in Sample}}} \times \frac{\text{Concentration}_{\text{IS in Sample}}}{\text{RF}_{\text{Analyte in ES}}}$$

(IS – internal standard, RF – response factor, ES – external standard)

## 8.2 VALIDATION OF METHODS

Validation of the methods used is an important component of laboratory technique. The methods used for the analysis of umbilical cord tissue have been validated and practised extensively (Cerolini *et al* 1996, Noble & Speake 1997, Speake *et al* 1999). The methods used for sample analysis undertaken at Yorkhill were validated as described in the following section.

### 8.2.1 Standards

From the published literature, the levels of fatty acids present in various human tissues were compared (Table 32). Non-esterified fatty acids (NEFA) in propanol were mixed together in concentrations similar to those found in human tissues. This produced a NEFA standard of known composition, for use in method validation and subsequent sample analysis as an external standard. Inclusion of a reference standard of "composition similar to that of the fatty matter to be analysed" in the analysis process is recommended (International Union of Pure and Applied Chemistry 1977b). NEFA 15:0 and 17:0 were included in the standard for use as internal standards (International Union of Pure and Applied Chemistry 1977b). The external standard will be referred to as Standard H (Table 33).

All free fatty acids were obtained from Sigma-Aldrich (Dorset, U.K.).

| Tissue | RBC (non-pregnant women)[1] | Plasma (non-pregnant women)[1] | Umbilical cord arterial RBC[2] | Umbilical cord arterial plasma[2] | Umbilical cord venous plasma[2,3,4,5,6,7,] | Umbilical cord artery vessel wall[2,3,4,5,6,7,8] | Umbilical cord vein vessel wall[2,3,4,5,7,8] | Placenta[2] | Breast Milk[9] |
|---|---|---|---|---|---|---|---|---|---|
| Lipid fraction | Total lipids | Total lipids | PL | PL | PL | PL | PL | PL | Total lipids |
| Fatty Acid | | | | | | | | | |
| 10:0 | | | | | | | | | 0.77 |
| 12:0 | | | | | | 1.1 | | | 6 |
| 14:0 | | | | | 0.2 | 3 | 0.8 | | 8 |
| 15:0 | | | | | | 0.7 | 0.7 | | 0.27 |
| 16:0 | 28.9 | 25.3 | | | 26 | 18 | 20.5 | | 21 |
| 16:1n-7 | | 2.8 | | | 1.2 | 2.4 | 1.6 | | 1.06 |
| 17:0 | | | | | 0.4 | 1.1 | 1.1 | | |
| 18:0 | 14.0 | 6.5 | | | 13 | 13.5 | 16 | | 7 |
| 18:1n-9 | 17.6 | 22.3 | | | 11 | 15 | 11 | | 35 |
| 18:2n-6 | 12.9 | 33.8 | 5.9 | 7.5 | 8 | 1.17 | 2.3 | 9.5 | 12 |
| 18:3n-3 | | 0.5 | | | 0.9 | | | | 0.9 |
| 18:3n-6 | | | | | 0.2 | 0.1 | 0.1 | | |
| 20:0 | | | | | 0.9 | 0.5 | 0.4 | | |
| 20:1n-9 | | | | | 0.2 | 0.9 | 0.6 | | |
| 20:2n-6 | | | | | 0.35 | 1.3 | 0.42 | | |
| 20:2n-9 | | | | | 0.6 | 1.1 | 0.6 | | |
| 20:3n-6 | 1.5 | 1.3 | 2.1 | 4.4 | 4.8 | 1.5 | 2.2 | 4.3 | |
| 20:3n-9 | | | 0.6 | 0.9 | 1.0 | 3.1 | 0.9 | 0.2 | |
| 20:4n-3 | | | | | 0.09 | | 0.1 | | |
| 20:4n-6 | 15.6 | 1.4 | 14.0 | 18.6 | 15 | 12 | 16 | 21.1 | 0.7 |
| 20:5n-3 | 0.5 | 0.5 | 0.2 | 0.7 | 0.8 | 0.07 | | 0.1 | 0.3 |
| 21:0 | | | | | 0.2 | 1.3 | 0.45 | | |
| 22:0 | | | | | 0.9 | 0.9 | 0.75 | | |
| 22:1n-9 | | | | | 0.3 | 0.3 | 0.3 | | |
| 22:3n-9 | | | 0.3 | | 0.1 | 1.7 | 0.5 | | |
| 22:4n-6 | 2.4 | | 4.0 | 1.9 | 1.2 | 2.5 | 4.5 | 1.6 | |
| 22:5n-3 | 1.9 | | 1.8 | 1.1 | 1.0 | 0.3 | 0.7 | 1.3 | |
| 22:5n-6 | 0.2 | | 1.4 | 1.4 | 1.5 | 3.2 | 2.6 | 1.0 | |
| 22:6n-3 | 4.5 | 1.2 | 4.7 | 6.1 | 6 | 5 | 5.5 | 4.8 | 0.5 |
| 23:0 | | | | | | 0.2 | 0.1 | | |
| 24:0 | | | | | 0.9 | 1.8 | 1.3 | | |
| 24:1n-9 | | | | | 2.0 | 4 | 3.5 | | |
| 24:2n-6 | | | | | 0.58 | 0.2 | | | |
| 24:3n-3 | | | | | | 0.1 | 0.2 | | |

Table 32. Relative fatty acid levels (% fatty acids) in various human tissues.

[1] Berry *et al* 2001
[2] Al *et al* 1990
[3] Al *et al* 1995b
[4] Al *et al* 1995a
[5] Hornstra *et al* 1992
[6] Reddy *et al* 199)
[7] van Houwelingen *et al* 1995
[8] Hornstra *et al* 1989
[9] Auestad *et al* 1997

| Fatty Acid | Fatty acid concentration (nmol) in Standard equivalent to 1mg lipid | % Total Fatty Acids excluding C15:0 and C17:0 (including C15:0 and C17:0) | % Total Fatty Acids on Analysis (Mean) |
|---|---|---|---|
| C15:0 | 517 | - (4.98) | |
| C17:0 | 463 | - (4.98) | |
| C10:0 | 145 | 1.05 (1.00) | 0.65 |
| C12:0 | 125 | 1.05 (1.00) | 1.02 |
| C14:0 | 548 | 5.24 (4.98) | 6.25 |
| C16:0 | 1953 | 20.96 (19.92) | 21.98 |
| C16:1 | 197 | 2.09 (1.99) | 2.46 |
| C18:0 | 1320 | 15.72 (14.94) | 21.09 |
| C18:1 | 1773 | 20.96 (19.92) | 20.75 |
| C18:2 | 1339 | 15.72 (14.94) | 14.76 |
| C18:3 | 90 | 1.05 (1.00) | 4.26 |
| C20:0 | 80 | 1.05 (1.00) | 1.29 |
| C20:3n-6 | 163 | 2.09 (1.99) | 0.64 |
| C20:4n-6 | 82 | 5.24 (4.98) | 1.03 |
| C20:5n-3 | 83 | 1.05 (1.00) | 0.60 |
| C22:4n-6 | 151 | 2.09 (1.99) | 0.88 |
| C22:5n-3 | 30 | 0.42 (0.40) | 0.22 |
| C22:6n-3 | 305 | 4.19 (3.98) | 2.10 |

Table 33. Composition of non-esterified fatty acid standard prepared in the laboratory (Standard H).

### 8.2.2 Validation of Lipid Extraction Procedure

8.2.2(a) Sample Concentrating by Drying under Nitrogen

The use of nitrogen as a drying/sample concentrating agent was determined by comparing the drying of FAME of known composition under nitrogen at 45°C, to drying in a Heto vacuum centrifuge.

Values obtained for area % and concentration (nmole) were lower for the FAME samples dried in the vacuum centrifuge than for those dried under nitrogen. Volatile and polyunsaturated fatty acids, which generate smaller peak areas on GC-MS analysis, were most susceptible to loss when dried in the vacuum centrifuge.

Drying under nitrogen at 45°C was therefore considered an efficient method of sample concentrating, and incurred fewer losses than other available methods.

8.2.2(b) Extraction Yield Test

To determine the loss of fatty acids incurred during lipid extraction, *i.e.* extraction yield, samples which had undergone both extraction and derivatization procedures were compared to samples which were derivatized only.

Replicates of a NEFA standard of known composition (Standard H), a triacylglycerol standard (tri-15), and two NEFA (C15:0 and C17:0) were either a) extracted and derivatized or b) derivatized only. Extraction yield was determined by expressing the mean value for peak area or concentration (nmole) for the appropriate fatty acids in the extracted/derivatized samples as a percentage of the corresponding values in the derivatized only samples.

The mean values for peak area of fatty acids in the extracted/derivatized samples were lower than those in the samples derivatized only (mean extraction yield 53%, range 27-79%). Extraction yields were similar for NEFA and triacylglycerols, thus there were no apparent differential losses. The mean values for concentration of fatty acids in the extracted/derivatized samples were comparable to those in the derivatized only samples (54-114% extraction yield, mean 96%)).

Thus, despite losses incurred during extraction procedures, the inclusion of an internal standard allows appropriate values for fatty acid concentration to be obtained. Expression of individual peak areas as a % of total peak area (*i.e.* area %) is not affected by extraction yield.

### 8.2.3 Validation of FAME Derivatization Procedure

Lipids from a fish oil of known composition (R.P. Scherer Limited, Swindon, U.K.) were derivatized using either methanolic HCl, sodium methoxide or boron tri-fluoride. Results from GC-MS analysis of all fatty acids for all three methylating agents were compared, and found to be similar. The procedure was repeated using red blood cell samples, and similar conclusions reached. Thus the use of methanolic HCl yielded results comparable to those obtained with other methylating agents. Moreover, the use of methanolic HCl for derivatization was previously established in this laboratory (Berry *et al* 2001, Farquharson *et al* 1996). Methanolic HCl was therefore used as the methylating agent for all subsequent derivatization procedures performed at Yorkhill.

A lipid sample of certified composition (Supelco 37 Component FAME Mix (Supelco Cat No 47885-U, Sigma-Aldrich Company Ltd., Dorset, U.K.), was analysed by GC-MS to determine whether the results obtained following derivatization are comparable

to those of the manufacturer. The results obtained (area as % total fatty acids/ %TFA) were compared with, and found to be comparable to, the certified results supplied by the manufacturer. The derivatization protocol and GC-MS analysis technique were thus considered appropriate.

### 8.2.4 Validation of GC-MS Analytical Procedure

8.2.4(a) Linearity Tests - High and Low Concentration Standard Curves

To determine whether the actual amount of each fatty acid present in the extracted/derivatized sample will be reflected in the value for peak area obtained, and whether these values can be used to accurately calculate concentration, linearity tests were performed.

Aliquots of a NEFA standard of known concentration, all equal to 1mg of fat, but with different amounts of each fatty acid relative to the internal standard (*i.e.* relative amount), representing the standard at half, full and double concentrations, were derivatized and analysed by GC-MS. The relative amount of each fatty acid was plotted against either its corresponding peak area or concentration, and correlation analyses were performed.

Values obtained for peak area and concentration were correlated to "relative amount" (mean $R^2$ 0.99). Thus, the peak area for any fatty acid is related to its actual amount in the sample, and can be used to accurately calculate fatty acid concentration.

8.2.4(b) Analytical Precision for Measures of Peak Area and Concentration

Samples of RBC, plasma, placenta tissue and breast milk, to which internal standards (tri-15 and C17:0) were added, were extracted and derivatized in parallel with reagent blanks and a NEFA standard of known composition (Standard H). For the three replicates of each sample type, reagent blank and standard, the mean and standard deviation values for area % and concentration of selected fatty acids was calculated. The coefficient of variation (CV (%), ((SD/Mean) x 100)) for each fatty acid was obtained. The mean peak area or concentration was plotted against its CV (%) for all sample types, to illustrate precision of area % and concentration. Selected values for DHA CV (%) are plotted against DHA peak area (Figure 14) and concentration (Figure

15). The overall mean values for the mean CV (%) for the selected fatty acids are shown in Table 34.

| | CV (%) for Peak Area | CV (%) for Concentration |
|---|---|---|
| C15:0 | 3.6 | 2.7 |
| C16:0 | 2.6 | 1.6 |
| C17:0 | 1.6 | |
| C18:0 | 2.4 | 1.3 |
| C18:1 | 2.7 | 1.7 |
| C18:2 | 2.3 | 1.3 |
| C22:5n-3 | 17.2 | 17.0 |
| C22:6n3 | 8.7 | 8.7 |

Table 34. Mean coefficient of variation (CV) for all sample types and standards for selected fatty acids measured as both peak area and concentration.

For the major fatty acids, precision of analysis was 3% or better. Analytical precision was slightly worse for LCPUFA, due to the small amounts present in the samples, but was within acceptable limits.

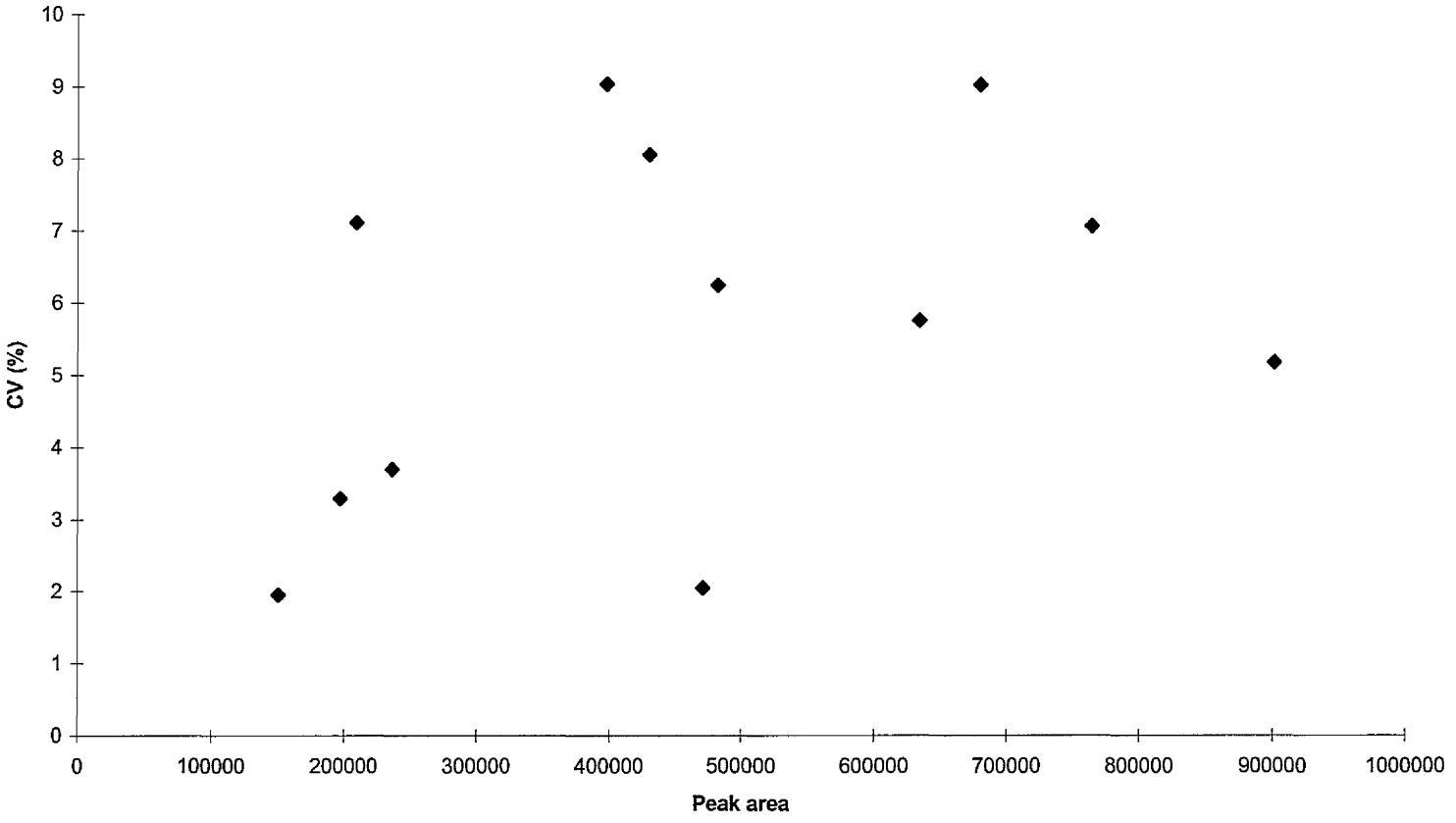



Figure 14. Analytical precision for DHA based on peak area.

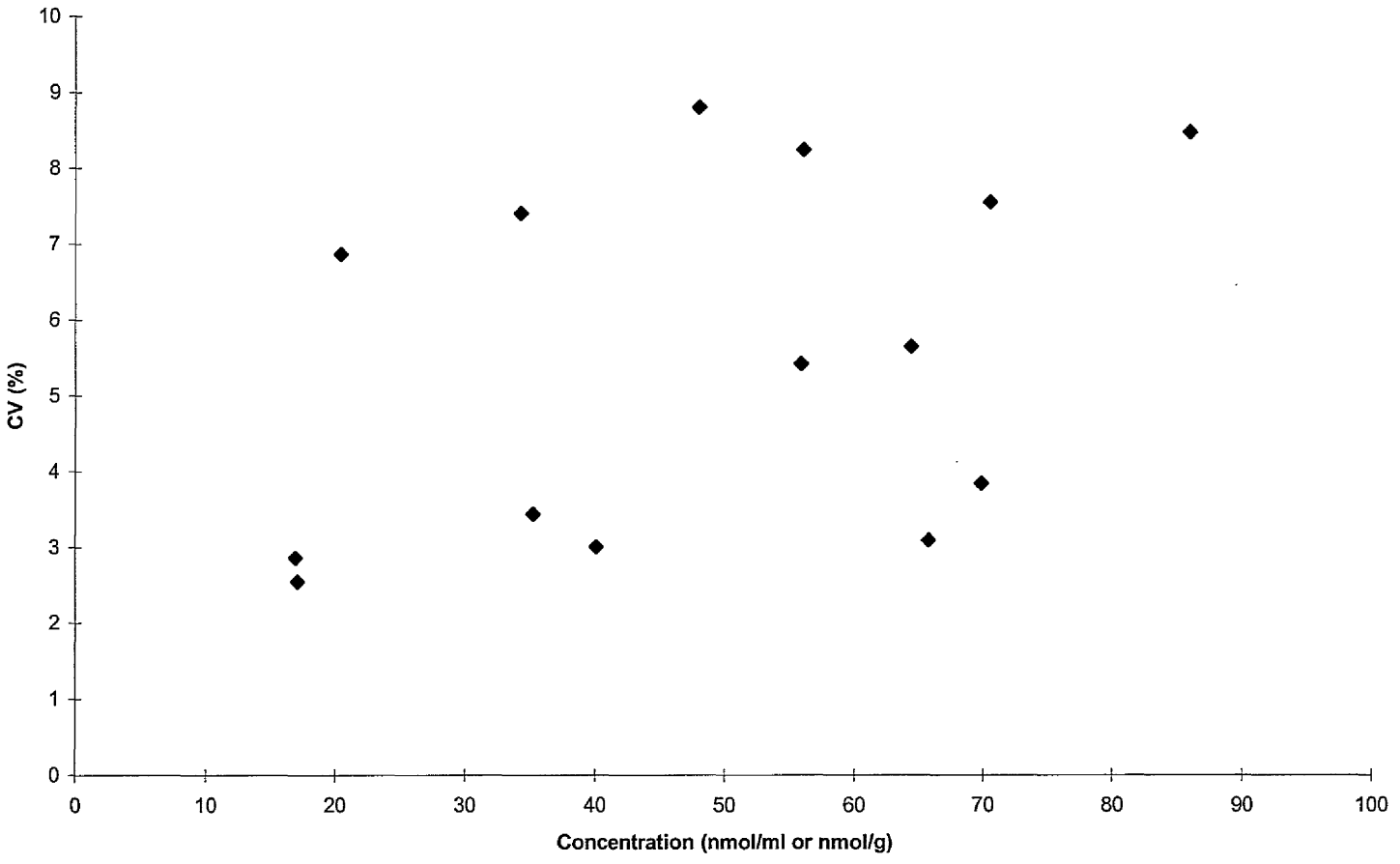



Figure 15. Analytical precision for DHA based on concentration.

## 8.3 COLLECTION, INITIAL PREPARATION AND STORAGE OF SAMPLES

### 8.3.1 Blood Samples: Red Blood Cells and Plasma

Venous maternal or umbilical cord blood (5ml) were collected in potassium EDTA (15mm x 54mm screw-top vials, Teklab, U.K.). Samples were centrifuged at 550 x *g* (2500rpm) for 5 minutes. Plasma was removed and transferred into a sterile vessel with no anticoagulant. Sterile saline (2.5ml) (0.15M NaCl) was added to red blood cells (RBC) and centrifuged at 550 x *g* for 5 minutes. The saline layer was removed and discarded. This process was repeated a further twice, such that RBC were washed in this manner a total of three times. Both plasma and RBC were stored at -70°C until analysis.

Fatty acid content does not change on storage of samples at -20°C in the intermediate term (12 months) (Olsen *et al* 1991), or at -80°C in the long term (12 years) (Zeleniuch-Jacquotte *et al* 2000), indicating the stability of samples stored at an appropriate temperature for prolonged periods.

### 8.3.2 Placental Tissue

Within two hours of delivery, a transverse section (from fetal to maternal side) of placental tissue (approximately $5cm^3$) was dissected free of decidua and major vessels, and stored in a sterile Universal container (30ml, Bibby Sterilin, R. and J. Wood, U.K.) on ice. Tissue was rinsed with sterile saline to remove surface blood clots and/or obstetric cream. Three aliquots of tissue (approximately 1g wet weight each) were dissected and stored in sterile Eppendorf tubes (1.5ml, Teklab, Durham, U.K.) at -70°C until homogenization. The remainder of the tissue was stored in the Universal container at -70°C for future reference.

Each aliquot of placental tissue was freeze-dried overnight (Micro Modulyar Freeze Drier, Edwards, U.K.). The aliquots were weighed to obtain the dry weight of tissue (approximately 0.2g). The weight of water in the original aliquot was determined by subtracting the dry weight from the wet weight; the weight of water was expressed as a percentage of the wet weight (mean 85%). The three aliquots of freeze dried tissue were removed from the Eppendorf tube, mixed together and ground to a homogeneous powder in a Potter-Elvehjem homogenizer (borosilicate glass mortar and PTFE/stainless steel pestle; Merck Ltd., Leics., U.K.); each Eppendorf tube was rinsed

with 1.5ml sterile saline to remove residual tissue and the contents added to the glass mortar. A further 1.5ml sterile saline was added to the powdered tissue in the glass mortar, and the final contents were homogenized, thus suspending approximately 3g of placental tissue (wet weight) in a total of 6ml sterile saline. The homogenate was stored at -70°C until analysis.

### 8.3.3 Umbilical Cord Tissue

Within two hours of delivery, approximately 5cm of umbilical cord, free of internal blood clots, was collected in a sterile Universal container and kept on ice, before rinsing with sterile saline (Velzing-Aarts *et al* 1999). Cord vessels were cannulated and flushed with sterile saline to remove any remaining blood. Cord tissue was stored in a fresh Universal container at -70°C until analysis.

Previous studies have analyzed the fatty acid composition of umbilical cord veins separately from umbilical cord arteries. This requires complex and time-consuming dissection. Moreover, dissection of the vessels free from Wharton's jelly is difficult and may be unnecessary: the small amount of lipid present in the jelly and the similarity of its fatty acid composition to that of the blood vessels, suggests that the presence of jelly material is unlikely to affect the fatty acid composition of the vessels (Hornstra *et al* 1989). Although previous studies have shown differences in the PUFA content of umbilical arteries compared to the umbilical vein, no difference was found in the DHA content of the vessels. The effect of maternal fish oil supplementation does not differ between umbilical arterial and venous tissue (van Houwelingen *et al* 1995). In addition, there is some evidence that umbilical cord tissue may vary as much with genetic differences as with maternal dietary patterns and fatty acid status (Hornstra *et al* 1992). Our study postulates that maternal DHA supplementation will increase the amount of DHA received by the fetus (via the umbilical vein) and hence fetal DHA status (assessed by umbilical arteries), thus one would expect all cord vessels to have a higher DHA status in the fish oil supplemented group. Thus concomitant analysis of venous and arterial cord vessels should indicate the overall status of the fetus. As a result, cord tissue was analyzed in its entirety, without dissection of vessels and Wharton's jelly, as a supplementary tissue, as opposed to the main indicator of fetal fatty acid status.

Umbilical cord tissue was homogenized using a mechanical homogenizer (Silverson, Chesham, U.K.) in 20ml of chloroform/methanol (1:1) containing BHT prior to esterification and derivatization, at the Scottish Agricultural College (see below).

### 8.3.4 Breast Milk

Breast milk samples (approximately 2ml) were manually expressed by mothers into a sterile Universal container, within seven days of delivery. Samples were stored at -70°C to prevent triacylglycerol hydrolysis by lipases. Samples were thawed at 4°C overnight, prior to analysis. This temperature prevents rapid thawing which activates lipolysis, and samples can be stored at 4°C for approximately one week before bacterial contamination becomes problematic (Jensen 1989b).

### 8.3.5 Sample Analyses

The fatty acid composition of RBC, plasma, placenta tissue and breast milk samples was analyzed in the University Department of Child Health laboratory at Yorkhill Hospital. Umbilical cord tissue samples were analyzed by Dr. Brian Speake at the Scottish Agricultural College (SAC) at Auchincruive, Ayr. The methods used in the analysis of all samples are described.

## 8.4 MATERIALS AND METHODS

### 8.4.1 Samples analysed at Yorkhill

8.4.1(a) Reagents

The solvents used were methanol and chloroform (Fisher Scientific, Leics., U.K.). The esterification agent used was anhydrous methanolic hydrochloric acid (methanolic HCL, MeOH/HCl, 3M) (Supelco, Sigma-Aldrich Company Limited, Dorset, U.K.): HCl cleaves esters while MeOH donates a methyl to form FAME.

The antioxidant butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol, BHT) (BDH Chemicals Limited, Poole, U.K.) was added to all solvents (50mg per L) to prevent lipid oxidation (Dodge and Phillips 1967), since polyunsaturated fatty acids rapidly oxidise on exposure to air.

The salts used were sodium chloride (NaCl, 9g per L deionised water *i.e.* 0.9%, 0.15M) (BDH Chemicals Limited, Poole, U.K.) and potassium chloride (KCl, 2.5g per

L deionised water *i.e.* 0.25%, 0.03M) (Sigma, Sigma-Aldrich Company limited, Dorset, U.K.).

All reagents used were analytical grade.

Oxygen-free nitrogen (BOC Gases Limited, Surrey, U.K.) was used as the evaporation agent to prevent lipid oxidation.

8.4.1(b) Standards

A 15:0 triacylglycerol (tri-15) standard (Sigma-Aldrich, Dorset, U.K.) was used as the internal standard to determine fatty acid concentration within samples (International Union of Pure and Applied Chemistry 1977b). The standard was required to be in the triacylglycerol form for lipid extraction. The desired concentration of tri-15 was 150nmole of tri-15 per mg lipid. For each sample type analysed, the amount of sample used in the extraction procedure was known to yield approximately 3mg of lipid. Therefore, 450nmole of tri-15 were added to a given amount of tissue assumed to contain 3mg of lipid, prior to extraction, thus equivalent to 150nmole per mg lipid. This was obtained using either 45µl of a 10mM solution or 90µl of a 5mM solution of tri-15 in propanol/hexane (1:1 v/v).

A non-esterified fatty acid 17:0 standard (Sigma-Aldrich, Dorset, U.K.) was used as an internal standard to aid quantification of loss during the derivatization/methylation procedure (International Union of Pure and Applied Chemistry 1977b). 450nmole of 17:0 were added per 2mg of lipid prior to derivatization, thus producing a concentration of 225nmoles of 17:0 per 1mg lipid. This was obtained using 12µl of a 10mg/ml solution of 17:0 in propanol.

A reference NEFA standard of known concentration was derivatized with each batch of samples as an external standard (International Union of Pure and Applied Chemistry 1977b). This standard was the NEFA standard used in the validation studies, which was of similar composition to the fatty acid levels found in various human tissues (Standard H). The purpose of this was three-fold: to determine repeatability of the results, to allow quantification of loss during derivatization procedures, and to allow

determination of fatty acid concentrations within each sample. The amount of external standard derivatized was equivalent to 1mg of lipid.

For each batch of samples, a reagent blank (*i.e.* all components, solvents *etc.* except sample) was included throughout the extraction/derivatization procedures and processed during GC-MS analysis, in order to check for memory in the glassware used during extraction/derivatization, or in the GC-MS column.

8.4.1(c) Glassware

All glass vials used during the extraction/derivatization process were obtained from Chromacol Limited (Herts., U.K.). Hewlett Packard GC-MS vials and inserts were used during analysis.

Glassware was cleaned using an alkali detergent, Decon 90 (Decon Laboratories Limited, Sussex, U.K.) All glassware was fully immersed in a Decon 90 solution of either 3% (if used for plasma and breast milk samples) or 5% (for RBC and placenta samples), and allowed to soak in solution for approximately 12 hours (usually overnight). On removal from Decon solution, glassware was rinsed thoroughly (at least three times) in free-flowing water, until all residue was removed, and then dried in a hot-air drier (LEEC).

8.4.1(d) Extraction Procedure

The procedure for lipid extraction was based on a modified Folch extraction in chloroform:methanol (2:1) with washing in KCl, as outlined by Dodge & Phillips (1967). The procedure for each sample type is described below.

*(i) Red Blood Cells (RBC)*

RBC samples were removed from storage at -70°C and thawed overnight at 4°C. RBC (1ml) were transferred to a clean vial (10ml screw cap) and 450nmole of tri-15 internal standard was added.

NaCl (0.5ml, 0.15M) was added to each sample to aid partitioning into solvent phase. Samples were mixed thoroughly and 2.5ml methanol containing BHT was added to rupture cell membranes. Samples were mixed thoroughly (WhirliMixer, Model No.

SGP202010J, Fisons Scientific Equipment, Leics., U.K.) and left to stand for 10 minutes.

Chloroform containing BHT (2.5ml) was added. Samples were mixed, left to stand for 10 minutes to allow phases to separate, and then centrifuged at 550 x *g* (2500rpm) for 5 minutes (Mistral 3000I, MSE, U.K.).

The resulting supernatant of methanol, chloroform and lipids was separated from the pellet of haem and protein and transferred to a fresh vial, to which 2.5ml of chloroform containing BHT was added. After mixing, 1.5ml 0.03M KCl was added to remove non-lipid components. The samples were mixed then centrifuged at 550 x *g* for 5 minutes. The mixture separated into two phases: an upper aqueous/methanol layer and a lower chloroform/lipid layer. The top layer was discarded, and the lower chloroform/lipid layer transferred to a pre-weighed vial, then placed under nitrogen at 45°C (Dri-Block DB-3, Techne (Cambridge) Limited, Cambridge, U.K.) to remove the chloroform.

*(ii) Plasma*

Plasma samples were removed from storage at -70°C and thawed overnight at 4°C. Plasma (1ml) was transferred to a clean vial and 450nmole of tri-15 internal standard was added.

Methanol containing BHT (1.25ml) was added and after thorough mixing 2.5ml chloroform containing BHT was added. The samples were mixed and left to stand for 10 minutes.

Chloroform containing BHT (2.5ml) was added to dissolve the lipids. Samples were mixed and left to stand for 10 minutes to allow phases to separate. The solution became bi-phasic, with a top aqueous/methanol layer and a dense lower layer of chloroform/lipids. KCl (0.75ml, 0.03M) was added to encourage the polar lipids into the chloroform and remove non-lipid contaminants. Samples were mixed, centrifuged at 550 x *g* for 5 minutes and the top aqueous/methanol layer discarded. The bottom layer was transferred into a fresh vial and 1.5ml methanol/water mixture (1:1) was added to dissolve protein. Samples were mixed and centrifuged at 550 x *g* for 5

minutes. The mixture again separated into two phases: an upper aqueous/methanol layer and a lower chloroform/lipid layer. The top layer was discarded, and the lower chloroform/lipid layer transferred to a pre-weighed vial, then dried under nitrogen at 45°C.

*(iii) Placenta*

Homogenised placenta samples were removed from storage at -70°C and thawed overnight at 4°C. Aliquots (1g) were transferred to a clean vial and 450nmole of tri-15 internal standard was added.

Since saline/NaCl had already been added to suspend the homogenised tissue, no further NaCl was added. Extraction then proceeded as for RBC samples, from the addition of methanol containing BHT onwards.

*(iv) Breast Milk*

Breast milk samples were removed from storage at -70°C and thawed overnight at 4°C. An aliquot (200ul) was transferred to a clean vial and 450nmole of tri-15 internal standard were added.

NaCl (400ul, 0.15M) was added to aid partitioning of lipids into solvent phase. Samples were mixed and 1.25ml methanol containing BHT was added. Following thorough mixing, 2.5ml chloroform containing BHT was added to dissolve lipids. Samples were mixed and left to stand for 10 minutes to allow phases to separate. The solution became bi-phasic with an upper aqueous/methanol layer and a lower chloroform/lipid layer. KCl (0.75ml, 0.03M) was added to encourage the polar lipid into the solvent phase and remove non-lipids. Samples were mixed and centrifuged at 550 x *g* for 5 minutes. The mixture again separated into two phases: an upper aqueous/methanol layer and a lower chloroform/lipid layer. The top layer was discarded, and the lower chloroform/lipid layer transferred to a pre-weighed vial, then dried under nitrogen at 45°C to remove the chloroform.

8.4.1(e) Derivatization (Methylation) Procedure

The pre-weighed vial containing the extracted lipid sample was reweighed to determine the yield of lipid, and chloroform added to produce a solution of 2mg lipid/100ul chloroform. Dissolution to a standard concentration facilitated recovery of a known quantity of lipid for analysis. The presence of another solvent also aids esterification of all lipid classes by methanolic HCl.

An aliquot of lipid/chloroform extract (100ul, 2mg lipid) was transferred to a fresh vial and 450nmole of 17:0 internal standard added. The external standard (Standard H, equivalent to 1mg lipid) was added to a fresh vial. Methanolic HCl (1ml, 3M) was added as esterification reagent to the samples and external standard.

Samples were placed in an oil bath (Multi-Blok Heater, Lab-Line, U.K.) at 100°C for 45 minutes and sealed with screw-caps and parafilm to prevent evaporation. On removal from the oil bath, samples were cooled to room temperature and 2.5ml distilled water was added to aid transfer of the residue. Petroleum spirit (1.5ml, BP 40-60°C) was added to aid extraction of the oily FAME from the water/HCl. After mixing and centrifuging at 550 x *g* for 5 minutes, the top petroleum spirit layer was transferred to a fresh vial. A further 1.5ml petroleum spirit was added to the original extract and mixed/centrifuged again. The top layer was then removed and added to the first petroleum spirit layer. The pooled petroleum spirit layers were evaporated to dryness under nitrogen at 45°C. Hexane (0.7ml) was added to dissolve the dried lipid. An aliquot was transferred to a 2ml glass vial and analysed by GC-MS; the remainder was stored at -70°C for future reference.

8.4.1(f) Gas Chromatography-Mass Spectrometry

Fatty acid methyl esters were identified and quantified using gas chromatography mass spectrometry (GC-MS). The GC (Hewlett Packard 5890 Series II) was used in split mode (ratio 20:1). The carrier gas was helium (Grade A 99.996% pure, BOC, Surrey, U.K.), at a flow rate of 1ml/min. The analytical column was a fused silica capillary column (BPX70, length 30m, internal diameter 0.25mm and film thickness 0.25μm; SGE Europe Ltd.). GC injector temperature was 250°C, and the injection volume was 2μl. The GC temperature programme was as follows: initial temperature of 120°C for

2 minutes, increasing by 4°C per minute to 180°C, then by 2°C per minute to 194°C, followed by 30°C per minute to 240°C which was maintained for 1 minute. The total GC run time for each sample was 26.53 minutes. The temperature of the transfer line between the GC and MS was 280°C. The MS (Hewlett Packard 5972) with electron impact ionisation, was operated in scanning mode.

### 8.4.2 Samples analysed at SAC, Ayr

The sample was homogenised using a mechanical homogenizer (Silverson, Chesham, U.K.) in 20ml of chloroform/methanol (1:1) containing BHT, then an additional 10ml of chloroform were added to produce a chloroform/methanol ratio of 2:1 (v/v). The homogenate was filtered through filter paper into a measuring cylinder and the filter paper washed with another 10ml chloroform/methanol (2:1). The total volume of the homogenate/extract was measured. KCl (0.88%) was added in a volume equal to 25% of the total volume. The sample was shaken by hand, and left at room temperature overnight to allow the layers to separate. The top aqueous/methanol layer was discarded, and the lower layer chloroform/lipid layer dried in a rotary evaporator (Buchi, Flawil, Switzerland). The lipids were dissolved in 10 ml chloroform, 5ml of which were removed and weighed in a flask to obtain the gravimetric weight of the total lipid. The remaining (non-dried) 5ml of chloroform/lipid were used for further analysis.

The solution of total lipids was added to 50ml flasks and 1ml internal standard added. The standard used was non-esterified 15:0 in methanol (0.3 mg/ml). The chloroform/lipid extract was dried by rotary evaporation. Fatty acids were converted to fatty acid methyl esters by refluxing at 60°C for 30 minutes with 4ml methanol/toluene/sulphuric acid (20:10:1). On cooling to room temperature, 10ml water and 10ml hexane were added. The sample was shaken, transferred to a test tube and the layers allowed to separate. The top hexane layer was transferred to another test tube containing a few grains of drying agent (sodium sulphate/sodium bicarbonate, 4:1), and left for 30 minutes at room temperature. The hexane layer was again transferred to another test tube and the volume reduced under nitrogen.

The samples (1μl) were injected into a Chrompack CP9001 GC containing a capillary column (Carbowax, length 30m, internal diameter 0.25mm, film thickness 0.25μm; Alltech, Carnforth, U.K.). The carrier gas was nitrogen. Initial oven temperature was 185°C, maintained at 185°C for 2minutes after injection, then increased at 5°C/min up to 230°C, held at 230°C for 24minutes. The GC was connected to a computer with an EZ Chrom Data System, which integrates the peaks and calculates % w/w of fatty acids. Standard fatty acid methyl ester mixtures containing all the fatty acids reported (Sigma, Sigma-Aldrich Company Limited, Dorset, U.K.) were run to identify retention times of each fatty acid peak. A proportion of the samples was also analysed by GC-MS (Fisons MD800) to confirm the identification.

### 8.4.3 Results

Since all the fatty acids in a lipid sample containing all the lipid classes (triacylglycerols, phospholipids *etc.*) present in the original tissue were measured, the results pertain to the total lipids of each tissue. Thus, total RBC lipids, total plasma lipids, total placenta lipids, total cord tissue lipids and total breast milk lipids were analysed.

Results were expressed both as relative and absolute amounts. The area of each fatty acid peak in the chromatograph was expressed as a percentage of the total peak area (as per Equation 1), thus each fatty acid is expressed as a percentage of total fatty acids (%TFA).

$$\% \text{ Fatty Acid A} = \frac{\text{Peak area A} \times 100}{\Sigma(\text{Peak area A} + \text{Peak area B}.....)}$$

Equation 1. Calculation of peak area as a percentage of total peak area.

For umbilical cord tissue, the fatty acids measured were different from those measured in the other tissues (Table 35). A total of 19 fatty acids were measured in RBC, plasma, placenta and breast milk samples, while 23 fatty acids were measured in the cord tissue. Of these, 15 fatty acids were commonly measured in all tissues. The results for cord tissue analysis are therefore expressed as per the 15 common fatty acids (*i.e.* each fatty acid as a % of 15 total fatty acids, compared to 19 total fatty acids). This illustrates the aforementioned problem of the dependency of relative

amounts on the species of fatty acids measured, and the difficulty encountered in comparing results from different analyses.

| Fatty Acids Measured in RBC, Plasma, Placenta and Breast Milk Samples | Fatty Acids Measured in Umbilical Cord Tissue Samples |
|---|---|
| 10:0 | |
| 12:0 | |
| 14:0 | 14:0 |
| 16:0 | 16:0 |
| 16:1n-7 | 16:1n-7 |
| | 17:0 |
| | 17:1n-7 |
| 18:0 | 18:0 |
| | 18:1n-7 |
| 18:1n-9 | 18:1n-9 |
| 18:2n-6 | 18:2n-6 |
| 18:3n-3 | 18:3n-3 |
| | g18:3n-6 |
| | 18:4n-3 |
| 20:0 | 20:0 |
| | 20:1n-9 |
| | 20:2n-6 |
| 20:3n-6 | 20:3n-6 |
| 20:4n-6 | 20:4n-6 |
| 20:5n-3 | 20:5n-3 |
| | 22:1n-9 |
| 22:4n-6 | 22:4n-6 |
| 22:5n-6 | 22:5n-6 |
| 24:0 | |
| 24:1n-9 | |
| 22:5n-3 | 22:5n-3 |
| 22:6n-3 | 22:6n-3 |

Table 35. Fatty acids measured in samples analysed at Yorkhill, compared to those measured in umbilical cord tissue samples analysed at SAC, Ayr.

The concentration of each fatty acid was calculated based on the inclusion of internal and external standards as follows:

$$\text{Response factor for FA} = \frac{\text{Area}_{\text{FA in ES}}}{\text{Area}_{\text{IS in ES}}} \times \frac{\text{Concentration}_{\text{IS in ES}}}{\text{Concentration}_{\text{FA in ES}}}$$

Equation 2. Calculation of response factor for each fatty acid (FA – fatty acid, IS - internal standard, ES – external standard).

$$\text{Concentration of FA} = \frac{\text{Area}_{\text{FA}}}{\text{Area}_{\text{IS in Sample}}} \times \frac{\text{Concentration}_{\text{IS in Sample}}}{\text{RF}_{\text{FA in ES}}}$$

Equation 3. Calculation of concentration for each fatty acid (FA – fatty acid, IS – internal standard, RF – response factor, ES – external standard).

Please note that for 22:5n-6 (DPA), 24:0 and 24:1, no standards were available for these fatty acids, preventing their inclusion in the external standard. Hence, the concentration of these fatty acids could not be calculated.

Concentrations were expressed in nmole per ml (nmol/ml) of RBC, plasma or breast milk, and per g wet weight tissue (nmol/g) for placenta. For RBC and plasma, 1ml of tissue was analysed and concentrations were therefore calculated directly. For breast milk, 200ul were analysed and correction to 1ml was achieved by multiplying concentrations obtained on calculation by a factor of 5. For placenta, approximately 1g homogenate was analysed; the wet and dry weight of tissue in the homogenate analysed was known. Thus concentrations were calculated for the amount of homogenate analysed and each sample multiplied by the appropriate factor to convert the weight of tissue present in the homogenate to 1g wet weight of tissue. Cord tissue samples were analysed off-site, and the calculation of concentrations is not included here.

The use of both relative and absolute methods of quantification permits the most comprehensive analysis and interpretation of the data. For each individual sample, the % TFA accounted for by each fatty acid is not directly related to the concentration of the fatty acid; for example, RBC DHA accounted for 4.1% TFA and had a concentration of 377nmol/ml in one sample, but was 5.8% TFA with a concentration of 219nmol/ml in another. Correlation of % TFA values with concentrations varies for the different fatty acids. The average correlation coefficients (r) for the correlation of area % TFA values with concentration for oleic acid (18:1n-9), AA (20:4n-6) and DHA were 0.3, 0.5 and 0.6 respectively. Moreover, correlation of % TFA and concentration varied with the tissue sampled, for example the mean correlation coefficient for DHA in RBC was 0.8, plasma 0.5, placenta 0.3 and breast milk 0.9. Previous authors (Al *et al* 1995b) have noted that the differences between supplemented and placebo groups in the relative amounts of fatty acids were not necessarily observed on consideration of absolute amounts.

Fatty acid metabolism occurs in membranes and is mediated by enzymes for which the fatty acids compete. The fatty acid present in the greatest amount within the membrane undergoes metabolism, thus it is the amount of the fatty acid relative to

those surrounding it, and not its actual amount within the entire cell or tissue, which determines metabolism (Al *et al* 1995b, Holman 1986). The expression of fatty acids in relative terms is therefore appropriate when considering metabolism. Conversely, the use of absolute quantification is useful when considering the nutritional implications of fatty acid composition, such as with human breast milk and/or infant formulae (Kohn *et al* 1996). Some authors have suggested that both relative and absolute measurements are required when considering maternal-fetal transfer of fatty acids (Al *et al* 1990, Matorras *et al* 1994).

By presenting the results of fatty acid analysis in both relative and absolute terms, it is possible to determine whether the amount of a particular fatty acid relative to others is influencing metabolism and possibly development, or whether an effect is observed above a certain threshold of fatty acid concentration.

## 8.5 STATISTICAL METHODS

### 8.5.1 Biochemical Variables

The results of fatty acid analyses (% TFA and concentrations) were expressed for individual fatty acids, as a sum of each PUFA class, and as the ratio of the PUFA classes. Fatty acids from both the n-6 and n-3 classes were summed (total n-6 and total n-3) in order to detect any differences not evident when considering the individual fatty acids. Several ratios have been employed when examining fatty acid status (BNF 1992). The polyunsaturated/saturated ratio (P/S) considers total PUFA relative to total saturates, but does not account for MUFA, whose contribution can be considered by using the ratio of total unsaturates to total saturates (U/S). However neither U/S nor P/S ratios account for the differences between the distinct metabolic families of PUFA (especially n-3 and n-6).

The ratio of 20:3n-9 to 20:4n-6 (triene/tetraene ratio) is useful when considering EFA deficiency, but limited when EFA are known to be sufficient. The n-6/n-3 ratio has many derivatives including the ratio of LA/αLA, which does not consider the "potency" of their respective longer chain derivatives (BNF 1992). Furthermore, it is of limited value when the study objective was to supplement with DHA. The ratio of total n-3 LCPUFA (20:5n-3 EPA, 22:5n-3 DPA and 22:6n-3 DHA) to AA (20:4n-6) may be a useful marker when studying the effect of high n-3 intake on AA levels

(Olsen *et al* 1991, 1995a, 1995b) but does not account for differential changes in total n-6 PUFA. Since the sums of total n-6 and total n-3 were calculated, the relative difference between these was of relevance, hence the ratio of n-6/n-3 was calculated.

### 8.5.2 Distribution of Data

Distribution of fatty acid values (% TFA and concentrations) were checked graphically (SPSS 9.0 for Windows) and by calculating whether approximately 70% of results fell within Mean ± SD (Fowler, Cohen & Jarvis 1998). Values were found to be non-normally distributed, indicating that the use of non-parametric statistical tests was the appropriate method of analysis. Median values are quoted throughout and 95% confidence intervals for the median were obtained by Wilcoxon Sign Ranked Confidence Intervals.

### 8.5.3 Effect of Variables on DHA

Parity, the interval between successive pregnancies and dietary fish intake have all been previously reported to influence DHA status. Mann-Whitney 2-sample rank tests were performed to detect the influence of these variables on DHA status in the whole study population, regardless of supplementation group. A significance value of $p<0.05$ was chosen. These variables were not considered further because the supplement and placebo groups were comparable in all underlying variables.

### 8.5.4 Analysis between Groups: Mann-Whitney Tests

Mann-Whitney 2-sample rank tests were used to detect significant differences between the fish oil and placebo groups. A significance level of $p<0.05$ was chosen for between group analyses because this was related to the initial calculation of sample size required to detect a 1SD of difference between groups in DHA status (see Chapter 6, Section 6.4 Sample Size Calculation).

### 8.5.5 Analysis within Groups (Paired Data): Sign Tests

For the longitudinal changes within each group, the null hypothesis was that the median difference over time was zero. To obtain the difference between samples over time, the values for the later samples were subtracted from those of the earlier samples. The same method was used to calculate the differences between maternal and umbilical cord values. The differences were found to be non-symmetrically

distributed, preventing the use of one-sample Wilcoxon Signed Rank tests of the median, which require the data to be approximately symmetrical in distribution (Altman 1991). Thus one-sample Sign tests of the median were performed to detect whether the difference was significantly different from the hypothesised median difference of zero (0.00).

Due to the large number of tests performed on the differences between maternal levels over time and between maternal and umbilical cord levels, only individual fatty acids were tested; the sums for total n-3 and total n-6 fatty acids, as well as the n-6/n-3 ratio, were not tested for changes within each group. Although correction of the significance level for multiple statistical testing with non-parametric statistics is not as formal a procedure as for parametric methods (*e.g.* Bonferroni correction), it was considered appropriate to lower the level of significance for the within group testing. Thus, due to the large number of tests performed, a significance level of $p<0.01$ was chosen for within group analyses.

### 8.5.6 Statistics Programme

All statistical analyses were performed using Minitab for Windows Version 10.51 software (1995, Minitab Inc., PA, U.S.A).

Chapter 9

# Results of Supplementation Study

## 9.1 DHA AND OTHER VARIABLES

### 9.1.1 DHA and Parity

Participants were divided into two groups on the basis of parity. Nulliparous women were those for whom the current pregnancy was their first and only; parous women were defined as those with any previously confirmed pregnancy, regardless of outcome or duration. Mann-Whitney tests were performed to test for differences in DHA status in all samples collected between the nulliparous and parous participants (Table 36).

Those with a previous obstetric history did not differ in maternal DHA status during gestation or at birth from those with no previous obstetric history. Placental tissue and breast milk DHA were also similar in both groups. Cord plasma DHA did, however, differ between the groups: DHA accounted for a higher % TFA (p=0.02), and was of a higher concentration (p=0.01), in cord plasma obtained from nulliparous women.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Nulliparous | | Parous | | p | Nulliparous | | Parous | | p |
| RBC 15 weeks | 2.9 | (n=41) | 2.7 | (n=55) | 0.23 | 179 | (n=41) | 125 | (n=55) | 0.63 |
| RBC 28 weeks | 3.8 | (n=28) | 3.9 | (n=35) | 0.56 | 192 | (n=28) | 187 | (n=35) | 0.92 |
| RBC Birth | 2.6 | (n=26) | 2.7 | (n=31) | 0.64 | 153 | (n=26) | 150 | (n=31) | 0.69 |
| RBC Cord | 4.4 | (n=23) | 4.1 | (n=33) | 0.12 | 282 | (n=23) | 219 | (n=33) | 0.09 |
| Plasma 15 weeks | 1.6 | (n=41) | 1.6 | (n=56) | 0.49 | 132 | (n=41) | 134 | (n=56) | 0.51 |
| Plasma 28 weeks | 1.9 | (n=28) | 1.8 | (n=35) | 0.26 | 213 | (n=28) | 181 | (n=35) | 0.13 |
| Plasma Birth | 1.3 | (n=26) | 1.4 | (n=33) | 0.96 | 158 | (n=26) | 163 | (n=33) | 0.82 |
| Plasma Cord | 3.6 | (n=23) | 2.8 | (n=33) | *0.02* | 701 | (n=23) | 350 | (n=33) | *0.01* |
| Placental Tissue | 3.6 | (n=21) | 3.6 | (n=32) | 0.74 | 6408 | (n=21) | 5171 | (n=32) | 0.32 |
| Cord Tissue | 6.3 | (n=20) | 5.9 | (n=33) | 0.28 | | | | | |
| Breast Milk | 0.3 | (n=10) | 0.2 | (n=16) | 0.77 | 3710 | (n=10) | 2711 | (n=16) | 0.98 |

Table 36. Median values for DHA as a % TFA and its concentration (nmol/ml for RBC, plasma and breast milk, nmol/g for placental tissue) in samples from nulliparous and parous participants.

### 9.1.2 DHA and Interval between Pregnancies

For parous participants, the interval between their last previous pregnancy and the current pregnancy was calculated in years. Classification was then made on the basis of whether the interval was of more or less than 1 year. Mann-Whitney tests were performed between those with an interval of 1 year or less between pregnancies, and those with more than 1 year between pregnancies (Table 37).

There were no differences in DHA status of RBC, plasma, tissue or breast milk samples between those whose last previous pregnancy was less than 1 year before the current pregnancy, and those whose last previous pregnancy was more than 1 year before.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 year or less | | More than 1 | | p | 1 year or less | | More than 1 | | p |
| RBC 15 weeks | 3.1 | (n=14) | 2.7 | (n=33) | 0.09 | 172 | (n=14) | 125 | (n=33) | 0.18 |
| RBC 28 weeks | 3.8 | (n=8) | 4.0 | (n=25) | 0.85 | 187 | (n=8) | 187 | (n=25) | 0.66 |
| RBC Birth | 2.50 | (n=6) | 2.8 | (n=22) | 0.19 | 95 | (n=6) | 161 | (n=22) | 0.12 |
| RBC Cord | 3.8 | (n=7) | 4.1 | (n=23) | 0.59 | 209 | (n=7) | 228 | (n=23) | 0.22 |
| Plasma 15 weeks | 1.6 | (n=15) | 1.8 | (n=33) | 0.49 | 109 | (n=15) | 140 | (n=33) | 0.20 |
| Plasma 28 weeks | 1.6 | (n=8) | 1.8 | (n=25) | 0.34 | 179 | (n=8) | 192 | (n=25) | 0.95 |
| Plasma Birth | 1.30 | (n=8) | 1.3 | (n=22) | 0.91 | 168 | (n=8) | 153 | (n=22) | 0.66 |
| Plasma Cord | 2.8 | (n=7) | 2.5 | (n=23) | 0.49 | 428 | (n=7) | 222 | (n=23) | 0.49 |
| Placental Tissue | 3.6 | (n=7) | 3.6 | (n=22) | 0.61 | 6990 | (n=7) | 5467 | (n=22) | 0.40 |
| Cord Tissue | 5.8 | (n=8) | 5.9 | (n=22) | 0.29 | | | | | |
| Breast Milk | 0.2 | (n=4) | 0.3 | (n=10) | 0.10 | 1603 | (n=4) | 2980 | (n=22) | 0.14 |

Table 37. Median values for DHA as a % TFA and its concentration (nmol/ml for RBC, plasma and breast milk, nmol/g for placental tissue) in samples from parous participants whose last previous pregnancy was 1 year or less, or more than 1 year, prior to the current pregnancy.

### 9.1.3 DHA and Frequency of Fish Consumption

Frequency of dietary fish intake was recorded at 15 weeks gestation, 28 weeks gestation and birth. At each time, fish intake frequency was classified as either: never or less than once per week, or once or more per week. Mann-Whitney tests were performed to determine differences in DHA status between those who consumed fish less than once per week, and those who consumed fish once or more per week at each time (Tables 38-40).

Consumption of fish once or more per week was associated with a higher % of DHA in both maternal RBC (p=0.003) and plasma (p=0.02) at 15 weeks. DHA concentration was not significantly higher at 15 weeks in those who consumed fish once or more per week. Moreover, weekly fish consumption was not associated with higher DHA status after supplementation began *i.e.* at either 28 weeks or birth.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Never or less than once per week | | Once or more per week | | p | Never or less than once per week | | Once or more per week | | p |
| RBC 15 weeks | 2.4 | (n=44) | 3.1 | (n=52) | *0.003* | 106 | (n=44) | 147 | (n=52) | 0.33 |
| Plasma 15 weeks | 1.6 | (n=45) | 1.8 | (n=52) | *0.02* | 124 | (n=45) | 144 | (n=52) | 0.14 |

Table 38. Median values for DHA as a % TFA and its concentration (nmol/ml) in samples from participants whose consumption of fish was classified as (1) never or less than once per week, or (2) once or more per week, at 15 weeks gestation.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Never or less than once per week | | Once or more per week | | p | Never or less than once per week | | Once or more per week | | p |
| RBC 28 weeks | 3.8 | (n=24) | 3.9 | (n=39) | 0.63 | 189 | (n=24) | 187 | (n=39) | 0.69 |
| Plasma 28 weeks | 1. 7 | (n=24) | 1.9 | (n=39) | 0.14 | 199 | (n=24) | 208 | (n=39) | 0.35 |

Table 39. Median values for DHA as a % TFA and its concentration (nmol/ml) in samples from participants whose consumption of fish was classified as (1) never or less than once per week, or (2) once or more per week, at 28 weeks gestation.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Never or less than once per week | | Once or more per week | | p | Never or less than one per week | | Once or more per week | | p |
| RBC Birth | 2.8 | (n=16) | 2.7 | (n=35) | 0.58 | 161 | (n=16) | 152 | (n=35) | 0.54 |
| RBC Cord | 4.3 | (n=17) | 4.3 | (n=34) | 0.65 | 233 | (n=17) | 262 | (n=34) | 0.29 |
| Plasma Birth | 1.4 | (n=17) | 1.3 | (n=36) | 0.75 | 146 | (n=17) | 179 | (n=36) | 0.10 |
| Plasma Cord | 2.5 | (n=17) | 3.1 | (n=34) | 0.07 | 222 | (n=17) | 528 | (n=34) | 0.07 |
| Placental Tissue | 3.6 | (n=17) | 3.7 | (n=32) | 0.35 | 6408 | (n=17) | 5939 | (n=32) | 0.89 |
| Cord Tissue | 5.8 | (n=16) | 6.3 | (n=32) | 0.23 | | | | | |
| Breast Milk | 0.20 | (n=8) | 0.3 | (n=16) | 0.28 | 2561 | (n=8) | 3503 | (n=16) | 0.69 |

Table 40. Median values for DHA as a % TFA and its concentration (nmol/ml for RBC, plasma and breast milk, nmol/g for placental tissue) in samples from participants whose consumption of fish was classified as (1) never or less than once per week, or (2) once or more per week, at time of birth.

### 9.1.4 DHA and Recent Fish Consumption

Most recent fish consumption prior to obtaining a maternal blood sample at both 15 weeks and 28 weeks gestation was determined as to whether it occurred within the 24-hour period prior to sampling. Thus, consumption was classified as either more than 24 hours before sampling, or 24 hours or less prior to sampling. Mann-Whitney tests were performed to determine any differences in DHA status between those who had consumed fish and those who had not, in the previous 24 hours (Tables 41 & 42).

Fish intake in the 24 hours prior to sampling was associated with a higher DHA status (both as a % TFA and a concentration) in maternal plasma at 15 weeks gestation. However, this effect was not observed at 28 weeks, following the commencement of supplementation.

The effect of recent fish intake on DHA status was not tested on the samples obtained at birth. The reasons for this were: (a) maternal blood samples were not always obtained within 24 hours of birth and therefore fish intake prior to delivery was not always equivalent to fish intake prior to sampling; (b) some mothers did not consume any food in the 24 hours prior to delivery, and (c) maternal recall of intake in the 24 hours preceding delivery/ sampling was often questionable, given the circumstances.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | More than 24 Hours | | 24 Hours or Less | | p | More than 24 Hours | | 24 Hours or Less | | p |
| RBC 15 weeks | 2.8 | (n=69) | 2.9 | (n=27) | 0.10 | 144 | (n=69) | 137 | (n=27) | 0.37 |
| Plasma 15 weeks | 1.6 | (n=70) | 1.9 | (n=27) | *0.004* | 122 | (n=70) | 154 | (n=27) | *0.005* |

Table 41. Median values for DHA as a % TFA and its concentration (nmol/ml) in samples from participants who had consumed fish more than 24 hours prior to sampling, or 24 hours or less before sampling at 15 weeks gestation.

| | % TFA | | | | | Concentration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | More than 24 Hours | | 24 Hours or Less | | p | More than 24 Hours | | 24 Hours or Less | | p |
| RBC 28 weeks | 3.8 | (n=35) | 4.2 | (n=12) | 0.63 | 177 | (n=35) | 198 | (n=12) | 0.68 |
| Plasma 28 weeks | 1.7 | (n=35) | 1.8 | (n=12) | 0.14 | 208 | (n=35) | 231 | (n=12) | 0.35 |

Table 42. Median values for DHA as a % TFA and its concentration (nmol/ml) in samples from participants who had consumed fish more than 24 hours prior to sampling, or 24 hours or less before sampling at 28 weeks gestation.

### 9.1.5 Concluding Remarks

DHA status in the study population varied with underlying factors as expected. Maternal DHA status has not been consistently related to parity in previous studies (Al *et al* 1997, van Houwelingen *et al* 1999); the effect of parity on neonatal status has been noted previously (Al *et al* 1997). The variation in plasma and RBC DHA with fish consumption has been demonstrated consistently (Anttolainen *et al* 1996, Ma *et al* 1995b, Romon *et al* 1995). These findings indicate that the relationship between DHA and parity or diet is physiologically appropriate and that the study population

are therefore "normal". Since there were no significant differences between the fish oil and placebo groups in these or other factors, such as anthropometry, gestational length *etc.*, these factors were not considered further by, for example, multivariate analysis.

## 9.2 ANALYSES BETWEEN FISH OIL AND PLACEBO GROUPS

### 9.2.1 RBC Fatty Acids: % Total Fatty Acids (Relative Levels) (Figure 16)

9.2.1(a) Maternal RBC at 15 weeks gestation (Table 43)

The two randomised groups were not significantly different in the % of total fatty acids (% TFA) for any fatty acids measured, in their baseline RBC samples. DHA accounted for median 2.8% TFA in the fish oil group, and 2.9% TFA in the placebo group (p=0.80).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C14:0 | 0.2 | (0.2, 0.3) | 0.0 - 0.7 | 0.0 | (0.1, 0.3) | 0.0 - 0.6 | 0.41 |
| C16:0 | 32.4 | (32.0, 33.8) | 27.1 - 40.8 | 32.9 | (32.5, 34.4) | 28.2 - 41.2 | 0.52 |
| C16:1n-7 | 0.1 | (0.1, 0.4) | 0.0 - 3.2 | 0.1 | (0.1, 0.3) | 0.0 - 3.9 | 0.56 |
| C18:0 | 16.8 | (16.5, 17.5) | 13.7 - 22.1 | 16.9 | (16.7, 17.4) | 15.3 - 19.9 | 0.88 |
| C18:1n-9 | 13.4 | (13.4, 14.5) | 11.0 - 18.5 | 13.2 | (13.0, 13.8) | 10.3 - 18.4 | 0.12 |
| C18:2n-6 | 9.1 | (8.8, 9.6) | 6.7 - 11.8 | 9.0 | (8.7, 9.3) | 6.8 - 12.0 | 0.49 |
| C18:3n-3 | 0.0 | (0.0, 0.0) | 0.0 - 0.6 | 0.0 | (0.0, 0.0) | 0.0 - 0.7 | |
| C20:0 | 0.5 | (0.3, 0.5) | 0.0 - 92.0 | 0.6 | (0.3, 0.6) | 0.0 - 0.8 | 0.19 |
| C20:3n-6 | 1.2 | (1.0, 1.2) | 0.0 - 2.2 | 1.0 | (1.0, 1.1) | 0.0 - 2.1 | 0.20 |
| C20:4n-6 | 10.9 | (10.6, 11.4) | 6.9 - 14.9 | 10.9 | (10.4, 11.4) | 6.4 - 13.8 | 0.89 |
| C20:5n-3 | 0.1 | (0.1, 0.2) | 0.0 - 0.7 | 0.0 | (0.1, 0.2) | 0.0 - 0.6 | 0.25 |
| C22:4n-6 | 1.9 | (1.6, 2.1) | 0.0 - 4.0 | 1.7 | (1.6, 2.1) | 0.4 - 4.1 | 0.74 |
| C22:5n-6 | 0.0 | (0.0, 0.1) | 0.0 - 0.4 | 0.0 | (0.0, 0.0) | 0.0 - 0.3 | |
| C24:0 | 3.7 | (3.1, 3.9) | 0.0 - 5.5 | 3.9 | (3.2, 4.1) | 0.4 - 6.4 | 0.56 |
| C24:1n-9 | 4.3 | (4.0, 4.7) | 1.1 - 6.9 | 4.6 | (4.1, 4.9) | 1.8 - 7.7 | 0.78 |
| C22:5n-3 | 1.0 | (0.9, 1.2) | 0.0 - 1.9 | 0.9 | (0.9, 1.1) | 0.0 - 1.7 | 0.60 |
| C22:6n-3 | 2.8 | (2.4, 3.0) | 1.0 - 6.7 | 2.9 | (2.5, 3.1) | 0.9 - 4.6 | 0.80 |
| Total n-3 | 3.9 | (3.4, 4.4) | 1.0 - 8.7 | 4.0 | (3.4, 4.3) | 1.1 - 6.6 | 0.86 |
| Total n-6 | 23.5 | (22.7, 23.9) | 18.0 - 26.8 | 23.0 | (22.2, 23.5) | 15.7 - 27.3 | 0.29 |
| Ratio n6/n3 | 5.8 | (5.6, 7.5) | 3.1 - 19.9 | 5.8 | (5.6, 7.2) | 3.5 - 19.1 | 0.92 |

Table 43. Percentage total fatty acids in maternal RBC total lipids at 15 weeks gestation for both groups (n=47 for fish oil group, n=49 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.1(b) Maternal RBC at 28 weeks gestation (Table 44)

Following supplementation for (mean) 13 weeks, the groups differed in DHA as a % TFA in their RBC. The fish oil group had a significantly higher % DHA (p=0.003), with a median of 4.2%, compared to the placebo group (median 3.3%).

The fish oil receiving mothers had a significantly higher % TFA (p=0.02) for total n-3 fatty acids (median 5.7%), compared to the placebo group (median 5.0% TFA). As a result, the fish oil group had a significantly lower (p=0.008) ratio of n6 to n3 fatty acids, with a median of 4.0, compared to 4.6 in the placebo group.

The treatment groups also differed significantly (p=0.02) in the % of arachidonic acid (AA, 20:4n-6). The fish oil group was significantly lower, with a median level of 10.6% TFA, in comparison to the placebo group (median 11.2%).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C14:0 | 0.4 | (0.4, 0.5) | 0.0 - 0.6 | 0.4 | (0.3, 0.4) | 0.0 - 0.7 | 0.29 |
| C16:0 | 31.4 | (30.8, 32.0) | 28.6 - 34.7 | 31.4 | (30.6, 31.6) | 28.8 - 36.1 | 0.53 |
| C16:1n-7 | 0.7 | (0.6, 0.8) | 0.0 - 3.9 | 0.6 | (0.5, 0.7) | 0.0 - 1.1 | 0.20 |
| C18:0 | 16.0 | (15.8, 16.4) | 14.5 - 17.9 | 16.1 | (15.8, 16.4) | 13.4 - 19.0 | 0.99 |
| C18:1n-9 | 12.9 | (12.6, 13.4) | 11.6 - 18.9 | 13.4 | (12.9, 13.6) | 11.2 - 18.4 | 0.22 |
| C18:2n-6 | 8.7 | (8.3, 9.2) | 6.9 - 12.7 | 8.7 | (8.3, 8.9) | 7.4 - 10.9 | 0.68 |
| C18:3n-3 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C20:0 | 0.6 | (0.5, 0.6) | 0.4 - 0.7 | 0.6 | (0.5, 0.6) | 0.0 - 0.8 | 0.63 |
| C20:3n-6 | 1.5 | (1.3, 1.5) | 0.7 - 2.0 | 1.4 | (1.4, 1.7) | 0.9 - 2.6 | 0.30 |
| C20:4n-6 | 10.6 | (10.1, 10.9) | 7.9 - 12.7 | 11.2 | (10.8, 11.5) | 9.0 - 12.6 | *0.02* |
| C20:5n-3 | 0.3 | (0.2, 0.3) | 0.0 - 0.6 | 0.2 | (0.2, 0.3) | 0.0 - 0.6 | 0.37 |
| C22:4n-6 | 1.9 | (1.7, 2.0) | 0.9 - 2.6 | 2.2 | (1.9, 2.3) | 0.8 - 3.3 | 0.07 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C24:0 | 4.1 | (3.8, 4.3) | 1.9 - 5.5 | 4.2 | (3.9, 4.3) | 2.2 - 5.3 | 0.78 |
| C24:1n-9 | 5.2 | (4.8, 5.3) | 3.6 - 8.8 | 5.1 | (4.9, 5.4) | 3.4 - 6.4 | 0.79 |
| C22:5n-3 | 1.3 | (1.2, 1.4) | 0.7 - 1.7 | 1.3 | (1.2, 1.5) | 0.0 - 1.9 | 0.34 |
| C22:6n-3 | 4.2 | (3.9, 4.4) | 2.3 - 5.6 | 3.3 | (3.2, 3.8) | 2.4 - 5.4 | *0.003* |
| Total n-3 | 5.7 | (5.3, 6.1) | 2.9 - 7.6 | 5.0 | (4.7, 5.5) | 2.4 - 7.3 | *0.02* |
| Total n-6 | 23.1 | (22.2, 23.4) | 17.2 - 24.9 | 23.5 | (22.8, 24.0) | 20.2 - 26.3 | 0.10 |
| Ratio n6/n3 | 4.0 | (3.8, 4.4) | 3.0 - 7.1 | 4.6 | (4.4, 5.3) | 2.9 - 9.5 | *0.01* |

Table 44. Percentage total fatty acids in maternal RBC total lipids at 28 weeks gestation for both groups (n=30 for fish oil group, n=33 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.1(c) Maternal RBC at Birth (Table 45)

At delivery, maternal RBC DHA accounted for 3.1% TFA in the fish oil group, and 2.4% in the placebo group. This difference was significant at p=0.01.

Total n-3 fatty acids accounted for a significantly higher % TFA (p=0.02) in the fish oil group (median 3.9% TFA) than the placebo group (median 3.3% TFA). The ratio of n-6 to n-3 fatty acids was therefore lower in the fish oil group, with a median of 4.9 compared to the placebo group median of 6.2 (p=0.01).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C14:0 | 0.3 | (0.2, 0.3) | 0.0 - 0.6 | 0.3 | (0.2, 0.4) | 0.0 - 0.8 | 0.19 |
| C16:0 | 35.1 | (34.1, 36.1) | 31.3 - 43.0 | 35.0 | (34.4, 36.6) | 30.8 - 40.6 | 0.67 |
| C16:1n-7 | 0.6 | (0.4, 0.7) | 0.0 - 4.2 | 0.6 | (0.5, 0.8) | 0.0 - 4.4 | 0.43 |
| C18:0 | 16.1 | (15.6, 16.7) | 12.8 - 18.3 | 16.1 | (14.9, 16.4) | 11.1 - 17.8 | 0.45 |
| C18:1n-9 | 14.7 | (14.4, 15.6) | 12.6 - 19.5 | 15.1 | (14.9, 16.4) | 13.5 - 19.4 | 0.10 |
| C18:2n-6 | 8.7 | (8.3, 8.9) | 6.0 - 11.5 | 8.5 | (8.2, 8.9) | 7.5 - 10.2 | 0.72 |
| C18:3n-3 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C20:0 | 0.5 | (0.5, 0.5) | 0.0 - 0.7 | 0.5 | (0.3, 0.5) | 0.0 - 0.8 | 0.17 |
| C20:3n-6 | 1.0 | (0.9, 1.2) | 0.4 - 1.6 | 1.0 | (0.9, 1.2) | 0.4 - 2.2 | 0.92 |
| C20:4n-6 | 8.5 | (7.5, 9.1) | 1.8 - 10.5 | 9.2 | (7.9, 9.6) | 3.9 - 11.5 | 0.27 |
| C20:5n-3 | 0.0 | (0.0, 0.1) | 0.0 - 0.3 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | 0.23 |
| C22:4n-6 | 1.2 | (0.9, 1.2) | 0.0 - 2.0 | 1.1 | (1.0, 1.3) | 0.0 - 2.0 | 0.52 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C24:0 | 3.6 | (3.5, 4.3) | 2.8 - 6.6 | 3.7 | (3.5, 4.2) | 2.5 - 6.6 | 0.88 |
| C24:1n-9 | 5.4 | (5.1, 5.9) | 2.9 - 9.2 | 5.0 | (4.8, 5.9) | 3.9 - 7.9 | 0.34 |
| C22:5n-3 | 0.9 | (0.7, 1.0) | 0.0 - 1.4 | 0.8 | (0.6, 0.9) | 0.0 - 1.4 | 0.49 |
| C22:6n-3 | 3.1 | (2.5, 3.5) | 0.3 - 4.8 | 2.4 | (2.0, 2.7) | 0.5 - 4.0 | *0.01* |
| Total n-3 | 3.9 | (3.3, 4.5) | 0.5 - 6.3 | 3.3 | (2.7, 3.6) | 0.7 - 5.3 | *0.02* |
| Total n-6 | 19.8 | (18.1, 20.3) | 9.7 - 22.3 | 19.9 | (18.5, 20.7) | 13.0 - 23.1 | 0.60 |
| Ratio n6/n3 | 4.9 | (4.5, 6.6) | 3.1 - 23.1 | 6.2 | (5.8, 7.6) | 3.7 - 17.6 | *0.01* |

Table 45. Percentage total fatty acids in maternal RBC total lipids at birth for both groups (n=29 for fish oil group, n=28 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.1(d) Umbilical Cord RBC (Table 46)

Umbilical cord RBC from the two groups did not differ significantly in the % TFA of DHA (fish oil group median 4.4%, placebo group median 4.1%, p=0.28), or indeed of other fatty acids.

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C14:0 | 0.4 | (0.2, 0.4) | 0.0 - 0.6 | 0.4 | (0.2, 0.4) | 0.0 - 0.7 | 0.55 |
| C16:0 | 34.8 | (34.4, 35.9) | 29.7 - 38.9 | 35.1 | (34.6, 36.2) | 32.2 - 41.0 | 0.63 |
| C16:1n-7 | 0.4 | (0.3, 0.4) | 0.0 - 4.6 | 0.3 | (0.3, 0.4) | 0.0 - 4.3 | 0.80 |
| C18:0 | 18.1 | (17.4, 18.5) | 13.2 - 20.0 | 18.1 | (17.4, 18.3) | 15.3 - 19.9 | 0.63 |
| C18:1n-9 | 11.0 | (10.8, 11.5) | 9.3 - 13.0 | 11.1 | (10.5, 11.5) | 8.8 - 13.7 | 0.76 |
| C18:2n-6 | 3.4 | (3.2, 4.0) | 2.2 - 6.4 | 3.5 | (3.3, 3.9) | 2.4 - 7.2 | 0.85 |
| C18:3n-3 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.4 | |
| C20:0 | 0.7 | (0.7, 0.8) | 0.0 - 1.0 | 0.8 | (0.7, 0.8) | 0.5 - 1.0 | 0.51 |
| C20:3n-6 | 2.0 | (1.9, 2.3) | 1.0 - 3.1 | 2.1 | (2.0, 2.4) | 1.2 - 3.0 | 0.50 |
| C20:4n-6 | 12.4 | (11.6, 12.7) | 9.2 - 14.5 | 12.4 | (11.8, 12.8) | 8.2 - 14.3 | 0.66 |
| C20:5n-3 | 0.0 | (0.0, 0.1) | 0.0 - 0.1 | 0.0 | (0.0, 0.1) | 0.0 - 0.3 | 0.88 |
| C22:4n-6 | 2.5 | (2.3, 2.7) | 1.5 - 3.6 | 2.7 | (2.5, 2.9) | 1.4 - 3.6 | 0.08 |
| C22:5n-6 | 0.3 | (0.2, 0.4) | 0.0 - 0.9 | 0.3 | (0.3, 0.5) | 0.0 - 1.0 | |
| C24:0 | 5.9 | (3.5, 6.1) | 0.6 - 8.1 | 5.6 | (3.2, 5.7) | 0.6 - 8.3 | 0.29 |
| C24:1n-9 | 4.1 | (3.9, 4.4) | 2.8 - 5.7 | 4.5 | (4.1, 4.7) | 3.2 - 5.9 | 0.13 |
| C22:5n-3 | 0.3 | (0.2, 0.4) | 0.0 - 0.9 | 0.3 | (0.2, 0.4) | 0.0 - 0.9 | 0.78 |
| C22:6n-3 | 4.4 | (4.1, 4.8) | 1.7 - 5.8 | 4.1 | (3.9, 4.5) | 1.8 - 6.2 | 0.28 |
| Total n-3 | 4.6 | (4.3, 5.2) | 1.7 - 6.2 | 4.7 | (4.1, 5.0) | 1.8 - 7.1 | 0.42 |
| Total n-6 | 20.8 | (20.1, 21.3) | 16.7 - 23.1 | 21.4 | (20.7, 21.8) | 14.0 - 2.4 | 0.22 |
| Ratio n6/n3 | 4.7 | (4.1, 4.8) | 3.0 - 11.6 | 4.6 | (4.4, 5.3) | 2.9 - 7.6 | 0.18 |

Table 46. Percentage total fatty acids in umbilical cord RBC total lipids for both groups (n=27 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

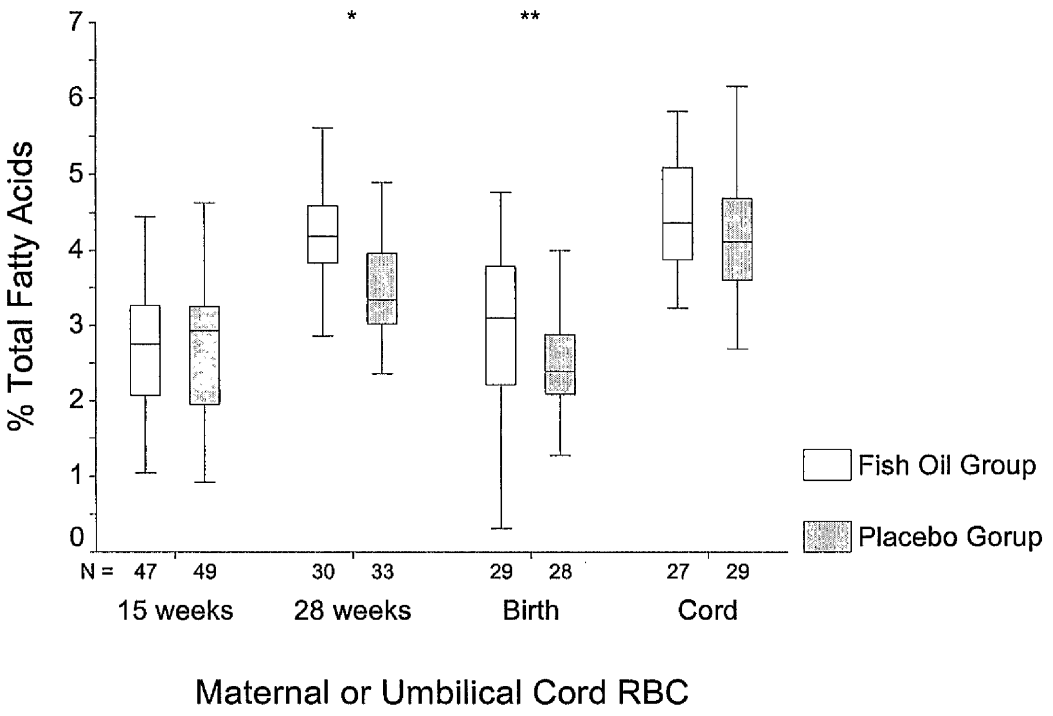



Figure 16. % DHA (median & quartiles) in RBC of both groups.

Significant differences between groups - * p=0.02, **p=0.01.

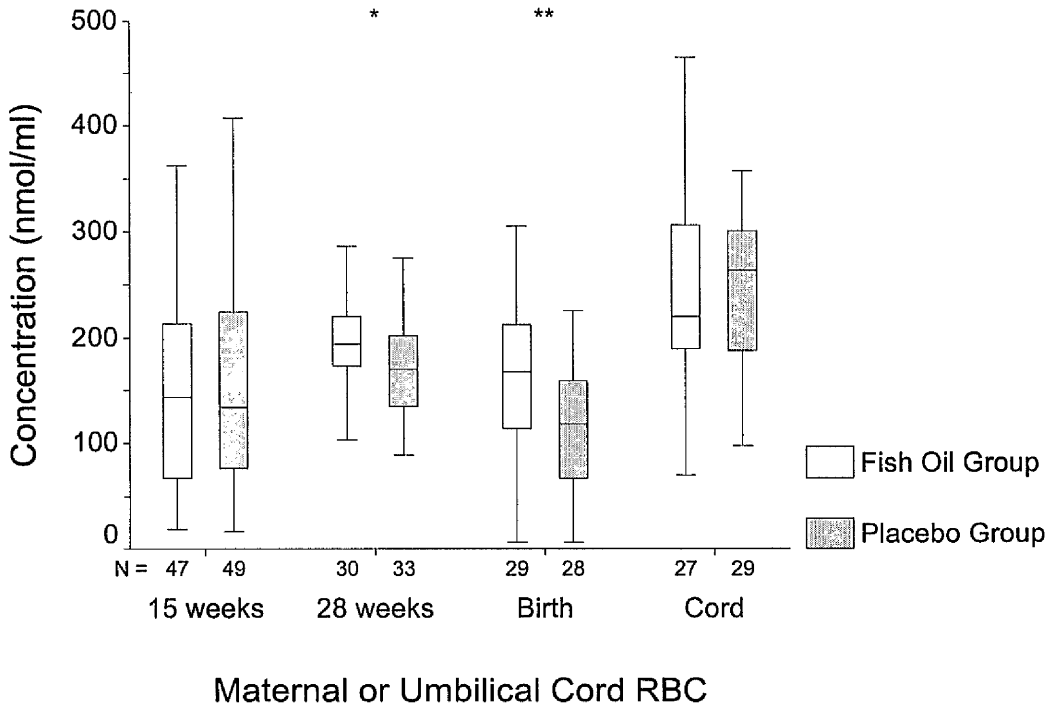



Figure 17. DHA concentration (median & quartiles) in RBC of both gropus. Significant differences between groups - *p=0.02, **p=0.02.

### 9.2.2 RBC Fatty Acids: Concentration (Absolute Levels) (Figure 17)

9.2.2(a) Maternal RBC at 15 weeks gestation (Table 47)

Baseline concentrations of all fatty acids measured were similar in the RBC of both treatment groups. RBC DHA had a concentration of 144 nmol/ml in the fish oil group, and 134 nmol/ml in the placebo group (p=0.98).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C12:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C14:0 | 7 | (5, 12) | 0 - 31 | 2 | (4, 8) | 0 - 37 | 0.38 |
| C16:0 | 855 | (850, 998) | 473 - 2522 | 873 | (840, 981) | 295 - 1912 | 0.87 |
| C16:1n-7 | 7 | (6, 11) | 0 - 92 | 5 | (3, 9) | 0 - 218 | 0.32 |
| C18:0 | 383 | (362, 419) | 184 - 1119 | 372 | (346, 406) | 117 - 751 | 0.51 |
| C18:1n-9 | 366 | (348, 418) | 235 - 759 | 353 | (327, 397) | 120 - 675 | 0.38 |
| C18:2n-6 | 272 | (238, 284) | 149 - 527 | 230 | (220, 267) | 82 - 471 | 0.41 |
| C18:3n-3 | 0 | (0, 0) | 0 - 45 | 0 | (0, 0) | 0 - 30 | |
| C20:0 | 10 | (7, 12) | 0 - 32 | 11 | (8, 12) | 0 - 23 | 0.64 |
| C20:3n-6 | 111 | (120, 255) | 0 - 837 | 142 | (136, 217) | 0 - 674 | 0.68 |
| C20:4n-6 | 326 | (302, 381) | 118 - 600 | 319 | (283, 374) | 84 - 646 | 0.59 |
| C20:5n-3 | 5 | (6, 15) | 0 - 57 | 0 | (3, 13) | 0 - 64 | 0.39 |
| C22:4n-6 | 151 | (116, 183) | 0 - 521 | 112 | (110, 186) | 11 - 489 | 0.96 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 55 | (47, 120) | 0 - 293 | 38 | (36, 123) | 0 - 294 | 1.00 |
| C22:6n-3 | 144 | (122, 179) | 19 - 363 | 134 | (122, 185) | 16 - 407 | 0.98 |
| Total n-3 | 205 | (177, 294) | 30 - 676 | 169 | (159, 273) | 23 - 695 | 0.66 |
| Total n-6 | 893 | (805, 1099) | 344 - 2295 | 827 | (758, 1048) | 196 - 1934 | 0.51 |
| Ratio n6/n3 | 5 | (4, 6) | 2 - 14 | 5 | (4, 6) | 2 - 20 | 0.96 |

Table 47. Total fatty acid concentrations (nmol/ml) in maternal RBC total lipids at 15 weeks gestation for both groups (n=47 for fish oil group, n=49 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.2(b) Maternal RBC at 28 weeks gestation (Table 48)

At 28 weeks gestation, maternal DHA concentration in RBC was significantly higher (p=0.02) in the fish oil group. The fish oil group had a median concentration of 194 nmol/ml of DHA, while the placebo group had a median value of 171 nmol/ml.

There were no other significant differences in RBC fatty acid concentrations between the groups at 28 weeks, including total n-3 fatty acids. However, the ratio of n-6 to n-3 fatty acids was significantly lower (p=0.01) in the fish oil group (median ratio 2.8) than the placebo group (median ratio 3.4).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C12:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C14:0 | 12 | (11, 14) | 0 - 20 | 11 | (9, 13) | 0 - 24 | 0.38 |
| C16:0 | 885 | (860, 917) | 701 - 1101 | 859 | (826, 911) | 533 - 1104 | 0.39 |
| C16:1n-7 | 16 | (14, 18) | 0 - 107 | 15 | (12, 17) | 0 - 29 | 0.28 |
| C18:0 | 369 | (353, 390) | 303 - 477 | 369 | (351, 384) | 162 - 508 | 0.93 |
| C18:1n-9 | 366 | (345, 392) | 279 - 504 | 365 | (346, 392) | 202 - 489 | 0.95 |
| C18:2n-6 | 253 | (239, 278) | 173 - 342 | 250 | (236, 262) | 132 - 334 | 0.50 |
| C18:3n-3 | 0 | (0, 1) | 0 - 3 | 0 | (0, 1) | 0 - 4 | |
| C20:0 | 11 | (10, 12) | 7 - 17 | 11 | (9, 12) | 0 - 19 | 0.99 |
| C20:3n-6 | 126 | (113, 140) | 67 - 180 | 134 | (116, 158) | 60 - 254 | 0.56 |
| C20:4n-6 | 264 | (250, 279) | 172 - 362 | 277 | (262, 298) | 121 - 366 | 0.17 |
| C20:5n-3 | 11 | (9, 14) | 0 - 23 | 11 | (7, 12) | 0 - 23 | 0.42 |
| C22:4n-6 | 105 | (91, 112) | 46 - 141 | 121 | (101, 127) | 33 - 181 | 0.12 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 58 | (51, 61) | 28 - 75 | 60 | (52, 66) | 0 - 90 | 0.34 |
| C22:6n-3 | 194 | (181, 211) | 103 - 297 | 171 | (153, 185) | 89 - 275 | *0.02* |
| Total n-3 | 257 | (244, 287) | 131 - 378 | 246 | (213, 262) | 104 - 364 | 0.12 |
| Total n-6 | 753 | (708, 788) | 493 - 1010 | 780 | (723, 839) | 371 - 1040 | 0.45 |
| Ratio n6/n3 | 3 | (3, 3) | 2 - 5 | 3 | (3, 4) | 2 - 6 | *0.01* |

Table 48. Total fatty acid concentrations (nmol/ml) in maternal RBC total lipids at 28 weeks gestation for both groups (n=30 for fish oil group, n=33 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.2(c) Maternal RBC at Birth (Table 49)

At the time of delivery, maternal RBC concentration of DHA remained significantly higher in the fish oil group (p=0.02), with a median value of 168 nmol/ml; the placebo group had a median of 118 nmol/ml. The summed concentrations of total n-3 fatty acids were higher (p=0.03) in the fish oil group with a total median of 207 nmol/ml, as opposed to 149 nmol/ml in the placebo group.

Differences in the concentrations of other fatty acids remained insignificant. However, the fish oil group did have a significantly lower (p=0.005) ratio of n-6 to n-3 fatty acids (median ratio 3.2) than the placebo group (median ratio 4.0).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 3 | |
| C12:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C14:0 | 10 | (5, 11) | 0 - 18 | 10 | (7, 12) | 0 - 23 | 0.46 |
| C16:0 | 1000 | (906, 1063) | 485 - 1408 | 971 | (827, 1013) | 254 - 1476 | 0.63 |
| C16:1n-7 | 16 | (10, 18) | 0 - 109 | 14 | (9, 17) | 0 - 112 | 0.68 |
| C18:0 | 380 | (337, 413) | 129 - 572 | 377 | (287, 397) | 70 - 514 | 0.45 |
| C18:1n-9 | 421 | (379, 466) | 198 - 676 | 432 | (368, 476) | 95 - 747 | 0.97 |
| C18:2n-6 | 251 | (227, 290) | 91 - 402 | 242 | (203, 270) | 64 - 411 | 0.36 |
| C18:3n-3 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 3 | |
| C20:0 | 11 | (8, 11) | 0 - 18 | 9 | (6, 10) | 0 - 15 | 0.17 |
| C20:3n-6 | 81 | (74, 116) | 21 - 196 | 91 | (69, 106) | 19 - 202 | 0.74 |
| C20:4n-6 | 225 | (190, 262) | 19 - 393 | 236 | (175, 260) | 34 - 372 | 0.96 |
| C20:5n-3 | 0 | (0, 7) | 0 - 19 | 0 | (0, 2) | 0 - 13 | 0.24 |
| C22:4n-6 | 57 | (51, 78) | 0 - 126 | 69 | (50, 82) | 0 - 133 | 0.85 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 40 | (31, 49) | 0 - 78 | 32 | (23, 41) | 0 - 74 | 0.19 |
| C22:6n-3 | 168 | (128, 194) | 6 - 305 | 118 | (92, 143) | 6 - 226 | *0.02* |
| Total n-3 | 207 | (164, 244) | 9 - 393 | 149 | (118, 185) | 11 - 298 | *0.03* |
| Total n-6 | 639 | (555, 742) | 131 - 1065 | 612 | (499, 709) | 117 - 1040 | 0.63 |
| Ratio n6/n3 | 3 | (3, 4) | 2 - 15 | 4 | (4, 5) | 2 - 12 | *0.005* |

Table 49. Total fatty acid concentrations (nmol/ml) in maternal RBC total lipids at birth for both groups (n=29 for fish oil group, n=28 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.2(d) Umbilical Cord RBC (Table 50)

Umbilical cord RBC from the two treatment groups were not significantly different in the concentrations of any fatty acids. Indeed, cord RBC from the placebo group were slightly higher (median 263 nmol/ml) in DHA concentration than cord RBC from the fish oil group (221 nmol/ml), but the difference was not significant (p=0.63).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C12:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C14:0 | 11 | (6, 12) | 0 - 19 | 12 | (7, 13) | 0 - 24 | 0.42 |
| C16:0 | 784 | (728, 906) | 532 - 1375 | 874 | (832, 989) | 359 - 4838 | 0.06 |
| C16:1n-7 | 13 | (11, 17) | 0 - 261 | 14 | (12, 17) | 0 - 169 | 0.76 |
| C18:0 | 328 | (304, 387) | 179 - 575 | 368 | (341, 389) | 0 - 501 | 0.29 |
| C18:1n-9 | 278 | (253, 330) | 179 - 579 | 306 | (283, 356) | 135 - 1947 | 0.14 |
| C18:2n-6 | 85 | (80, 106) | 46 - 161 | 98 | (87, 118) | 27 - 554 | 0.43 |
| C18:3n-3 | 0 | (0, 0) | 0 - 3 | 0 | (0, 0) | 0 - 12 | |
| C20:0 | 12 | (11, 16) | 0 - 28 | 16 | (14, 18) | 6 - 38 | 0.12 |
| C20:3n-6 | 482 | (416, 584) | 153 - 939 | 573 | (500, 662) | 188 - 1244 | 0.18 |
| C20:4n-6 | 349 | (321, 426) | 239 - 734 | 420 | (384, 465) | 154 - 690 | 0.06 |
| C20:5n-3 | 2 | (1, 4) | 0 - 13 | 2 | (1, 6) | 0 - 41 | 0.96 |
| C22:4n-6 | 195 | (175, 251) | 85 - 515 | 251 | (217, 295) | 111 - 598 | 0.14 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 26 | (16, 33) | 0 - 110 | 19 | (11, 33) | 0 - 66 | 0.78 |
| C22:6n-3 | 221 | (207, 283) | 70 - 465 | 263 | (225, 288) | 98 - 478 | 0.63 |
| Total n-3 | 255 | (228, 310) | 70 - 481 | 296 | (243, 311) | 104 - 542 | 0.81 |
| Total n-6 | 1150 | (1008, 1371) | 613 - 2121 | 1373 | (1199, 1525) | 480 - 3086 | 0.10 |
| Ratio n6/n3 | 5 | (4, 5) | 3 - 9 | 5 | (5, 6) | 3 - 8 | 0.07 |

Table 50. Total fatty acid concentrations (nmol/ml) in umbilical cord RBC total lipids for both groups (n=27 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

### 9.2.3 Plasma Fatty Acids: % Total Fatty Acids (Relative Levels) (Figure 18)

9.2.3(a) Maternal Plasma at 15 weeks gestation (Table 51)

Initial values of % TFA did not differ significantly in the plasma samples of the treatment groups. DHA accounted for 1.5% TFA in the fish oil group, and 1.7% TFA in the placebo group (p=0.15).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C12:0 | 0.1 | (0.1, 0.2) | 0.0 - 0.6 | 0.1 | (0.1, 0.1) | 0.0 - 1.2 | |
| C14:0 | 1.4 | (1.3, 1.6) | 0.6 - 2.4 | 1.2 | (1.1, 1.4) | 0.3 - 3.0 | 0.13 |
| C16:0 | 29.7 | (29.2, 30.6) | 24.0 - 35.0 | 30.2 | (29.4, 30.4) | 25.3 - 34.2 | 0.91 |
| C16:1n-7 | 2.3 | (2.1, 2.6) | 0.9 - 4.6 | 1.9 | (1.8, 2.2) | 1.1 - 4.3 | 0.17 |
| C18:0 | 7.4 | (7.2, 7.8) | 5.8 - 10.0 | 7.5 | (7.1, 7.6) | 5.0 - 10.8 | 0.44 |
| C18:1n-9 | 20.4 | (20.0, 21.5) | 15.4 - 26.7 | 19.8 | (19.1, 20.8) | 14.6 - 25.8 | 0.14 |
| C18:2n-6 | 27.3 | (25.4, 28.1) | 18.9 - 38.0 | 27.3 | (26.7, 28.7) | 18.9 - 36.9 | 0.25 |
| C18:3n-3 | 0.5 | (0.5, 0.6) | 0.1 - 0.9 | 0.5 | (0.4, 0.5) | 0.2 - 1.1 | 0.38 |
| C20:0 | 0.3 | (0.3, 0.4) | 0.0 - 0.6 | 0.3 | (0.3, 0.4) | 0.2 - 0.6 | 0.78 |
| C20:3n-6 | 1.6 | (1.4, 1.7) | 0.9 - 2.4 | 1.5 | (1.5, 1.7) | 0.8 - 3.3 | 0.81 |
| C20:4n-6 | 5.4 | (4.9, 5.7) | 3.3 - 7.8 | 5.4 | (5.2, 5.8) | 3.1 - 9.0 | 0.38 |
| C20:5n-3 | 0.3 | (0.3, 0.4) | 0.0 - 2.0 | 0.3 | (0.3, 0.4) | 0.0 - 1.2 | 0.59 |
| C22:4n-6 | 0.1 | (0.1, 0.2) | 0.0 - 0.4 | 0.2 | (0.1, 0.2) | 0.0 - 0.4 | 0.79 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C24:0 | 0.2 | (0.2, 0.3) | 0.0 - 0.7 | 0.3 | (0.2, 0.3) | 0.0 - 0.8 | 0.29 |
| C24:1n-9 | 0.9 | (0.7, 0.9) | 0.0 - 1.5 | 0.9 | (0.8, 1.0) | 0.1 - 1.8 | 0.19 |
| C22:5n-3 | 0.2 | (0.2, 0.2) | 0.0 - 0.5 | 0.2 | (0.2, 0.2) | 0.0 - 0.4 | 0.49 |
| C22:6n-3 | 1.5 | (1.4, 1.8) | 0.8 - 2.9 | 1.7 | (1.6, 1.9) | 0.7 - 3.2 | 0.15 |
| Total n-3 | 2.5 | (2.4, 3.0) | 1.2 - 5.6 | 2.7 | (2.5, 3.0) | 1.6 - 4.7 | 0.57 |
| Total n-6 | 35.0 | (32.5, 35.4) | 25.9 - 42.4 | 35.1 | (34.0, 36.0) | 26.0 - 43.7 | 0.23 |
| Ratio n6/n3 | 13.4 | (12.0, 14.9) | 5.8 - 30.1 | 12.7 | (12.1, 14.6) | 7.7 - 21.5 | 0.98 |

Table 51. Percentage total fatty acids in maternal plasma total lipids at 15 weeks gestation for both groups (n=48 for fish oil group, n=49 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.3(b) Maternal Plasma at 28 weeks (Table 52)

There was no significant difference in DHA between groups, with medians of 1.8% TFA and 1.6% TFA for the fish oil group and placebo groups respectively (p=0.09).

By 28 weeks, the placebo group had a significantly higher % TFA (p=0.01) of dihomo-γ-linolenic acid (DHGLA, 20:3n-6), compared to the fish oil group (median 1.5% *vs.* median 1.4%). The fish oil group had a significantly higher (p=0.05) % of total n-3 fatty acids, with a median of 3.1% compared to 2.6% in the placebo group. The ratio of n-6 to n-3 fatty acids was lower (p=0.04) in the fish oil group (median ratio 10.1) than the placebo group (median ratio 12.2).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Media** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C12:0 | 0.1 | (0.1, 0.2) | 0.0 - 0.1 | 0.1 | (0.1, 0.1) | 0.1 - 0.7 | |
| C14:0 | 1.6 | (1.4, 1.8) | 0.7 - 1.7 | 1.6 | (1.4, 1.7) | 1.4 - 2.3 | 0.43 |
| C16:0 | 31.6 | (30.5, 31.9) | 16.4 - 31.6 | 30.7 | (30.0, 31.6) | 30.0 - 36.4 | 0.48 |
| C16:1n-7 | 2.5 | (2.2, 3.0) | 0.9 - 2.9 | 2.5 | (2.3, 2.9) | 2.3 - 4.7 | 0.90 |
| C18:0 | 6.6 | (6.3, 6.9) | 3.4 - 6.6 | 6.5 | (6.3, 6.6) | 6.3 - 8.0 | 0.61 |
| C18:1n-9 | 21.5 | (20.4, 22.9) | 9.8 - 22.1 | 21.1 | (20.7, 22.1) | 20.7 - 25.3 | 0.59 |
| C18:2n-6 | 25.4 | (24.4, 27.3) | 18.3 - 27.7 | 27.1 | (25.6, 27.7) | 25.6 - 31.4 | 0.33 |
| C18:3n-3 | 0.6 | (0.7, 0.7) | 0.3 - 0.7 | 0.6 | (0.5, 0.7) | 0.5 - 1.3 | 0.26 |
| C20:0 | 0.3 | (0.3, 0.4) | 0.2 - 0.4 | 0.3 | (0.3, 0.4) | 0.3 - 0.6 | 0.43 |
| C20:3n-6 | 1.4 | (1.3, 1.5) | 0.9 - 1.7 | 1.5 | (1.5, 1.7) | 1.5 - 2.2 | *0.01* |
| C20:4n-6 | 4.2 | (4.0, 4.8) | 2.4 - 5.0 | 4.6 | (4.4, 5.0) | 4.4 - 7.6 | 0.24 |
| C20:5n-3 | 0.3 | (0.3, 0.5) | 0.2 - 0.4 | 0.3 | (0.3, 0.4) | 0.3 - 1.8 | 0.14 |
| C22:4n-6 | 0.1 | (0.1, 0.1) | 0.0 - 0.1 | 0.1 | (0.1, 0.1) | 0.1 - 0.4 | 0.90 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C24:0 | 0.1 | (0.1, 0.1) | 0.0 - 0.1 | 0.1 | (0.1, 0.1) | 0.1 - 0.4 | 0.41 |
| C24:1n-9 | 0.8 | (0.7, 0.9) | 0.1 - 0.8 | 0.7 | (0.5, 0.8) | 0.5 - 1.2 | 0.29 |
| C22:5n-3 | 0.2 | (0.2, 0.2) | 0.0 - 0.2 | 0.2 | (0.1, 0.2) | 0.1 - 0.4 | 0.37 |
| C22:6n-3 | 1.8 | (1.7, 2.1) | 1.0 - 1.9 | 1.6 | (1.5, 1.9) | 1.5 - 2.9 | 0.09 |
| Total n-3 | 3.1 | (2.9, 3.4) | 2.0 - 3.2 | 2.6 | (2.4, 3.2) | 2.4 - 5.7 | *0.05* |
| Total n-6 | 31.7 | (30.1, 33.4) | 23.7 - 34.2 | 33.3 | (32.1, 34.2) | 32.1 - 38.0 | 0.13 |
| Ratio n6/n3 | 10.1 | (9.6, 11.2) | 7.0 - 14.2 | 12.2 | (11.1, 14.2) | 11.1 - 27.0 | *0.02* |

Table 52. Percentage total fatty acids in maternal plasma total lipids at 28 weeks gestation maternal plasma for both groups (n=30 for fish oil group, n=33 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.3(c) Maternal Plasma at Birth (Table 53)

Maternal plasma DHA as a % TFA was not significantly different at the time of delivery between groups: the fish oil group had median 1.4% DHA, the placebo group 1.1% (p=0.06).

Maternal plasma % of palmitic acid (16:0) was significantly lower (p=0.04) in the fish oil group (median 32.5%) than the placebo group (median 33.7%).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C14:0 | 1.0 | (0.9, 1.1) | 0.5 - 1.8 | 1.0 | (0.9, 1.1) | 0.3 - 1.7 | 0.22 |
| C16:0 | 32.5 | (31.9, 33.1) | 28.9 - 35.5 | 33.7 | (32.8, 34.5) | 29.0 - 38.4 | *0.04* |
| C16:1n-7 | 2.6 | (2.4, 3.1) | 1.6 - 4.6 | 3.0 | (2.8, 3.5) | 1.7 - 5.5 | 0.10 |
| C18:0 | 6.4 | (6.0, 6.5) | 4.8 - 7.7 | 6.1 | (5.8, 6.3) | 4.3 - 8.6 | 0.12 |
| C18:1n-9 | 23.1 | (22.4, 24.9) | 18.0 - 31.2 | 23.8 | (22.8, 24.9) | 19.1 - 30.3 | 0.67 |
| C18:2n-6 | 26.2 | (24.3, 27.1) | 18.7 - 32.1 | 24.2 | (23.0, 25.4) | 19.2 - 28.8 | 0.08 |
| C18:3n-3 | 0.5 | (0.4, 0.6) | 0.0 - 1.3 | 0.5 | (0.4, 0.6) | 0.2 - 1.1 | 0.96 |
| C20:0 | 0.3 | (0.1, 0.3) | 0.0 - 0.4 | 0.2 | (0.1, 0.3) | 0.0 - 0.4 | 0.34 |
| C20:3n-6 | 1.2 | (1.1, 0.3) | 0.7 - 2.0 | 1.3 | (1.1, 1.3) | 0.8 - 1.9 | 0.64 |
| C20:4n-6 | 3.7 | (3.5, 4.3) | 1.9 - 6.3 | 3.9 | (3.7, 4.2) | 2.5 - 5.3 | 0.63 |
| C20:5n-3 | 0.2 | (0.2, 0.3) | 0.0 - 0.6 | 0.2 | (0.1, 0.2) | 0.0 - 0.6 | 0.23 |
| C22:4n-6 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | 0.0 | (0.0, 0.1) | 0.0 - 0.1 | 0.80 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C24:0 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | 0.70 |
| C24:1n-9 | 0.6 | (0.5, 0.7) | 0.0 - 1.1 | 0.6 | (0.5, 0.7) | 0.0 - 1.0 | 0.64 |
| C22:5n-3 | 0.1 | (0.1, 0.1) | 0.0 - 0.2 | 0.1 | (0.1, 0.1) | 0.0 - 0.2 | 0.42 |
| C22:6n-3 | 1.4 | (1.2, 1.6) | 0.2 - 2.2 | 1.1 | (1.0, 1.4) | 0.6 - 2.6 | 0.06 |
| Total n-3 | 2.4 | (1.9, 2.5) | 0.2 - 3.7 | 1.9 | (1.7, 2.2) | 0.8 - 3.9 | 0.07 |
| Total n-6 | 30.7 | (29.5, 32.1) | 24.5 - 37.0 | 29.5 | (28.1, 30.7) | 23.3 - 34.5 | 0.17 |
| Ratio n6/n3 | 13.9 | (12.7, 17.2) | 9.5 - 124.7 | 15.1 | (14.1, 20.5) | 6.8 - 31.5 | 0.16 |

Table 53. Percentage total fatty acids in maternal plasma total lipids at birth for both groups (n=30 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.3(d) Umbilical Cord Plasma (Table 54)

Plasma samples from the umbilical cord of the two groups did not differ significantly in the percentage contribution of any fatty acids. Cord plasma from both the fish oil and placebo groups had a DHA level of 3.0% TFA.

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.2 | |
| C14:0 | 1.0 | (0.9, 1.0) | 0.3 - 1.7 | 1.0 | (1.0, 1.1) | 0.7 - 1.5 | 0.10 |
| C16:0 | 33.9 | (33.3, 34.5) | 30.5 - 37.2 | 33.9 | (33.4, 35.0) | 31.4 - 39.1 | 0.72 |
| C16:1n-7 | 4.4 | (4.2, 5.0) | 2.6 - 6.9 | 4.7 | (4.4, 5.2) | 2.1 - 7.8 | 0.53 |
| C18:0 | 11.2 | (10.6, 11.7) | 6.8 - 13.1 | 10.8 | (10.0, 11.2) | 6.0 - 12.2 | 0.11 |
| C18:1n-9 | 19.1 | (18.1, 19.7) | 13.9 - 24.9 | 17.7 | (17.4, 19.7) | 15.0 - 23.4 | 0.47 |
| C18:2n-6 | 10.9 | (10.5, 13.3) | 7.7 - 23.9 | 11.2 | (10.4, 12.0) | 7.8 - 26.3 | 0.77 |
| C18:3n-3 | 0.1 | (0.1, 0.2) | 0.0 - 0.8 | 0.1 | (0.1, 0.2) | 0.0 - 1.0 | 0.81 |
| C20:0 | 0.6 | (0.5, 0.7) | 0.0 - 1.0 | 0.6 | (0.5, 0.6) | 0.0 - 0.9 | 0.47 |
| C20:3n-6 | 2.6 | (2.4, 2.9) | 0.4 - 3.8 | 2.5 | (2.3, 2.9) | 1.4 - 4.3 | 0.63 |
| C20:4n-6 | 10.6 | (9.1, 11.2) | 1.9 - 14.3 | 11.1 | (9.8, 11.7) | 4.1 - 14.8 | 0.38 |
| C20:5n-3 | 0.3 | (0.2, 0.3) | 0.0 - 1.1 | 0.2 | (0.1, 0.2) | 0.0 - 0.8 | 0.07 |
| C22:4n-6 | 0.4 | (0.2, 0.4) | 0.0 - 0.8 | 0.4 | (0.3, 0.5) | 0.0 - 1.3 | 0.24 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.2 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | |
| C24:0 | 0.2 | (0.1, 0.4) | 0.0 - 1.0 | 0.2 | (0.1, 0.4) | 0.0 - 1.0 | 0.78 |
| C24:1n-9 | 1.1 | (0.8, 1.3) | 0.0 - 2.2 | 0.9 | (0.8, 1.2) | 0.0 - 2.1 | 0.64 |
| C22:5n-3 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.91 |
| C22:6n-3 | 3.0 | (2.5, 3.4) | 0.4 - 6.0 | 3.0 | (2.3, 3.2) | 1.0 - 5.1 | 0.49 |
| Total n-3 | 3.3 | (3.1, 3.9) | 0.4 - 6.8 | 3.3 | (2.7, 3.6) | 1.1 - 6.2 | 0.41 |
| Total n-6 | 25.8 | (24.5, 26.2) | 17.8 - 31.6 | 26.0 | (24.7, 26.9) | 18.6 - 31.9 | 0.46 |
| Ratio n6/n3 | 7.5 | (6.6, 8.7) | 3.9 - 62.5 | 8.2 | (7.4, 10.7) | 4.2 - 18.6 | 0.19 |

Table 54. Percentage total fatty acids in umbilical cord plasma total lipids for both groups (n=27 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

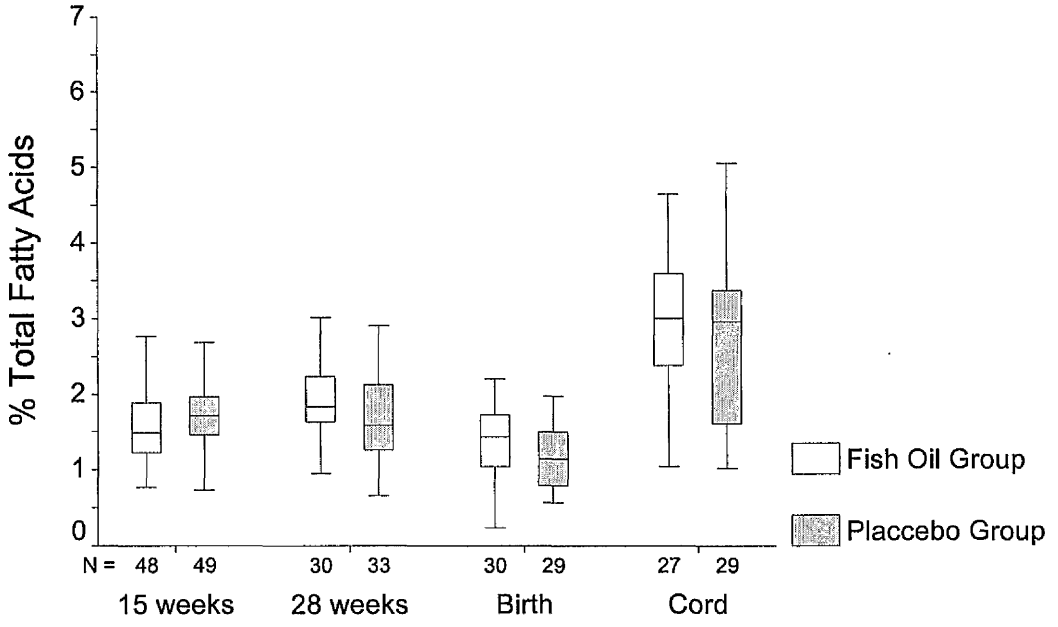



Figure 18. % DHA (median & quartiles) in plasma of both groups.

No significant differences between groups.

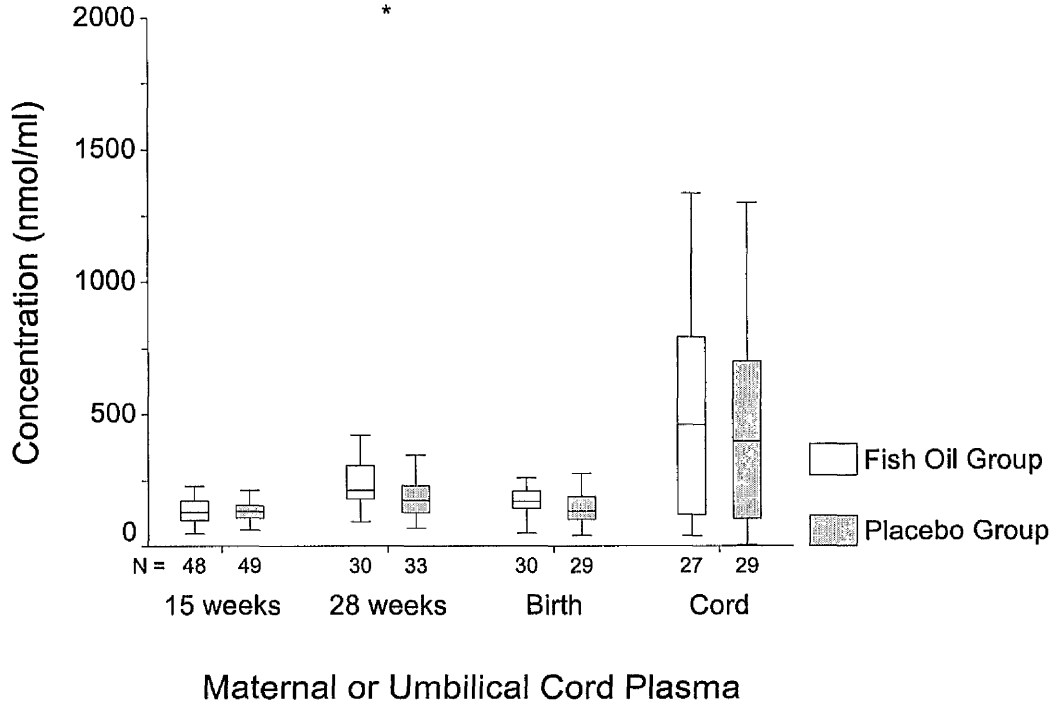



Figure 19. DHA concentration (median & quartiles) in plasma of both groups. Significant difference between groups - *p=0.019.

### 9.2.4 Plasma Fatty Acids: Concentration (Absolute Levels) (Figure 19)

9.2.4(a) Maternal Plasma at 15 weeks gestation (Table 55)

The concentrations of DHA in the plasma of the treatment groups were remarkably similar at baseline: the fish oil group had a median DHA concentration of 132 nmol/ml, while the placebo group had a corresponding median of 134 nmol/ml (p=0.98). There were no differences between the groups in the concentrations of total n-3 or total n-6 fatty acids, or in the n-6/n-3 ratio.

Baseline plasma samples differed between groups in the concentrations of saturated fatty acids. The fish oil group had a higher median concentration than the placebo group for the saturates: myristic acid (14:0) (p= 0.02, 92 nmol/ml *vs.* 77 nmol/ml); palmitic acid (16:0) (p= 0.02, 1953 nmol/ml *vs.* 1618 nmol/ml); and stearic acid (18:0) (p= 0.02, 356 nmol/ml *vs.* 322 nmol/ml).

The fish oil group also had higher concentrations of the monounsaturates palmitoleic acid (16:1n-9) (p=0.03, median 125 nmol/ml *vs.* 101 nmol/ml), and oleic acid (18:1n-9) (p=0.004, median 1221 nmol/ml *vs.* 1054 nmol/ml).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 9 | 0 | (0, 0) | 0 - 9 | |
| C12:0 | 8 | (8, 13) | 0 - 50 | 5 | (4, 8) | 0 - 118 | |
| C14:0 | 92 | (85, 108) | 27 - 190 | 77 | (66, 90) | 14 - 195 | *0.02* |
| C16:0 | 1953 | (1785, 2017) | 1028 - 2806 | 1618 | (1563, 1803) | 837 - 2846 | *0.02* |
| C16:1n-7 | 125 | (113, 149) | 46 - 242 | 101 | (91, 116) | 38 - 238 | *0.03* |
| C18:0 | 356 | (345, 395) | 228 - 562 | 322 | (298, 348) | 157 - 642 | *0.02* |
| C18:1n-9 | 1221 | (1136, 1328) | 659 - 2142 | 1054 | (950, 1129) | 154 - 1897 | *0.004* |
| C18:2n-6 | 1579 | (1477, 1726) | 715 - 2458 | 1437 | (1350, 1565) | 880 - 2468 | 0.09 |
| C18:3n-3 | 61 | (52, 66) | 11 - 98 | 50 | (44, 57) | 14 - 110 | 0.06 |
| C20:0 | 13 | (12, 14) | 0 - 28 | 12 | (11, 13) | 4 - 28 | 0.15 |
| C20:3n-6 | 272 | (257, 311) | 140 - 511 | 238 | (234, 296) | 116 - 540 | 0.27 |
| C20:4n-6 | 237 | (218, 260) | 110 - 412 | 220 | (206, 249) | 113 - 386 | 0.35 |
| C20:5n-3 | 23 | (21, 33) | 0 - 109 | 24 | (21, 29) | 0 - 101 | 0.93 |
| C22:4n-6 | 12 | (10, 17) | 0 - 40 | 13 | (10, 15) | 0 - 31 | 0.70 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 14 | (13, 17) | 0 - 37 | 13 | (11, 15) | 0 - 29 | 0.24 |
| C22:6n-3 | 132 | (123, 152) | 48 - 230 | 134 | (124, 148) | 62 - 241 | 0.98 |
| Total n-3 | 233 | (217, 262) | 121 - 396 | 229 | (203, 247) | 52 - 413 | 0.36 |
| Total n-6 | 2141 | (2007, 2286) | 1135 - 3119 | 1867 | (1829, 2138) | 1133 - 3293 | 0.09 |
| Ratio n6/n3 | 9 | (8, 10) | 5 - 20 | 9 | (8, 10) | 6 - 15 | 0.93 |

Table 55. Total fatty acid concentrations (nmol/ml) in maternal plasma total lipids at 15 weeks gestation for both groups (n=48 for fish oil group, n=49 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.4(b) Maternal plasma at 28 weeks gestation (Table 56)

Following supplementation, the differences between groups in saturated and monounsaturated fatty acids were no longer significant. The fish oil group had higher concentrations of two n-3 polyunsaturates, EPA (eicosapentaenoic acid/ timnodonic acid, 20:5n-3) and DHA, and consequently, total n-3 fatty acids.

The difference in EPA concentration between the fish oil group (median 33 nmol/ml) and the placebo group (median 27 nmol/ml) was significant at p=0.04. Median DHA concentration in the fish oil group was 215 nmol/ml; compared to the placebo group median of 176 nmol/ml, this difference was significant at p=0.02. Total n-3 fatty acids had a median concentration of 359 nmol/ml in the fish oil group, and 275 nmol/ml in the placebo group (p=0.006). The ratio of n-6 to n-3 fatty acids was lower (p=0.003) in the fish oil group (median ratio 7), compared to the placebo group (median ratio 8).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 9 | 0 | (0, 0) | 0 - 25 | |
| C12:0 | 18 | (12, 24) | 0 - 69 | 11 | (8, 15) | 0 - 79 | |
| C14:0 | 157 | (140, 179) | 73 - 254 | 148 | (124, 159) | 54 - 220 | 0.20 |
| C16:0 | 2807 | (2655, 3084) | 1786 - 4401 | 2546 | (2425, 2939) | 1196 - 4375 | 0.10 |
| C16:1n-7 | 226 | (179, 238) | 77 - 368 | 183 | (165, 221) | 48 - 477 | 0.35 |
| C18:0 | 425 | (375, 456) | 243 - 653 | 374 | (339, 403) | 211 - 532 | 0.07 |
| C18:1n-9 | 1621 | (1464, 1790) | 973 - 2631 | 1501 | (1317, 1580) | 773 - 2651 | 0.14 |
| C18:2n-6 | 1886 | (1679, 2103) | 891 - 8163 | 1645 | (1498, 1881) | 820 - 2715 | 0.16 |
| C18:3n-3 | 82 | (68, 110) | 19 - 285 | 66 | (54, 82) | 15 - 184 | 0.10 |
| C20:0 | 19 | (18, 23) | 12 - 67 | 19 | (17, 22) | 7 - 45 | 0.50 |
| C20:3n-6 | 343 | (322, 382) | 191 - 776 | 365 | (335, 414) | 214 - 562 | 0.44 |
| C20:4n-6 | 254 | (244, 310) | 126 - 757 | 303 | (253, 308) | 155 - 483 | 0.86 |
| C20:5n-3 | 33 | (30, 45) | 14 - 349 | 27 | (22, 37) | 0 - 128 | *0.04* |
| C22:4n-6 | 9 | (8, 15) | 0 - 37 | 9 | (6, 13) | 0 - 38 | 0.66 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 18 | (15, 21) | 4 - 95 | 15 | (12, 19) | 0 - 48 | 0.18 |
| C22:6n-3 | 215 | (205, 265) | 93 - 572 | 176 | (155, 217) | 70 - 460 | *0.02* |
| Total n-3 | 359 | (334, 426) | 212 - 1302 | 275 | (256, 354) | 98 - 624 | *0.01* |
| Total n-6 | 2499 | (2286, 2772) | 1431 - 9722 | 2387 | (2124, 2589) | 1261 - 3584 | 0.36 |
| Ratio n6/n3 | 7 | (6, 7) | 4 - 11 | 8 | (7, 9) | 3 - 16 | *0.03* |

Table 56. Total fatty acid concentrations (nmol/ml) in maternal plasma total lipids at 28 weeks gestation for both groups (n=30 for fish oil group, n=33 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.4(c) Maternal Plasma at Birth (Table 57)

Maternal plasma concentrations of all fatty acids did not differ significantly between treatment groups at the time of delivery. Plasma from the fish oil group did have a higher concentration of DHA (median 172 nmol/ml) than the placebo group (median 135 nmol/ml), but the difference was not significant (p=0.11).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 0 | |
| C12:0 | 0 | (0, 4) | 0 - 14 | 0 | (0, 4) | 0 - 16 | |
| C14:0 | 78 | (70, 96) | 21 - 235 | 96 | (77, 107) | 18 - 172 | 0.37 |
| C16:0 | 2749 | (2404, 3032) | 929 - 4638 | 2572 | (2400, 2963) | 1460 - 6282 | 0.90 |
| C16:1n-7 | 178 | (155, 216) | 60 - 425 | 217 | (186, 244) | 71 - 396 | 0.12 |
| C18:0 | 445 | (380, 467) | 168 - 634 | 413 | (359, 448) | 169 - 777 | 0.49 |
| C18:1n-9 | 1853 | (1610, 2107) | 621 - 3406 | 1750 | (1594, 2003) | 946 - 5282 | 0.77 |
| C18:2n-6 | 2006 | (1830, 2305) | 817 - 3577 | 1830 | (1640, 2157) | 1089 - 3732 | 0.25 |
| C18:3n-3 | 60 | (53, 79) | 0 - 202 | 63 | (57, 82) | 21 - 127 | 0.64 |
| C20:0 | 13 | (8, 15) | 0 - 28 | 13 | (8, 15) | 0 - 25 | 0.85 |
| C20:3n-6 | 296 | (255, 340) | 101 - 527 | 308 | (275, 356) | 167 - 679 | 0.43 |
| C20:4n-6 | 251 | (219, 289) | 107 - 455 | 258 | (227, 288) | 122 - 531 | 0.74 |
| C20:5n-3 | 23 | (17, 31) | 0 - 91 | 19 | (12, 26) | 0 - 61 | 0.32 |
| C22:4n-6 | 0 | (0, 8) | 0 - 17 | 0 | (0, 7) | 0 - 23 | 0.91 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 12 | (8, 16) | 0 - 54 | 10 | (6, 13) | 0 - 24 | 0.35 |
| C22:6n-3 | 172 | (152, 207) | 29 - 1954 | 135 | (119, 179) | 41 - 346 | 0.11 |
| Total n-3 | 281 | (237, 336) | 30 - 2184 | 232 | (206, 295) | 70 - 510 | 0.25 |
| Total n-6 | 2569 | (2360, 2919) | 1025 - 4107 | 2384 | (2171, 2805) | 1377 - 4817 | 0.40 |
| Ratio n6/n3 | 9 | (8, 11) | 2 - 89 | 10 | (9, 14) | 5 - 20 | 0.16 |

Table 57. Total fatty acid concentrations (nmol/ml) in maternal plasma total lipids at birth for both groups (n=30 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

9.2.4(d) Umbilical Cord Plasma (Table 58)

Fatty acid concentrations in umbilical cord plasma from the fish oil group did not differ significantly from those of the placebo group. DHA concentrations were higher in the cord plasma of the fish oil group (median 460 nmol/ml) compared to the placebo group (median 397 nmol/ml), but the difference was not significant (p=0.50).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 0 | 0 | (0, 0) | 0 - 10 | |
| C12:0 | 0 | (0, 1) | 0 - 6 | 0 | (0, 1) | 0 - 10 | |
| C14:0 | 22 | (19, 26) | 3 - 69 | 26 | (23, 34) | 3 - 95 | 0.10 |
| C16:0 | 833 | (710, 932) | 362 - 1908 | 827 | (770, 1016) | 94 - 2773 | 0.45 |
| C16:1n-7 | 73 | (69, 95) | 40 - 178 | 87 | (83, 109) | 12 - 153 | 0.07 |
| C18:0 | 198 | (172, 230) | 55 - 313 | 208 | (183, 232) | 24 - 381 | 0.83 |
| C18:1n-9 | 401 | (350, 463) | 175 - 1204 | 398 | (368, 506) | 38 - 1821 | 0.72 |
| C18:2n-6 | 265 | (233, 313) | 78 - 1398 | 256 | (233, 346) | 24 - 2282 | 0.96 |
| C18:3n-3 | 3 | (2, 5) | 0 - 41 | 3 | (2, 4) | 0 - 90 | 0.82 |
| C20:0 | 11 | (8, 12) | 0 - 25 | 10 | (8, 11) | 0 - 19 | 0.99 |
| C20:3n-6 | 76 | (61, 101) | 5 - 269 | 89 | (82, 136) | 15 - 256 | 0.12 |
| C20:4n-6 | 147 | (109, 157) | 11 - 204 | 165 | (138, 183) | 18 - 271 | 0.17 |
| C20:5n-3 | 8 | (5, 10) | 0 - 15 | 5 | (4, 7) | 0 - 14 | 0.17 |
| C22:4n-6 | 10 | (6, 12) | 0 - 20 | 12 | (9, 15) | 0 - 27 | 0.12 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 4 | (3, 14) | 0 - 34 | 8 | (5, 13) | 0 - 39 | 0.87 |
| C22:6n-3 | 460 | (354, 660) | 40 - 1335 | 397 | (268, 555) | 5 - 1299 | 0.50 |
| Total n-3 | 467 | (358, 685) | 40 - 1384 | 416 | (281, 583) | 5 - 1346 | 0.47 |
| Total n-6 | 519 | (435, 577) | 151 - 1654 | 559 | (493, 692) | 61 - 2601 | 0.27 |
| Ratio n6/n3 | 1 | (1, 3) | 0 - 7 | 2 | (1, 5) | 0 - 13 | 0.14 |

Table 58. Total fatty acid concentrations (nmol/ml) in umbilical cord plasma total lipids for both groups (n=27 for fish oil group, n=29 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

**9.2.5 Placental Tissue Fatty Acids: % Total Fatty Acids (Relative Levels)** (Figure 20, Table 59)

DHA accounted for remarkably similar % TFA in the placental tissue of the two treatment groups. Placenta from the fish oil group had median 3.6% DHA, while that from the placebo group had median 3.5% DHA (p=0.11).

Myristic acid (14:0) in placenta tissue from the fish oil group (median 0.41%) was of a lower % TFA (p=0.02) than in the placebo group (0.45%).

The ratio of n-6 to n-3 fatty acids was lower in the fish oil group (median 8.0) than the placebo group (median 8.5), a difference significant at p=0.04.

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.2 | |
| C12:0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | |
| C14:0 | 0.4 | (0.3, 0.4) | 0.0 - 0.6 | 0.5 | (0.4, 0.5) | 0.2 - 0.7 | *0.02* |
| C16:0 | 31.7 | (31.2, 32.8) | 26.9 - 35.3 | 32.5 | (31.9, 33.0) | 28.1 - 34.1 | 0.23 |
| C16:1n-7 | 0.8 | (0.8, 1.0) | 0.5 - 1.5 | 0.9 | (0.8, 1.0) | 0.5 - 3.0 | 0.56 |
| C18:0 | 14.8 | (14.5, 15.4) | 13.5 - 21.7 | 14.4 | (14.2, 14.9) | 13.4 - 17.1 | 0.15 |
| C18:1n-9 | 10.1 | (9.8, 10.4) | 8.6 - 11.3 | 10.4 | (10.2, 10.6) | 9.2 - 11.8 | 0.21 |
| C18:2n-6 | 9.9 | (9.4, 10.4) | 6.1 - 12.4 | 9.6 | (9.4, 10.2) | 8.2 - 12.6 | 0.52 |
| C18:3n-3 | 0.1 | (0.0, 0.1) | 0.0 - 0.6 | 0.0 | (0.0, 0.1) | 0.0 - 0.4 | 0.18 |
| C20:0 | 0.3 | (0.2, 0.5) | 0.0 - 1.0 | 0.3 | (0.2, 0.3) | 0.0 - 1.2 | 0.13 |
| C20:3n-6 | 4.4 | (4.2, 5.0) | 3.5 - 6.4 | 4.5 | (4.4, 5.0) | 3.3 - 7.0 | 0.80 |
| C20:4n-6 | 18.2 | (17.7, 19.1) | 14.7 - 21.9 | 18.9 | (18.5, 19.4) | 17.2 - 21.3 | 0.12 |
| C20:5n-3 | 0.1 | (0.1, 0.2) | 0.0 - 0.4 | 0.1 | (0.1, 0.1) | 0.0 - 0.4 | 0.67 |
| C22:4n-6 | 1.0 | (0.9, 1.0) | 0.4 - 1.4 | 1.0 | (0.9, 1.1) | 0.6 - 2.8 | 0.33 |
| C22:5n-6 | 0.1 | (0.1, 0.1) | 0.0 - 0.3 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.26 |
| C24:0 | 1.4 | (1.2, 1.7) | 0.4 - 5.0 | 1.2 | (1.1, 1.4) | 0.1 - 2.1 | 0.16 |
| C24:1n-9 | 1.0 | (0.9, 1.3) | 0.1 - 3.7 | 1.1 | (1.0, 1.2) | 0.7 - 2.0 | 0.53 |
| C22:5n-3 | 0.5 | (0.4, 0.5) | 0.3 - 0.8 | 0.5 | (0.4, 0.5) | 0.2 - 0.8 | 0.72 |
| C22:6n-3 | 3.6 | (3.6, 4.0) | 2.1 - 5.5 | 3.5 | (3.1, 3.7) | 2.1 - 5.3 | 0.11 |
| Total n-3 | 4.3 | (4.1, 4.7) | 2.8 - 6.5 | 4.1 | (3.6, 4.4) | 2.4 - 5.8 | 0.07 |
| Total n-6 | 34.5 | (33.7, 34.8) | 26.5 - 35.7 | 34.6 | (34.1, 35.2) | 32.8 - 37.4 | 0.34 |
| Ratio n6/n3 | 8.0 | (7.4, 8.2) | 5.5 - 10.2 | 8.5 | (8.1, 9.9) | 5.7 - 14.3 | *0.04* |

Table 59. Percentage total fatty acids in placental tissue total lipids for both groups (n=26 for fish oil group, n=27 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

**9.2.6 Placental Tissue Fatty Acids: Concentration (Absolute Levels)** (Figure 21, Table 60)

Placental tissue DHA concentrations were high for both groups (median 6707 nmol/g in the fish oil group, 5190 nmol/g in the placebo group), although the difference between them was not significant (p=0.12).

Myristic acid (14:0) concentration was lower (p=0.04) in the placental tissue of the fish oil group (median 58 nmol/g) than the placebo group (median 67 nmol/g).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0 | (0, 0) | 0 - 37 | 0 | (0, 0) | 0 - 38 | |
| C12:0 | 0 | (0, 1) | 0 - 3 | 0 | (0, 2) | 0 - 8 | |
| C14:0 | 58 | (42, 64) | 0 - 113 | 67 | (59, 75) | 39 - 101 | *0.04* |
| C16:0 | 5082 | (4678, 5353) | 3663 - 7213 | 4781 | (4530, 5170) | 3347 - 6617 | 0.43 |
| C16:1n-7 | 93 | (87, 119) | 56 - 210 | 102 | (90, 113) | 41 - 332 | 0.98 |
| C18:0 | 1599 | (1525, 1771) | 1191 - 3425 | 1531 | (1437, 1680) | 1078 - 2571 | 0.29 |
| C18:1n-9 | 1454 | (1310, 1528) | 1013 - 2275 | 1374 | (1287, 1500) | 929 - 2042 | 0.82 |
| C18:2n-6 | 1510 | (1423, 1630) | 1131 - 2053 | 1385 | (1333, 1592) | 1078 - 1959 | 0.35 |
| C18:3n-3 | 8 | (4, 17) | 0 - 103 | 6 | (3, 8) | 0 - 51 | 0.34 |
| C20:0 | 17 | (13, 27) | 2 - 77 | 14 | (10, 17) | 0 - 72 | 0.16 |
| C20:3n-6 | 615 | (581, 726) | 331 - 1090 | 609 | (552, 702) | 347 - 1187 | 0.70 |
| C20:4n-6 | 1315 | (1156, 1402) | 799 - 1775 | 1254 | (1159, 1364) | 832 - 1635 | 0.85 |
| C20:5n-3 | 35 | (25, 90) | 0 - 215 | 33 | (21, 63) | 0 - 195 | 0.63 |
| C22:4n-6 | 130 | (111, 163) | 47 - 284 | 143 | (122, 168) | 54 - 262 | 0.56 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 200 | (177, 279) | 969 - 471 | 176 | (166, 231) | 83 - 371 | 0.50 |
| C22:6n-3 | 6717 | (5051, 7471) | 1726 - 12024 | 5190 | (4223, 6090) | 1798 - 9579 | 0.12 |
| Total n-3 | 6973 | (5340, 7775) | 1860 - 12561 | 5636 | (4437, 6356) | 1971 - 9742 | 0.12 |
| Total n-6 | 3598 | (3357, 3880) | 2369 - 4566 | 3390 | (3239, 3806) | 2576 - 4838 | 0.56 |
| Ratio n6/n3 | 1 | (0, 1) | 0 - 1 | 1 | (1, 1) | 0 - 1 | 0.17 |

Table 60. Total fatty acid concentrations (nmol/g) in placental tissue total lipids for both groups (n=26 for fish oil group, n=27 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

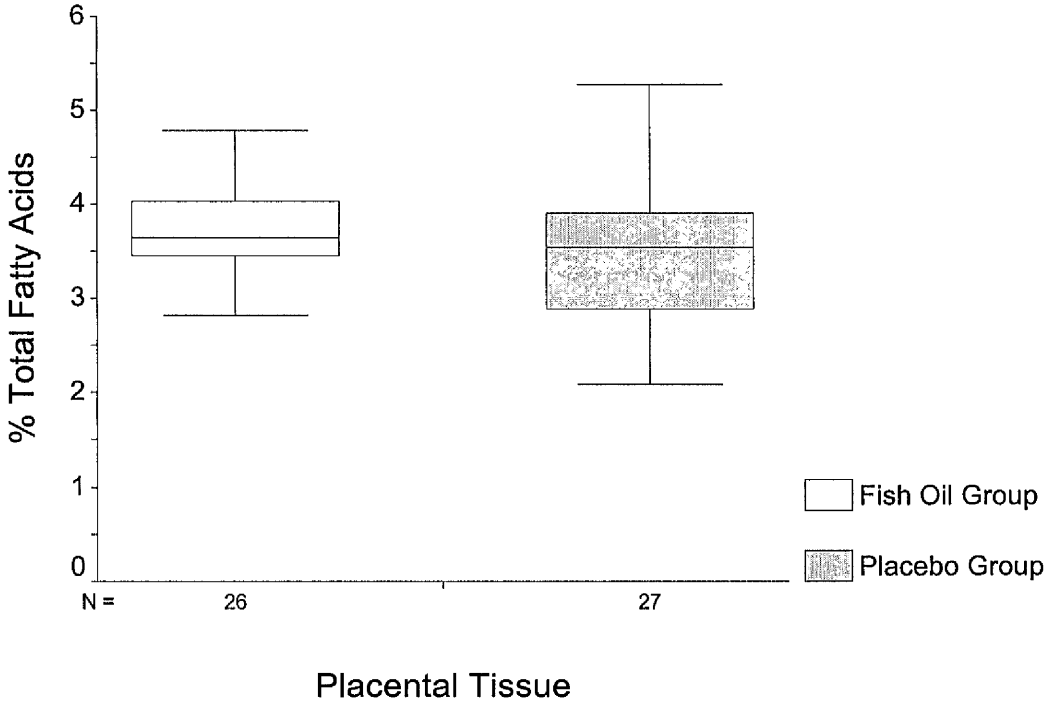



Figure 20. % DHA (median & quartiles) in placental tissue of both groups. No significant difference between groups.

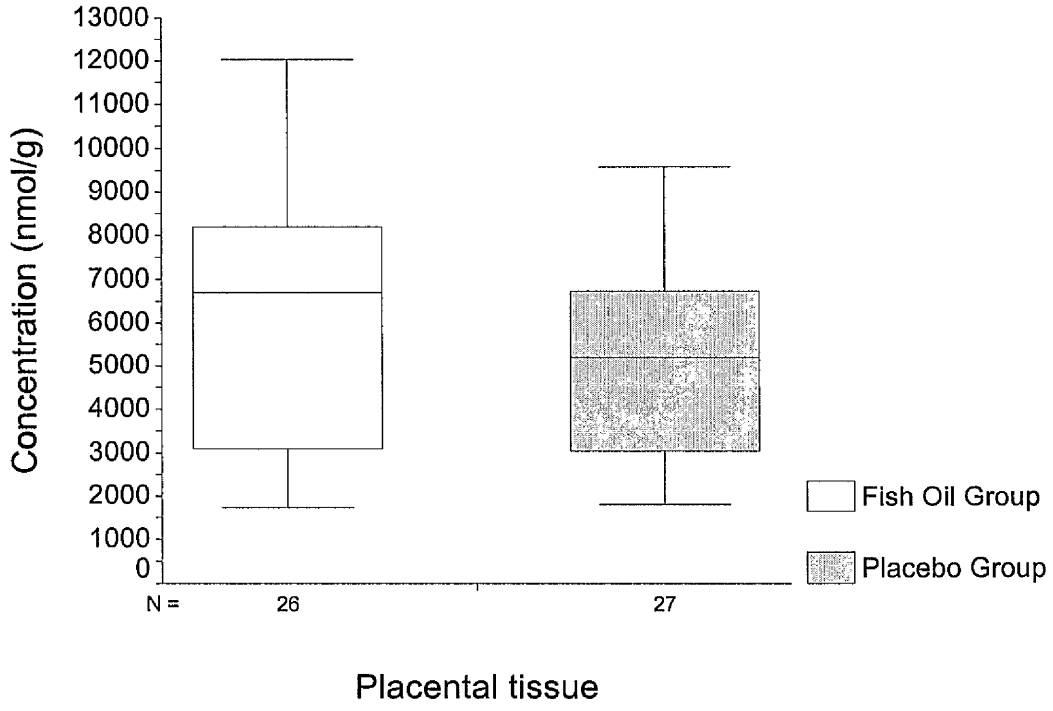



Figure 21. DHA concentration (median & quartiles) in placental tissue of both groups. No significant difference between groups.

### 9.2.7 Umbilical Cord Tissue Fatty Acids: % Total fatty Acids (Relative Levels)

(Figure 22, Table 61)

Fish oil and placebo groups had no significant differences in the % TFA measured in cord tissue samples. DHA accounted for median 6.3% TFA in cord tissue from the fish oil group, and median 5.9% TFA in that from the placebo group (p=0.19).

| | Fish Oil Group | | | Placebo Group | | | p |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | | | | | | | |
| C12:0 | | | | | | | |
| C14:0 | 0.7 | (0.7, 0.9) | 0.4 - 1.4 | 0.8 | (0.7, 0.9) | 0.2 - 1.2 | 0.68 |
| C16:0 | 21.9 | (21.5, 22.8) | 19.7 - 24.8 | 22.3 | (21.2, 22.8) | 17.7 - 24.8 | 0.92 |
| C16:1n-7 | 10.2 | (1.1, 1.3) | 1.0 - 1.6 | 1.2 | (1.2, 1.3) | 1.0 - 1.6 | 0.54 |
| C18:0 | 19.3 | (18.9, 19.8) | 18.0 - 21.1 | 19.1 | (18.9, 19.9) | 17.3 - 21.7 | 0.98 |
| C18:1n-9 | 15.7 | (15.3, 17.0) | 13.8 - 21.3 | 17.0 | (16.3, 17.7) | 14.2 - 20.5 | 0.13 |
| C18:2n-6 | 4.2 | (4.1, 4.5) | 3.5 - 5.6 | 4.2 | (4.0, 4.5) | 3.5 - 5.8 | 0.60 |
| C18:3n-3 | 0.6 | (0.4, 0.7) | 0.0 - 1.4 | 0.5 | (0.5, 0.7) | 0.1 - 1.2 | 0.99 |
| C20:0 | 0.9 | (0.7, 1.4) | 0.3 - 2.7 | 0.6 | (0.6, 1.3) | 0.3 - 2.8 | 0.61 |
| C20:3n-6 | 2.7 | (1.6, 3.3) | 0.0 - 5.2 | 3.0 | (1.5, 3.1) | 0.0 - 5.6 | 0.65 |
| C20:4n-6 | 16.3 | (15.9, 16.8) | 13.9 - 18.4 | 16.5 | (16.0, 16.8) | 14.0 - 19.0 | 0.76 |
| C20:5n-3 | 0.3 | (0.3, 0.4) | 0.1 - 0.7 | 0.3 | (0.2, 0.5) | 0.1 - 1.0 | 0.96 |
| C22:4n-6 | 5.9 | (5.6, 6.0) | 4.8 - 6.5 | 5.8 | (5.5, 6.1) | 4.7 - 7.5 | 0.75 |
| C22:5n-6 | 2.5 | (2.4, 2.8) | 1.9 - 3.5 | 2.7 | (2.5, 2.9) | 1.7 - 3.6 | 0.57 |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 0.6 | (0.5, 0.6) | 0.3 - 0.8 | 0.6 | (0.5, 0.7) | 0.3 - 1.1 | 0.70 |
| C22:6n-3 | 6.3 | (5.9, 6.5) | 4.5 - 7.4 | 5.9 | (5.7, 6.2) | 4.6 - 7.7 | 0.19 |
| Total n-3 | 7.7 | (7.3, 8.1) | 5.9 - 9.2 | 7.5 | (7.1, 7.8) | 5.7 - 9.8 | 0.38 |
| Total n-6 | 32.3 | (30.4, 32.5) | 27.0 - 35.9 | 30.9 | (30.0, 32.1) | 26.5 - 36.6 | 0.65 |
| Ratio n6/n3 | 4.0 | (3.9, 4.3) | 3.3 - 5.7 | 4.1 | (3.9, 4.5) | 3.1 - 5.3 | 0.76 |

Table 61. Percentage total fatty acids in umbilical cord tissue total lipids for both groups (n=26 for fish oil group, n=27 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

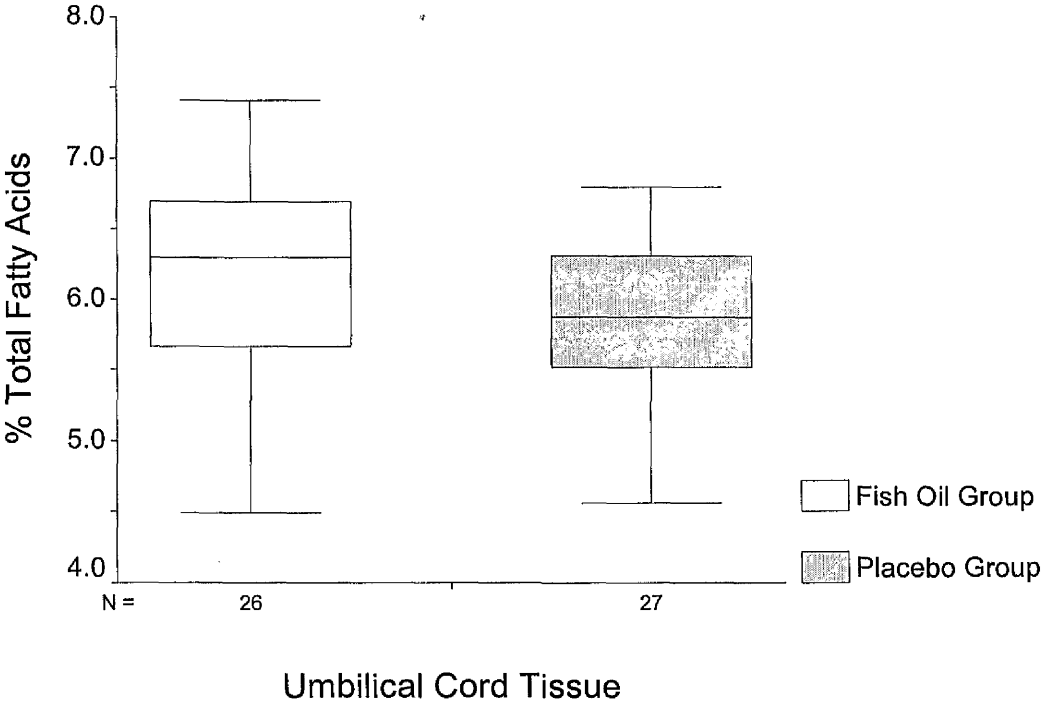



Figure 22. % DHA (median & quartiles) in umbilical cord tissue of both groups. No significant difference between groups.

**9.2.8 Breast Milk Fatty Acids: % Total Fatty Acids (Relative Levels)** (Figure 23, Table 62)

The contribution of DHA to % TFA was similar in the breast milk samples from the two treatment groups. Breast milk from the fish oil group contained DHA as median 0.2% TFA; milk from the placebo group had 0.3% DHA (p=0.88).

Palmitic acid (16:0) accounted for a significantly lower (p=0.03) % TFA in the breast milk of the fish oil group (median 29.6%) than the placebo group (median 31.0%).

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 0.1 | (0.1, 0.3) | 0.0 - 0.6 | 0.0 | (0.0, 0.1) | 0.0 - 0.4 | 0.20 |
| C12:0 | 3.6 | (2.3, 4.3) | 1.1 - 5.4 | 2.2 | (1.7, 3.6) | 0.9 - 5.6 | 0.25 |
| C14:0 | 7.0 | (5.8, 7.7) | 4.5 - 8.6 | 5.9 | (5.3, 7.7) | 4.2 - 10.7 | 0.43 |
| C16:0 | 29.6 | (28.2, 30.4) | 26.7 - 31.5 | 31.0 | (30.3, 31.8) | 28.4 - 33.4 | *0.03* |
| C16:1n-7 | 2.3 | (2.0, 2.7) | 1.3 - 4.1 | 2.3 | (2.1, 3.0) | 1.9 - 4.0 | 0.86 |
| C18:0 | 8.4 | (8.0, 8.8) | 6.5 - 10.6 | 8.0 | (7.5, 8.7) | 6.8 - 10.0 | 0.38 |
| C18:1n-9 | 36.0 | (34.5, 37.1) | 32.9 - 38.9 | 36.8 | (34.5, 38.1) | 30.1 - 39.3 | 0.19 |
| C18:2n-6 | 11.1 | (9.9, 11.9) | 8.3 - 14.0 | 10.5 | (9.3, 10.9) | 6.7 - 12.5 | 0.21 |
| C18:3n-3 | 0.9 | (0.8, 1.0) | 0.6 - 1.3 | 0.9 | (0.8, 1.0) | 0.3 - 1.2 | 0.90 |
| C20:0 | 0.3 | (0.3, 0.4) | 0.2 - 0.5 | 0.3 | (0.3, 0.3) | 0.0 - 0.4 | 0.31 |
| C20:3n-6 | 0.4 | (0.3, 0.6) | 0.2 - 0.8 | 0.5 | (0.4, 0.6) | 0.3 - 0.7 | 0.57 |
| C20:4n-6 | 0.4 | (0.3, 0.5) | 0.3 - 0.7 | 0.5 | (0.5, 0.6) | 0.3 - 0.7 | 0.10 |
| C20:5n-3 | 0.0 | (0.0, 0.0) | 0.0 - 0.1 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C22:4n-6 | 0.1 | (0.1, 0.3) | 0.0 - 0.4 | 0.2 | (0.1, 0.3) | 0.0 - 0.4 | 0.34 |
| C22:5n-6 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | 0.0 | (0.0, 0.0) | 0.0 - 0.0 | |
| C24:0 | 0.0 | (0.0, 0.1) | 0.0 - 0.2 | 0.0 | (0.0, 0.1) | 0.0 - 0.1 | 0.83 |
| C24:1n-9 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.49 |
| C22:5n-3 | 0.1 | (0.1, 0.3) | 0.0 - 0.6 | 0.1 | (0.1, 0.2) | 0.0 - 0.3 | 0.74 |
| C22:6n-3 | 0.2 | (0.2, 0.6) | 0.1 - 1.1 | 0.3 | (0.2, 0.3) | 0.1 - 0.5 | 0.88 |
| Total n-3 | 1.4 | (1.2, 1.7) | 0.9 - 2.8 | 1.4 | (1.1, 1.5) | 0.3 - 1.7 | 0.86 |
| Total n-6 | 11.8 | (10.8, 13.1) | 9.0 - 15.1 | 11.6 | (10.4, 12.2) | 7.6 - 13.4 | 0.41 |
| Ratio n6/n3 | 8.9 | (7.7, 10.1) | 4.7 - 12.0 | 8.8 | (7.9, 10.9) | 5.7 - 33.2 | 0.94 |

Table 62. Percentage total fatty acids in breast milk total lipids for both groups (n=12 for fish oil group, n=14 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

**9.2.9 Breast Milk Fatty Acids: Concentration (Absolute Levels)** (Figure 24, Table 63)

DHA concentrations were slightly, and non-significantly (p=0.78) higher in the breast milk of the placebo group (median 2943 nmol/ml), compared to the fish oil group (median 2561 nmol/ml).

The placebo group had a significantly higher (p=0.03) concentration of AA (20:4n-6) in breast milk (median 119 nmol/ml) than did the fish oil group (median 194 nmol/ml). However, the concentrations of total n-3 and total n-6 fatty acids did not differ between the groups, nor did the ratio of n-6 to n-3 fatty acids.

| | Fish Oil Group | | | Placebo Group | | | *p* |
|---|---|---|---|---|---|---|---|
| | **Median** | **95% CI** | **Range** | **Median** | **95% CI** | **Range** | |
| C10:0 | 209 | (117, 792) | 0 - 2488 | 120 | (39, 445) | 0 - 1581 | 0.36 |
| C12:0 | 1873 | (1352, 3891) | 627 - 6562 | 1977 | (1245, 3401) | 265 - 6320 | 0.09 |
| C14:0 | 3803 | (2513, 4948) | 1503 - 6171 | 4827 | (3181, 5947) | 1178 - 10000 | 0.49 |
| C16:0 | 16158 | (12423, 20464) | 7169 - 26511 | 22292 | (16309, 25937) | 7796 - 31938 | 0.08 |
| C16:1n-7 | 973 | (699, 1369) | 397 - 3214 | 1231 | (946, 1762) | 440 - 2704 | 0.19 |
| C18:0 | 3311 | (2615, 4238) | 1893 - 5598 | 3966 | (3104, 4876) | 1523 - 6539 | 0.19 |
| C18:1n-9 | 17346 | (13527, 21752) | 8337 - 32552 | 22013 | (17628, 26052) | 8409 - 34685 | 0.13 |
| C18:2n-6 | 5559 | (4497, 7840) | 2548 - 10660 | 6726 | (5526, 7979) | 3190 - 9530 | 0.55 |
| C18:3n-3 | 459 | (347, 747) | 260 - 1235 | 583 | (427, 721) | 231 - 955 | 0.49 |
| C20:0 | 97 | (80, 148) | 59 - 208 | 134 | (90, 163) | 0 - 223 | 0.43 |
| C20:3n-6 | 229 | (182, 369) | 57 - 675 | 361 | (252, 447) | 86 - 715 | 0.16 |
| C20:4n-6 | 119 | (88, 169) | 36 - 268 | 194 | (145, 213) | 57 - 245 | *0.03* |
| C20:5n-3 | 0 | (0, 68) | 0 - 186 | 0 | (0, 0) | 0 - 0 | |
| C22:4n-6 | 55 | (28, 191) | 9 - 420 | 115 | (78, 191) | 0 - 297 | 0.17 |
| C22:5n-6 | | | | | | | |
| C24:0 | | | | | | | |
| C24:1n-9 | | | | | | | |
| C22:5n-3 | 213 | (118, 556) | 0 - 2343 | 340 | (232, 553) | 0 - 948 | 0.41 |
| C22:6n-3 | 2561 | (1614, 5249) | 669 - 16466 | 2943 | (1979, 4360) | 260 - 6000 | 0.78 |
| Total n-3 | 3594 | (2222, 6216) | 988 - 19830 | 3893 | (2661, 5561) | 557 - 7564 | 0.70 |
| Total n-6 | 6018 | (4901, 8419) | 2661 - 11182 | 7387 | (5987, 8707) | 3362 - 10286 | 0.59 |
| Ratio n6/n3 | 2 | (1, 3) | 1 - 4 | 2 | (2, 4) | 1 - 8 | 0.98 |

Table 63. Total fatty acid concentrations (nmol/ml) in breast milk total lipids for both groups (n=12 for fish oil group, n=14 for placebo group). *p* values were obtained on performing Mann-Whitney tests between the groups. 95% CI – Wilcoxon confidence interval for the median.

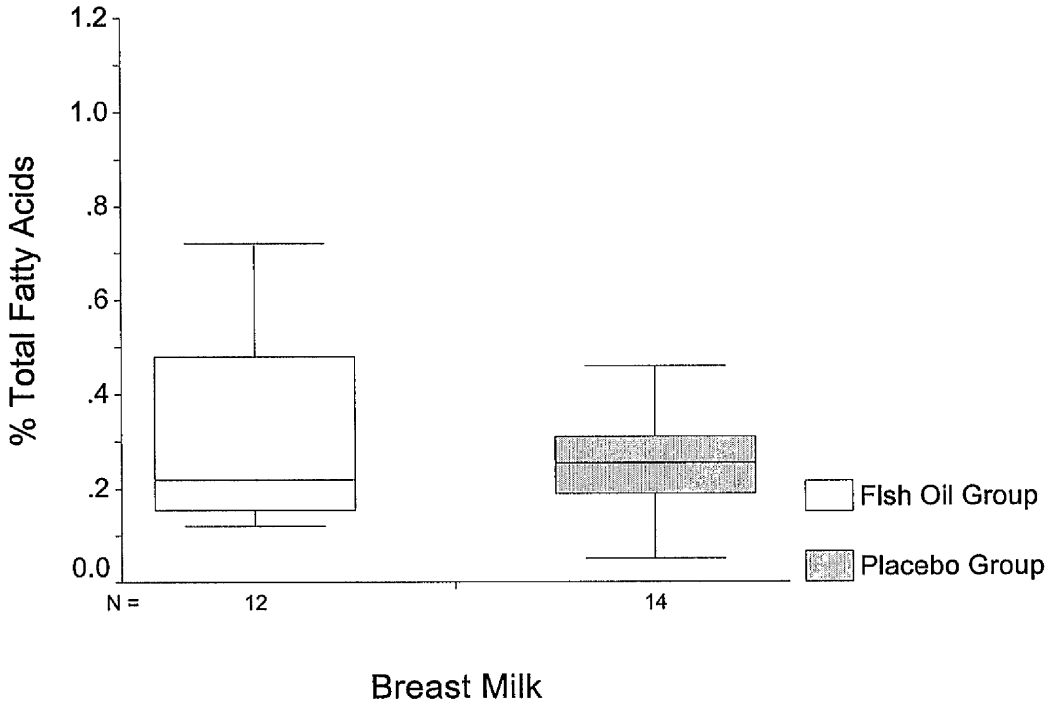



Figure 23. % DHA (median & quartiles) in breast milk of both groups. No significant difference between groups


Figure 24. DHA concentration (median & quartiles) in breast milk of both groups. No significant difference between groups.

## 9.3 ANALYSES WITHIN GROUPS: LONGITUDINAL CHANGE AND THE RELATIONSHIP BETWEEN MATERNAL AND FETAL STATUS

### 9.3.1 RBC Fatty Acids: % Total Fatty Acids (Relative Levels)

9.3.1(a) Fish Oil Group: Maternal RBC at 15 and 28 weeks gestation (Figure 25)

By 28 weeks gestation, maternal DHA had increased ($p<0.0001$) as a % TFA in maternal RBC in the fish oil group, from a median of 2.9% to 4.2%.

Myristic acid (14:0) increased in the fish oil group between 15 and 28 weeks ($p=0.004$), from a baseline of 0.2% TFA to 0.4% TFA. RBC at 28 weeks also had increased ($p=0.002$) palmitoleic acid (16:1n-7), elevated from 0.1% TFA to 0.7% TFA. Linoleic acid (LA, 18:2n-6) significantly decreased ($p=0.001$) in the fish oil group by 28 weeks from a median 9.5% to 8.6%.

9.3.1(b) Fish Oil Group: Maternal RBC at 28 weeks and Birth (Figure 25)

By delivery, maternal RBC DHA status had declined in the fish oil group ($p<0.0001$) from median 4.1% TFA to 3.4% TFA, a median decline of 0.8% TFA. Thus, at delivery, RBC DHA status of fish oil supplemented mothers was (median) 84% of their status at 28 weeks. Although maternal RBC DHA was of a higher median % TFA at birth (3.1%) than at 15 weeks (2.9%), the difference was not significant ($p=0.57$).

AA (20:4n-6, $p<0.0001$), EPA (20:5n-3, $p<0.0001$), adrenic acid (22:4n-6, $p<0.0001$) and DPA (22:5n-3, $p=0.0001$) all decreased between 28 weeks and birth, to levels which were also significantly lower than at 15 weeks (all $p<0.01$). The fish oil group also declined in DHGLA (20:3n-6, $p<0.0001$) between 28 weeks and birth. Palmitate (16:0, $p<0.0001$) and oleate (18:1n-9, $p=0.0005$) increased from 28 weeks to birth.

9.3.1(c) Fish Oil Group: Maternal RBC at Birth and Fetal Umbilical Cord RBC (Figure 25)

Cord RBC from infants in the fish oil group had a significantly higher % TFA ($p<0.0001$) of DHA than maternal RBC samples at the time of delivery. DHA accounted for median 4.3% TFA in cord RBC, and 3.1% TFA in maternal RBC at birth. Cord DHA (median 4.4% TFA) was also significantly higher ($p=0.0003$) than maternal 15 weeks DHA (median 2.9% TFA), but there was no significant difference

(p=0.54) between cord (median 4.3% TFA) and maternal 28 weeks (median 4% TFA) % DHA.

Cord RBC was higher in stearic acid (18:0, p<0.0001), eicosanoic acid (20:0, p=0.0005), DHGLA (20:3n-6, p<0.0001), AA (20:4n-6, p<0.0001), and adrenic acid (22:4n-6, p<0.0001) as % TFA than maternal RBC at birth. Lignoceric acid (24:0) was also higher in cord than maternal RBC, but the difference only tended to significance (p=0.02).

Cord RBC was lower in % contributions of oleic acid (18:1n-9, p<0.0001), LA (18:2n-6, p<0.0001), nervonic acid (24:1n-9, p<0.0001), and DPA (22:5n-3, p<0.0001) than maternal RBC at birth.

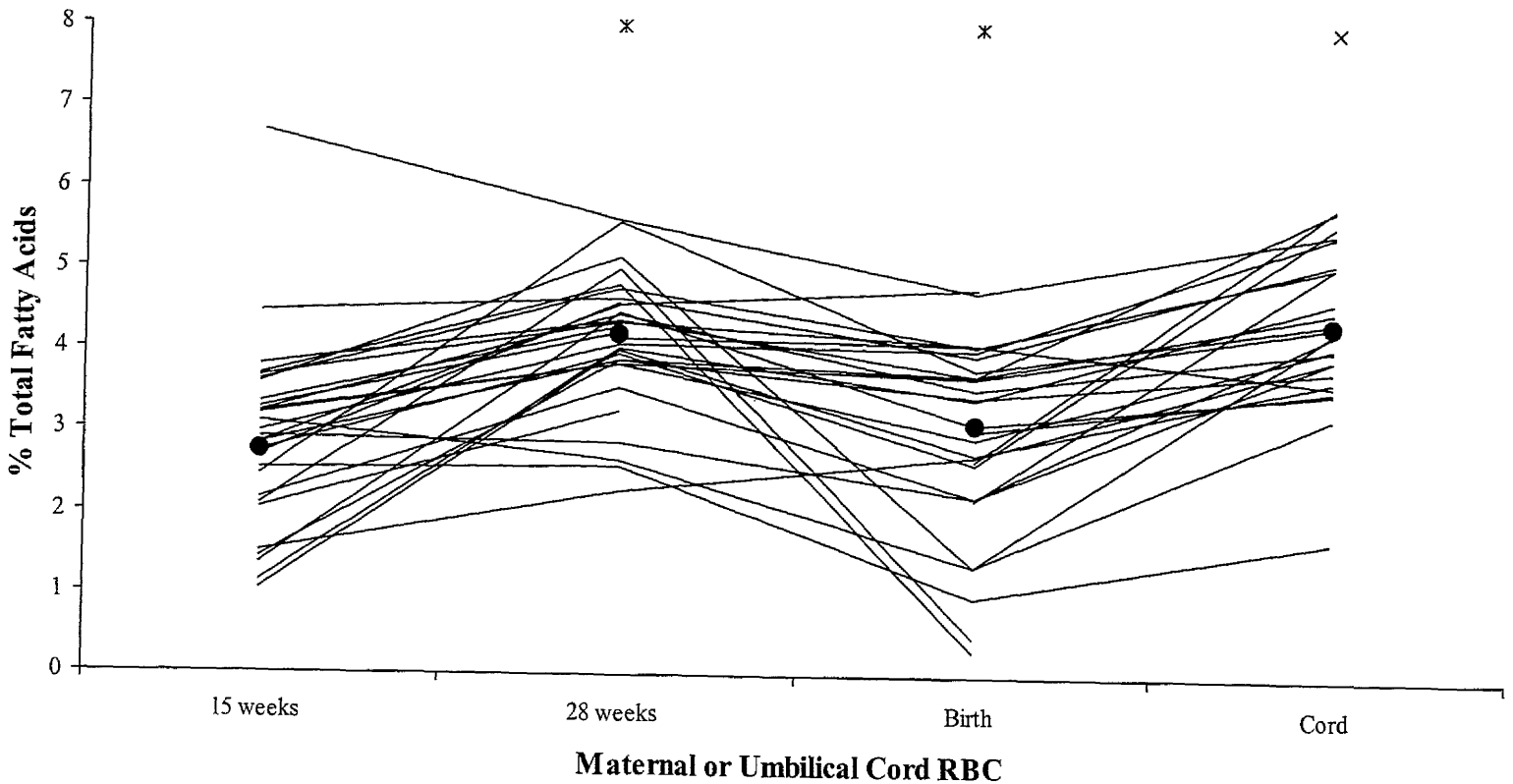



Figure 25. Longitudinal changes in % DHA in RBC of fish oil group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

9.3.1(d) Placebo Group: Maternal RBC at 15 and 28 weeks gestation (Figure 26)

By 28 weeks gestation, the placebo group had increased ($p=0.0005$) their % TFA of RBC DHA from median 2.8% to 3.4%, a median increase of 25% of their 15 weeks level.

Fatty acids of chain length 14:0 (myristic, $p=0.005$), 16:1n-7 (palmitoleic, $p=0.002$), 20:3n-6 (DHGLA, $p<0.0001$), 20:5n-3 (EPA, $p=0.002$), and 22:5n-3 (DPA, $p=0.002$) all accounted for significantly higher % TFA in RBC at 28 weeks than 15 weeks in the placebo group.

Fatty acids of chain length 16:0 (palmitic, $p=0.007$) and 18:0 (stearic, $p=0.001$) declined as % TFA in placebo group RBC between 15 weeks and 28 weeks.

9.3.1(e) Placebo Group: Maternal RBC at 28 weeks and Birth (Figure 26)

Maternal RBC DHA declined ($p=0.0002$) from a median of 3.3% TFA at 28 weeks to 2.4% TFA at delivery, a median decrease of 1.4% TFA, 41% of the 28 weeks level. The difference between maternal DHA at 15 weeks (median 2.8% TFA) and birth (median 2.4% TFA) was not significant ($p=0.85$).

Palmitic acid (16:0), which had decreased as a % TFA between 15 weeks and 28 weeks, significantly increased ($p=0.0002$) between 28 weeks and delivery, to % similar to those at 15 weeks. Conversely, after an increase between 15 and 28 weeks, DHGLA (20:3n-6, $p<0.0001$), EPA (20:5n-3, $p=0.0004$), and DPA (22:5n-3, $p=0.0002$) decreased between 28 weeks and birth to levels comparable to those at 15 weeks.

Oleic acid (18:1n-9) increased between 28 weeks and delivery ($p<0.0001$). AA (20:4n-6, $p<0.0001$) and adrenic acid (22:4n-6, $p=0.0002$) decreased between 28 weeks and delivery, to lower than at baseline 15 weeks ($p=0.0002$ and 0.004 respectively).

9.3.1(f) Placebo Group: Maternal RBC at Birth and Fetal Umbilical Cord RBC (Figure 26)

RBC cord DHA was 74% higher than maternal DHA at birth ($p<0.0001$). Cord DHA had a median value of 4.1% TFA and maternal DHA at birth was median 2.4% TFA. Cord DHA was also of a significantly higher % TFA than maternal DHA at 15 weeks (median 4.1% TFA *vs.* 2.8% TFA, $p<0.0001$), and at 28 weeks (median 4.2% TFA *vs.* 3.4% TFA, $p=0.003$).

Percentage contributions of palmitoleic acid (16:1n-7, $p=0.003$), oleic acid (18:1n-9, $p<0.0001$), LA (18:2n-6, $p<0.0001$), nervonic acid (24:1n-9, $p=0.0003$), and DPA (22:5n-3, $p=0.006$) were all lower in cord RBC than maternal RBC at birth. Stearic acid (18:0, $p<0.0001$), arachidic acid (20:0, $p<0.0001$), DHGLA (20:3n-6, $p<0.0001$), AA (20:4n-6, $p<0.0001$), and adrenic acid (22:4n-6, $p<0.0001$) were all higher in cord RBC than maternal RBC at birth.

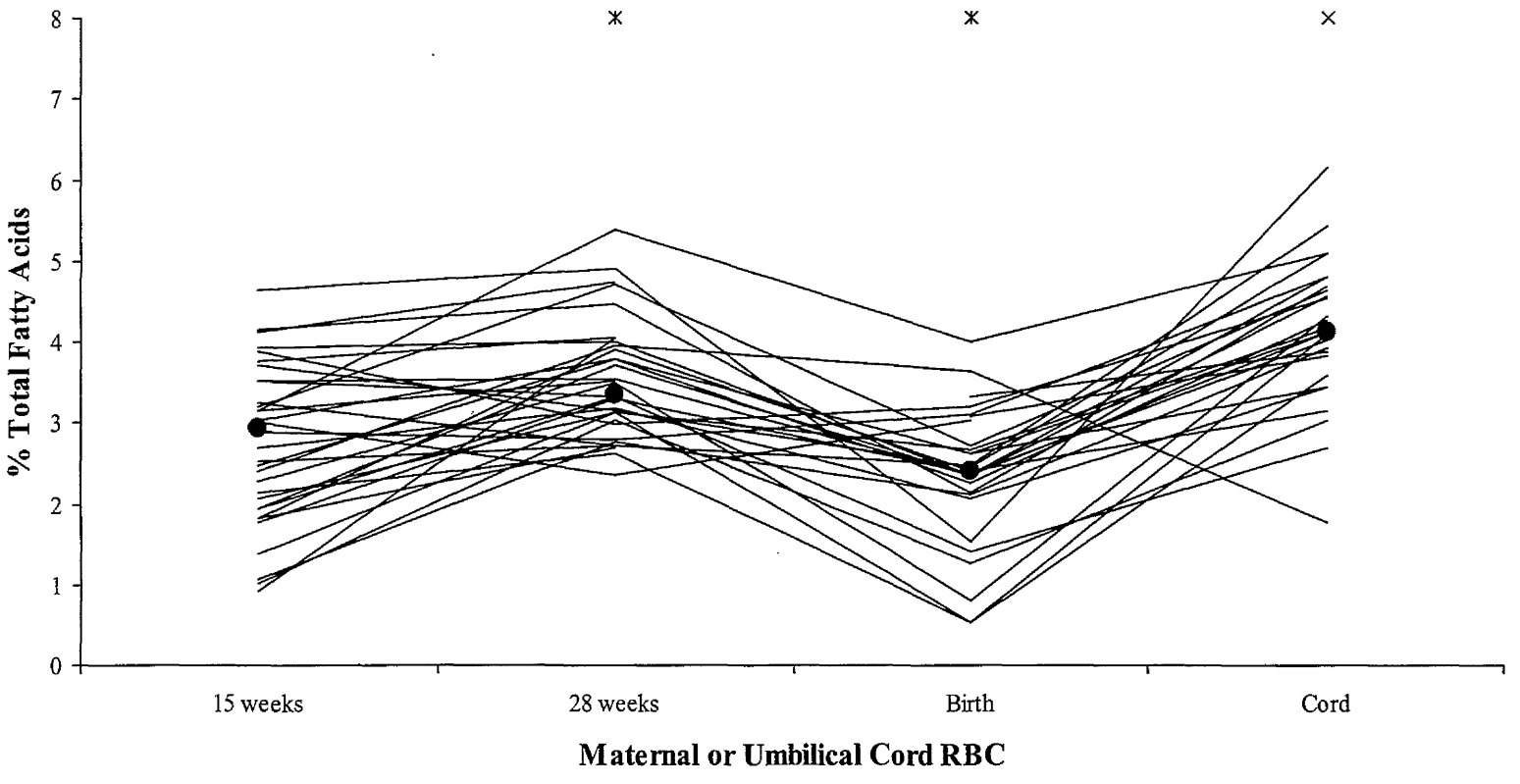



Figure 26. Longitudinal changes in % DHA in RBC of placebo group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

### 9.3.2 RBC Fatty Acids: Concentration (Absolute Levels)

9.3.2(a) Fish Oil Group: Maternal RBC at 15 and 28 weeks gestation (Figure 27)

The concentration of DHA in maternal RBC at 15 weeks (median 178 nmol/ml) was non-significantly different (p=0.46) from that at 28 weeks (median 195 nmol/ml).

By 28 weeks, the fish oil group had an increased (p=0.0005) concentration of 16:1n-7 (palmitoleic acid) in their RBC, having increased their 15 weeks level by 9 nmol/ml, or 126%.

9.3.2(b) Fish Oil Group: Maternal RBC at 28 weeks and Birth (Figure 27)

The difference between maternal DHA concentration at 28 weeks (median 195 nmol/ml) and at birth (median 176 nmol/ml) was not significant (p=0.56). The concentration at birth (median 171 nmol/ml) remained similar to that at 15 weeks (median 172 nmol/ml) (p=0.57).

The concentration of EPA (20:5n-3) decreased in RBC between 28 weeks and birth from a median of 11 nmol/ml to 0.0 nmol/ml (p=0.0003). (Concentrations of 20:5n-3 at 15 weeks and birth also differed, but only tended to significance at p=0.03).

There were no other significant differences in fatty acid concentrations in the fish oil group between 28 weeks and birth, although 14:0 (myristic acid) was lower (p=0.03) and 16:0 (palmitic acid) was higher (p=0.03) at birth.

9.3.2(c) Fish Oil Group: Maternal RBC at Birth and Fetal Umbilical Cord RBC (Figure 27)

Cord RBC (median 228 nmol/ml) had a higher concentration of DHA than maternal RBC at birth (median 173 nmol/ml) (p=0.0002). The median difference of 68 nmol/ml between maternal and cord levels corresponded to a maternal level equivalent to 76% of the cord level. Although cord DHA concentration was significantly different from maternal levels at birth at p<0.01, it was only tending to a significant difference from maternal concentrations at 15 weeks (p=0.02, median 221 nmol/ml *vs.* 178 nmol/ml) and 28 weeks (p=0.02, median 227 nmol/ml *vs.* 193 nmol/ml). Maternal DHA concentration at 28 weeks was equivalent to 85% of that in cord RBC.

Cord RBC also had higher concentrations of DHGLA (20:3n-6, $p<0.0001$), AA (20:4n-6, $p<0.0001$), adrenic acid (22:4n-6, $p<0.0001$), but lower concentrations of palmitic acid (16:0, $p=0.015$), oleic acid (18:1n-9, $p<0.0001$) and LA (18:2n-6, $p<0.0001$).

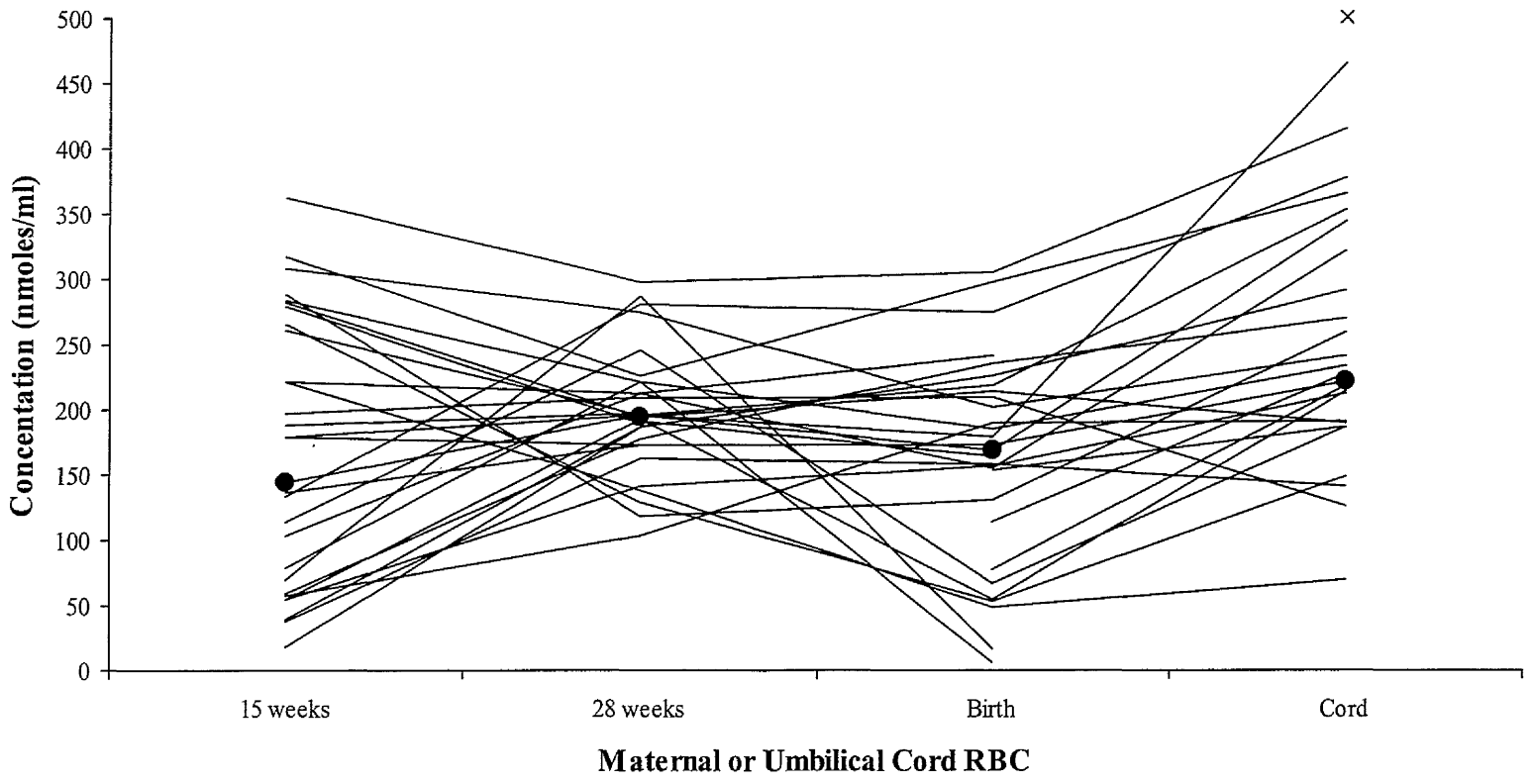



Figure 27. Longitudinal changes in DHA concentration in RBC of fish oil group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

9.3.2(d) Placebo Group: Maternal RBC at 15 and 28 weeks gestation (Figure 28)
RBC concentrations of DHA did not differ significantly at 15 weeks (median 128 nmol/ml) and 28 weeks (173 nmol/ml) (p=0.38).

The concentration of 16:1n-7 (palmitoleic acid) increased between 15 and 28 weeks in the RBC of the placebo group (p=0.008). The difference (a median of 11.08 nmol/ml) was a 543% increase on the 15 weeks level.

Myristic acid (14:0) was higher at 15 weeks, but significance was only 0.04.

9.3.2(e) Placebo Group: Maternal RBC at 28 weeks and Birth (Figure 28)
At the time of delivery, maternal RBC DHA concentration had decreased from its level at 28 weeks (median 171 nmol/ml) to a median of 115 nmol/ml, by a median of 56 nmol/ml (p=0.004). DHA at birth (median 118 nmol/ml) did not, however, differ in concentration from 15 weeks (median 128 nmol/ml) (p=0.85).

The concentration of EPA (20:5n-3) decreased from a median 11 nmol/ml to 0.0 nmol/ml (p=0.0004). Maternal RBC was also lower in DHGLA (20:3n-6, p=0.0009), adrenic acid (22:4n-6, p=0.003), and DPA (22:5n-3, p=0.0009) at birth than 28 weeks. However, these decreases did not cause the fatty acids to differ significantly from their baseline 15 weeks levels, although the difference between maternal 15 weeks and birth adrenic acid (22:4n-6) tended to significance, with p=0.03. Concentrations of palmitate (16:0) were higher at birth but with significance of only 0.02, while concentrations of AA (20:4n-6) were lower (p=0.04).

9.3.2(f) Placebo Group: Maternal RBC at Birth and Fetal Umbilical Cord RBC (Figure 28)
DHA concentrations were significantly higher in cord then maternal RBC at birth (p<0.0001). Cord RBC (median 265 nmol/ml) were higher by a median of 149 nmol/ml than maternal RBC (median 117 nmol/ml); median maternal values were thus only 44% of median cord values. Moreover, cord RBC DHA concentration (median 264 nmol/ml) was also higher than maternal 28 weeks DHA (median 173 nmol/ml) (p=0.0001), by a median of 72 nmol/ml. Although maternal DHA was maximal at 28 weeks, it was still only 65% of cord levels. The difference (159

nmol/ml) between DHA concentrations in maternal 15 weeks RBC (median 128 nmol/ml) and cord RBC (median 264 nmol/ml) RBC was also significant ($p=0.01$).

Cord RBC was also higher in concentrations of arachidic acid (20:0, $p<0.0001$), DHGLA (20:3n-6, $p<0.0001$), AA (20:4n-6, $p<0.0001$), and adrenic acid (22:4n-6, $p<0.0001$), but lower in LA (18:2n-6, $p<0.0001$). Oleic acid (18:1n-9) was also lower in cord, but not significantly at $p<0.01$ ($p=0.02$).

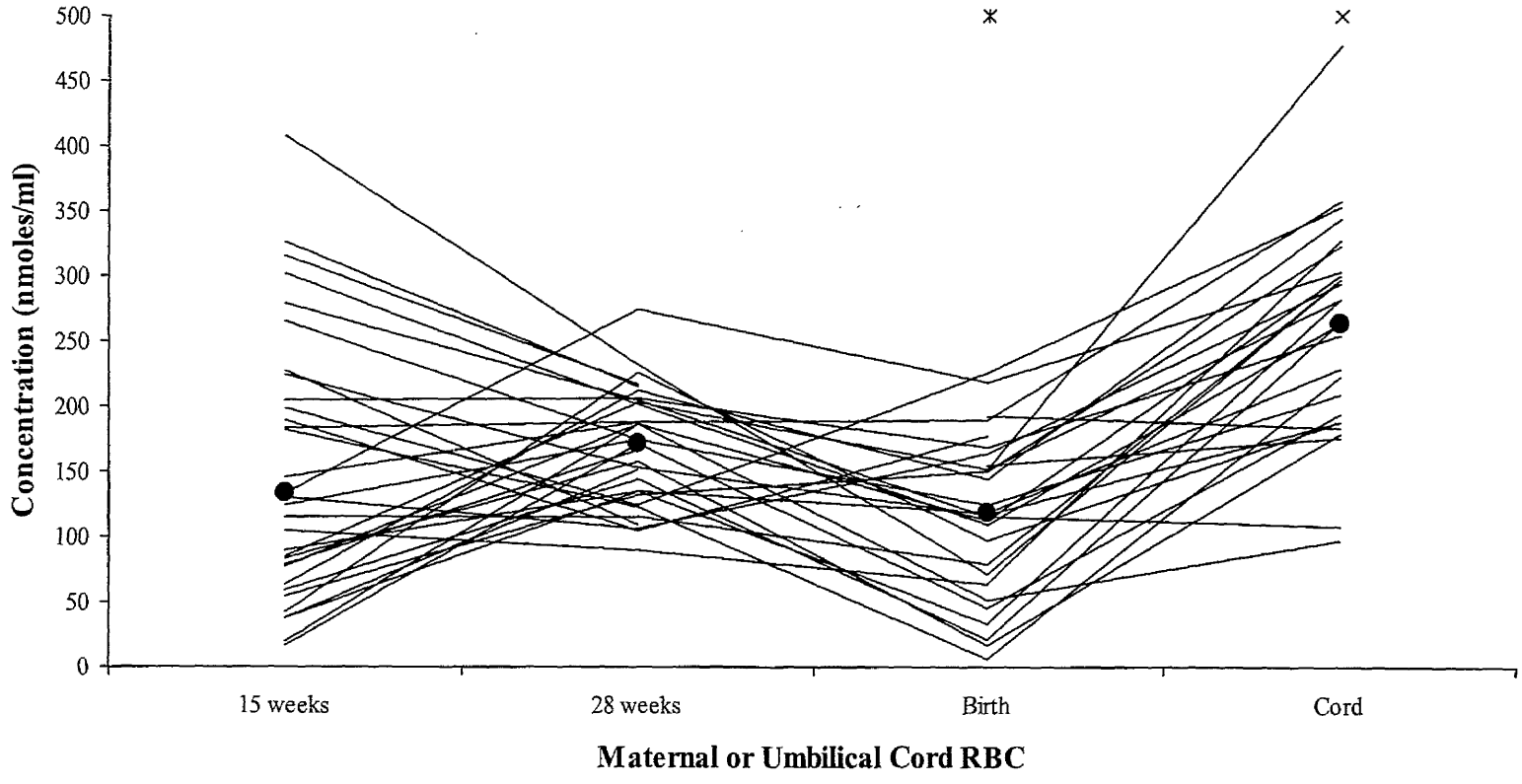



Figure 28. Longitudinal changes in DHA concentration in RBC of placebo group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

### 9.3.3 Plasma Fatty Acids: % Total Fatty Acids (Relative Levels)

9.3.3(a) Fish Oil Group: Maternal Plasma at 15 and 28 weeks gestation (Figure 29)

There was no change in DHA as a % TFA in plasma of the fish oil group between 15 and 28 weeks. The difference in 15 weeks DHA (median 1.7% TFA) and 28 weeks DHA (median 1.8% TFA) was non-significant (p=0.26).

Palmitoleic acid (16:1n-7, p=0.01) did increase in plasma between 15 weeks and 28 weeks, while stearic (18:0, p=0.0005) and lignoceric (24:0, p=0.0009) acids both decreased.

9.3.3(b) Fish Oil Group: Maternal Plasma at 28 weeks and Birth (Figure 29)

Maternal DHA significantly decreased (p<0.0001) between 28 weeks and birth, from a median of 1.8% TFA to 1.4% TFA. This represented a median decline of 28% in its level at 28 weeks; despite this, maternal birth plasma DHA (median 1.4% TFA) was not significantly different (p=0.06) from that at 15 weeks (median 1.7% TFA).

Myristic acid (14:0, p=0.0003), arachidic acid (20:0, p=0.0001) DHGLA (20:3n-6, p=0.002), adrenic acid (22:4n-6, p=0.004), lignoceric acid (24:0, p=0.004) and DPA (22:5n-3, p=0.0002) were of lower % TFA at birth than 28 weeks, and had decreased to values significantly lower than those observed at baseline 15 weeks (all p<0.01). Nervonic acid (24:1n-9) tended to be higher (p=0.02) at 28 weeks than birth, and was significantly higher at 15 weeks than birth (p=0.0001). Both αLA (18:3n-3, p=0.006) and EPA (20:5n-3, p=0.0003) also decreased between 28 weeks and birth, but did not decrease significantly below baseline levels.

9.3.3(c) Fish Oil Group: Maternal Plasma at Birth and Fetal Umbilical Cord Plasma (Figure 29)

DHA was significantly higher in cord plasma than maternal plasma at birth (p<0.0001). Maternal plasma DHA was a median of 1.4% TFA while cord DHA was a median of 3.0% TFA. Cord DHA was of a significantly higher % TFA than in maternal plasma at 15 weeks (median 3.0% *vs.* 1.7% TFA, p=0.0003), and at 28 weeks (median 3.0% *vs.* 1.8% TFA, p<0.0001).

Palmitoleic acid (16:1n-7, $p<0.0001$), stearic acid (18:0, $p<0.0001$), arachidic acid (20:0, $p<0.0001$), DHGLA (20:3n-6, $p<0.0001$), AA (20:4n-6, $p<0.0001$), adrenic acid (22:4n-6, $p<0.0001$), lignoceric acid (24:0, $p=0.002$), and nervonic acid (24:1n-9, $p=0.009$) were all of a higher % TFA in cord plasma than maternal plasma at birth. Of these, only cord 24:0 and 24:1n-9 were not significantly higher than in maternal plasma at 28 weeks, (although the difference between maternal 28 weeks and cord plasma in 24:1n-9 tended to significance ($p=0.02$)).

Oleic acid (18:1n-9, $p<0.0001$), LA (18:2n-6, $p<0.0001$), and αLA (18:3n-3, $p=0.0005$) were all lower in cord plasma than maternal plasma at delivery (and also at 28 weeks, all $p<0.01$).

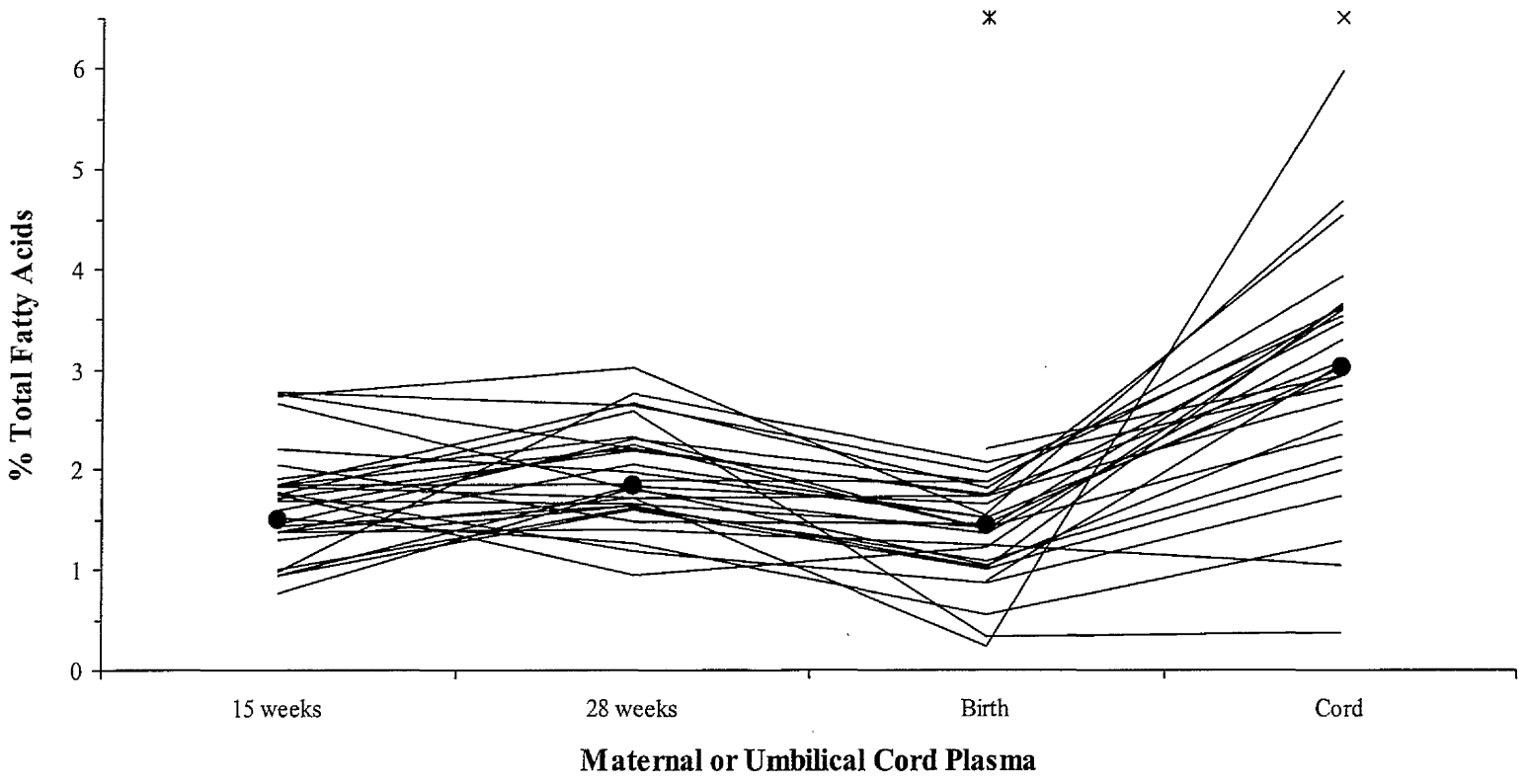



Figure 29. Longitudinal changes in % DHA in plasma of fish oil group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

9.3.3(d) Placebo Group: Maternal Plasma at 15 and 28 weeks gestation (Figure 30)
DHA as a % TFA did not change in the plasma of placebo receiving mothers between 15 weeks (median 1.7% TFA) and 28 weeks (median 1.6% TFA) (p=1.0).

Relative amounts of stearic acid (18:0, p=0.0001) and AA (20:4n-6, p=0.002) were lower in plasma from the placebo group at 28 weeks than 15 weeks. Maternal αLA (18:3n-3) increased over this time from a median of 0.5% TFA to 0.6% TFA (p=0.007), a median increase of 0.2% TFA *i.e.* 32% of 15 week level.

Although not significant at p<0.01, palmitoleic acid (16:1n-7) was higher at 28 weeks than 15 weeks (p=0.02), and nervonic acid (24:1n-9) was lower at 28 weeks (p=0.02).

9.3.3(e) Placebo Group: Maternal Plasma at 28 weeks and Birth (Figure 30)
Plasma DHA decreased from a median of 1.6% TFA at 28 weeks to median 1.2% TFA by birth in the placebo group. This (median) 33% decrease in 28 weeks level was significant at p<0.0001. Relative levels at 15 weeks (median 1.6% TFA) and birth (median 1.2% TFA) also differed significantly (p=0.0003).

AA (20:4n-6) continued to decrease until the time of birth to % TFA lower than at 28 weeks (p=0.0005) and hence 15 weeks (p<0.0001).

Myristic acid (14:0, p=0.0001), arachidic acid (20:0, p<0.0001), DHGLA (20:3n-6, p<0.0001), EPA (20:5n-3, p=0.0001), lignoceric acid (24:0, p=0.003) and DPA (22:5n-3, p<0.0001) all decreased from 28 weeks to birth in maternal plasma. The decrease in 20:0, 20:3n-6, 24:0 and 22:5n-3 caused these fatty acids to be of lower % TFA than at 15 weeks (all p<0.01).

By birth, maternal palmitic (16:0, p=0.009) and oleic (18:1n-9, p=0.009) acids had increased from their levels at 28 weeks.

LA (18:2n-6, p=0.03) and adrenic acid (22:4n-6, p=0.02) were also higher at 28 weeks than birth, although not significantly at p<0.01.

9.3.3(f) Placebo Group: Maternal Plasma at Birth and Fetal Umbilical Cord Plasma (Figure 30)

DHA was of a significantly higher % TFA ($p<0.0001$) in cord plasma (median 3.0%) than maternal plasma at birth (median 1.2%), with maternal levels a median of 58% below that of cord plasma. Cord DHA was also significantly lower than maternal DHA at 15 weeks (median 3.0% TFA *vs.* 1.6% TFA, $p<0.0001$), and at 28 weeks (median 2.9% TFA *vs.* 1.7% TFA, $p=0.003$).

Cord plasma was also higher than maternal plasma in its % of: palmitoleic acid (16:1n-7, $p<0.0001$); stearic acid (18:0, $p<0.0001$); arachidic acid (20:0, $p<0.0001$); DHGLA (20:3n-6, $p<0.0001$); AA (20:4n-6, $p<0.0001$); adrenic acid (22:4n-6, $p<0.0001$); lignoceric acid (24:0, $p=0.004$), and nervonic acid (24:1n-9, $p=0.006$). All these fatty acids, except 24:0 and 24:1n-9, were higher in cord than maternal 28 weeks plasma.

Cord plasma was lower in oleic acid (18:1n-9, $p<0.0001$), LA (18:2n-6, $p<0.0001$), and αLA (18:3n-3, $p<0.0001$) than maternal birth plasma (and also maternal 28 weeks plasma).

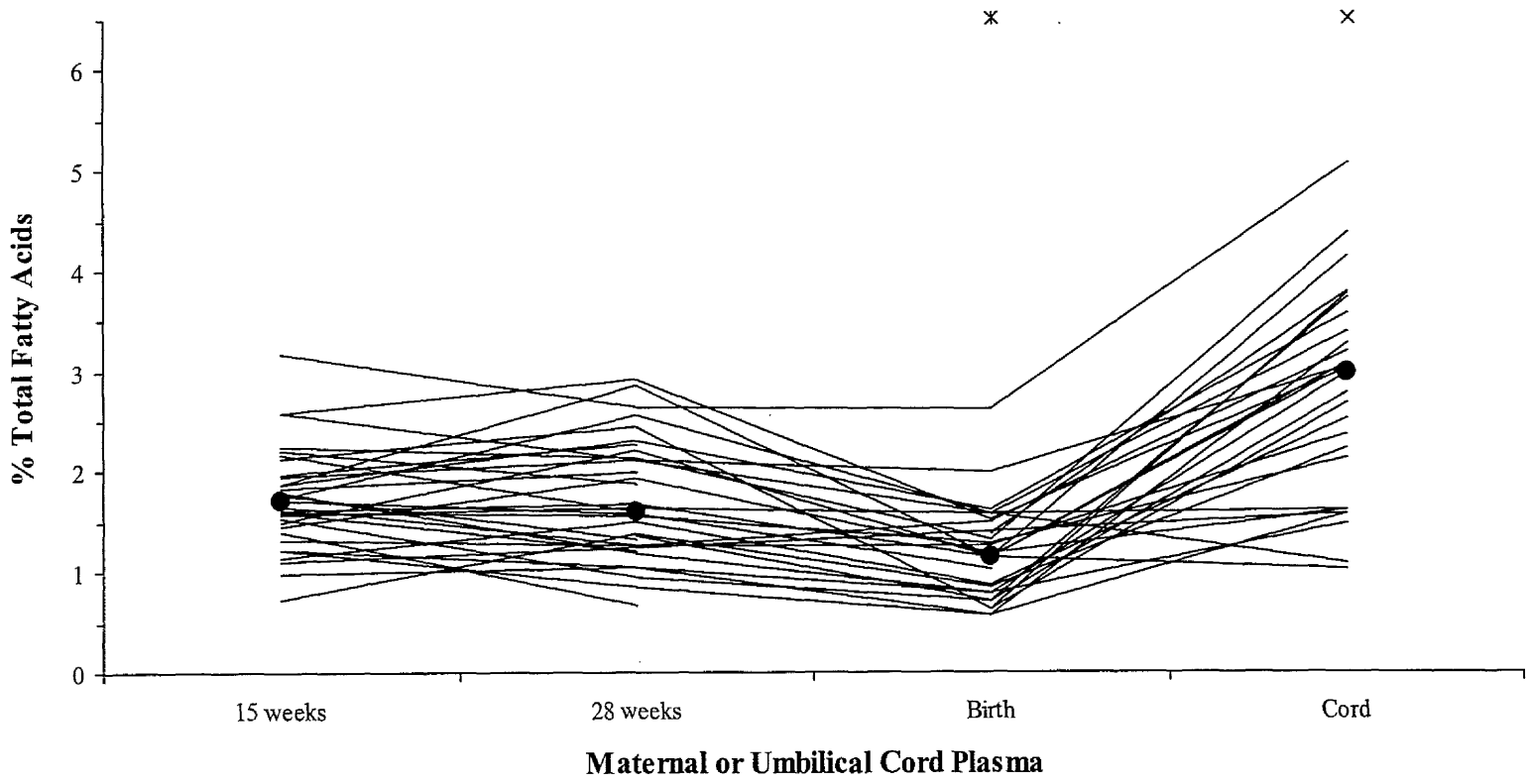



Figure 30. Longitudinal changes in % DHA in plasma of placebo group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

### 9.3.4 Plasma Fatty Acids: Concentration (Absolute Levels)

9.3.4(a) Fish Oil Group: Maternal Plasma at 15 and 28 weeks gestation (Figure 31)

The concentration of DHA in maternal plasma increased significantly ($p=0.0005$) in the fish oil group from a median of 135 nmol/ml at 15 weeks to 216 nmol/ml at 28 weeks.

Concentrations of myristic acid (14:0, $p<0.0001$), palmitic acid (16:0, $p<0.0001$), palmitoleic acid (16:1n-7, $p<0.0001$), stearic acid (18:0, $p=0.008$), oleic acid (18:1n-9, $p=0.002$), arachidic (20:0, $p<0.0001$), DHGLA (20:3n-6, $p=0.008$) and EPA (20:5n-3, $p=0.002$) all increased in the plasma of the fish oil group between 15 and 28 weeks.

9.3.4(b) Fish Oil Group: Maternal Plasma at 28 weeks and Birth (Figure 31)

By birth, maternal DHA concentrations were not significantly different from those at 28 weeks, although tending to significance at $p=0.02$, with 28 weeks concentrations higher (median 216 nmol/ml *vs.* 171 nmol/ml). Maternal DHA concentrations at birth (median 173 nmol/ml) were higher than at 15 weeks (median 135 nmol/ml), but, again, this was not significant with $p=0.02$.

Myristic (14:0) and arachidic (20:0) acids, which both increased between 15 and 28 weeks, significantly decreased between 28 weeks and birth ($p<0.0001$ for both), to levels similar to their original 15 weeks levels. DHGLA (20:3n-6, $p=0.02$), EPA (20:5n-3, $p=0.02$) and adrenic acid (22:4n-6, $p=0.04$) were also higher at 28 weeks than birth, but with a significance level of $p<0.01$, were not significantly so.

9.3.4(c) Fish Oil Group: Maternal Plasma at Birth and Fetal Umbilical Cord Plasma (Figure 31)

The concentration of DHA was not significantly different in maternal plasma at birth to that in cord plasma, only tending to significance at $p=0.03$, with cord concentrations higher (median 436 nmol/ml *vs.* 172 nmol/ml). Cord concentrations were also higher than maternal levels at 15 weeks (median 460 nmol/ml *vs.* 133 nmol/ml), and at 28 weeks (465 nmol/ml *vs.* 217 nmol/ml); again, these differences were non-significant ($p=0.05$ and 0.06, respectively).

Cord plasma was lower in concentrations of myristic acid (14:0), palmitic acid(16:0) palmitoleic acid (16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), LA (18:2n-6), αLA (18:3n-3), DHGLA (20:3n-6), AA (20:4n-6) (all $p<0.0001$) and EPA (20:5n-3) ($p=0.004$). All of these cord fatty acids were also lower (all $p<0.0001$) than maternal 15 and 28 weeks levels, except for 16:1n-7 which was lower than maternal levels at 28 weeks but not 15 weeks.

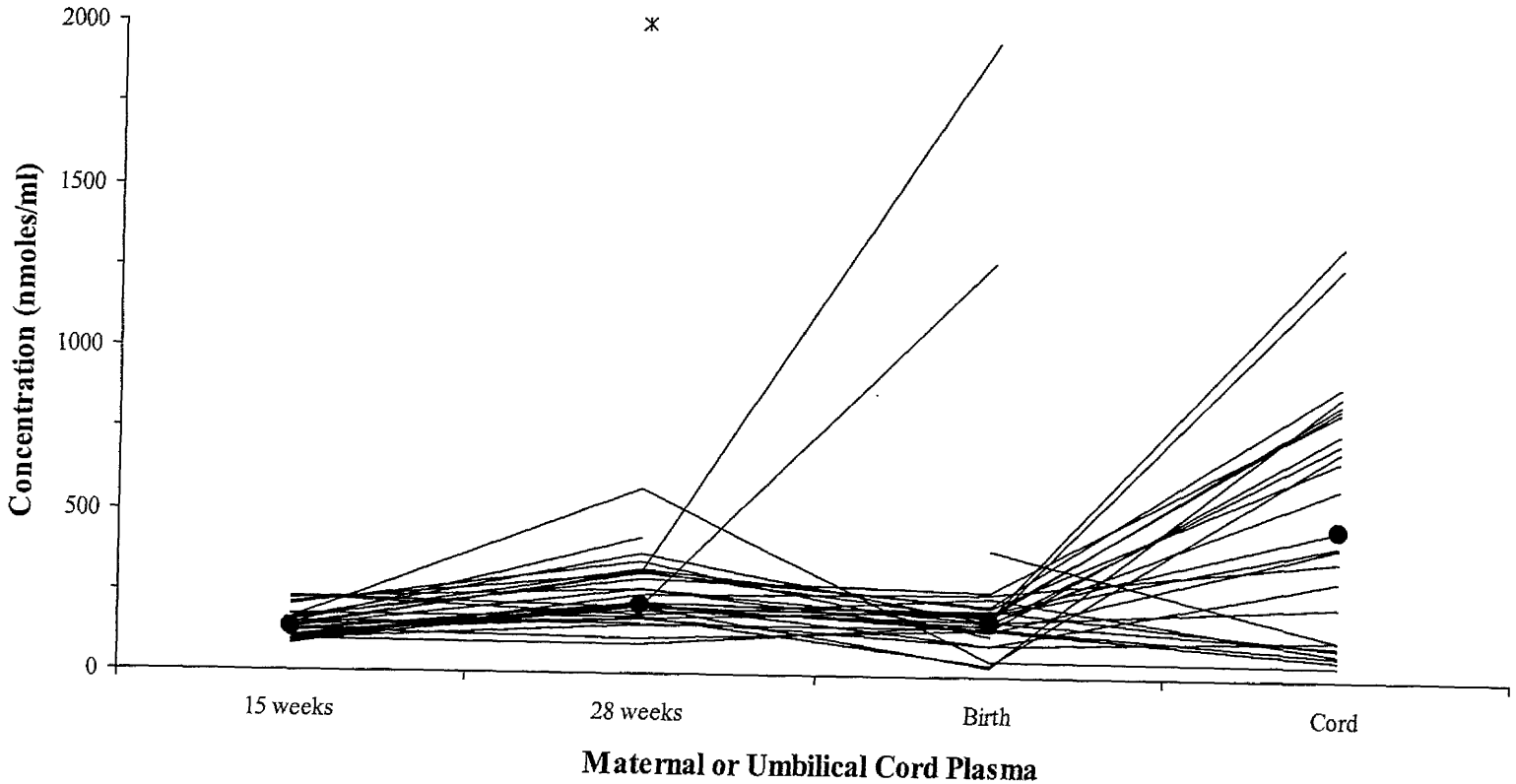



Figure 31. Longitudinal changes in DHA concentration in plasma of fish oil group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

9.3.4(d) Placebo Group: Maternal Plasma at 15 and 28 weeks gestation (Figure 32)
Maternal DHA increased in concentration between 15 and 28 weeks, from median 138 nmol/ml to 179 nmol/ml, a median increase of 59 nmol/ml ($p=0.002$).

The group also had significant increases in their concentrations of myristic acid (14:0, $p<0.0001$), palmitic acid (16:0, $p<0.0001$), palmitoleic acid (16:1n-7, $p<0.0001$), oleic acid (18:1n-9, $p<0.0001$), arachidic acid (20:0, $p<0.0001$), DHGLA (20:3n-6, $p=0.0005$), and AA (20:4n-6, $p=0.0005$).

9.3.4(e) Placebo Group: Maternal Plasma at 28 weeks and Birth (Figure 32)
There was no significant difference between DHA concentrations at 28 weeks (median 172 nmol/ml) and birth (median 131 nmol/ml) in the placebo group ($p=0.56$). DHA concentrations at birth (median 137 nmol/ml) were similar to those at 15 weeks (median 137 nmol/ml) ($p=0.34$).

Placebo group concentrations of myristic (14:0) and arachidic (20:0) acids increased between 15 and 28 weeks, but then decreased between 28 weeks and birth (both $p=0.009$), to levels not significantly different to those as 15 weeks. Indeed, the difference in 14:0 between 15 and 28 weeks was 41% of the 28 weeks value, and its difference between 28 weeks and birth was 39% of the 28 weeks value. For 20:0, the increment between 15 and 28 weeks was 47% of the 28 weeks value, and the corresponding decrease was 38% of the 28 weeks value. Thus it can be seen that the magnitude of change for both of these fatty acids between 15 weeks and 28 weeks was similar to that between 28 weeks to birth, but of the opposite direction, accounting for the significant differences observed between time points.

Oleic acid (18:1n-9) continued to increase ($p=0.003$) in the placebo group between 28 weeks and birth, having already increased between 15 and 28 weeks.

9.3.4(f) Placebo Group: Maternal Plasma at Birth and Fetal Umbilical Cord Plasma (Figure 32)
The placebo group had no significant difference between DHA concentrations in maternal plasma at birth (median 137 nmol/ml) and cord plasma (median 413 nmol/ml) ($p=0.18$). Moreover, cord concentrations (median 413 nmol/ml) did not

differ significantly from maternal 28 weeks (median 172 nmol/ml) concentrations ($p=0.17$). There was, however, a significant difference between concentrations in cord plasma (median 374 nmol/ml) and maternal 15 weeks plasma (median 141 nmol/median) ($p=0.01$).

Myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), LA (18:2n-6), αLA (18:3n-3), DHGLA (20:3n-6), AA (20:4n-6) and EPA (20:5n-3) were all of a lower concentration in cord plasma than maternal plasma at birth (all $p<0.0001$). All these cord fatty acids were also significantly lower ($p<0.01$) than maternal concentrations at 15 and 28 weeks, except cord 16:1n-7 which was only lower than maternal 28 weeks level.

Placebo group cord plasma had a higher concentration of adrenic acid (22:4n-6) than maternal birth plasma ($p=0.006$).

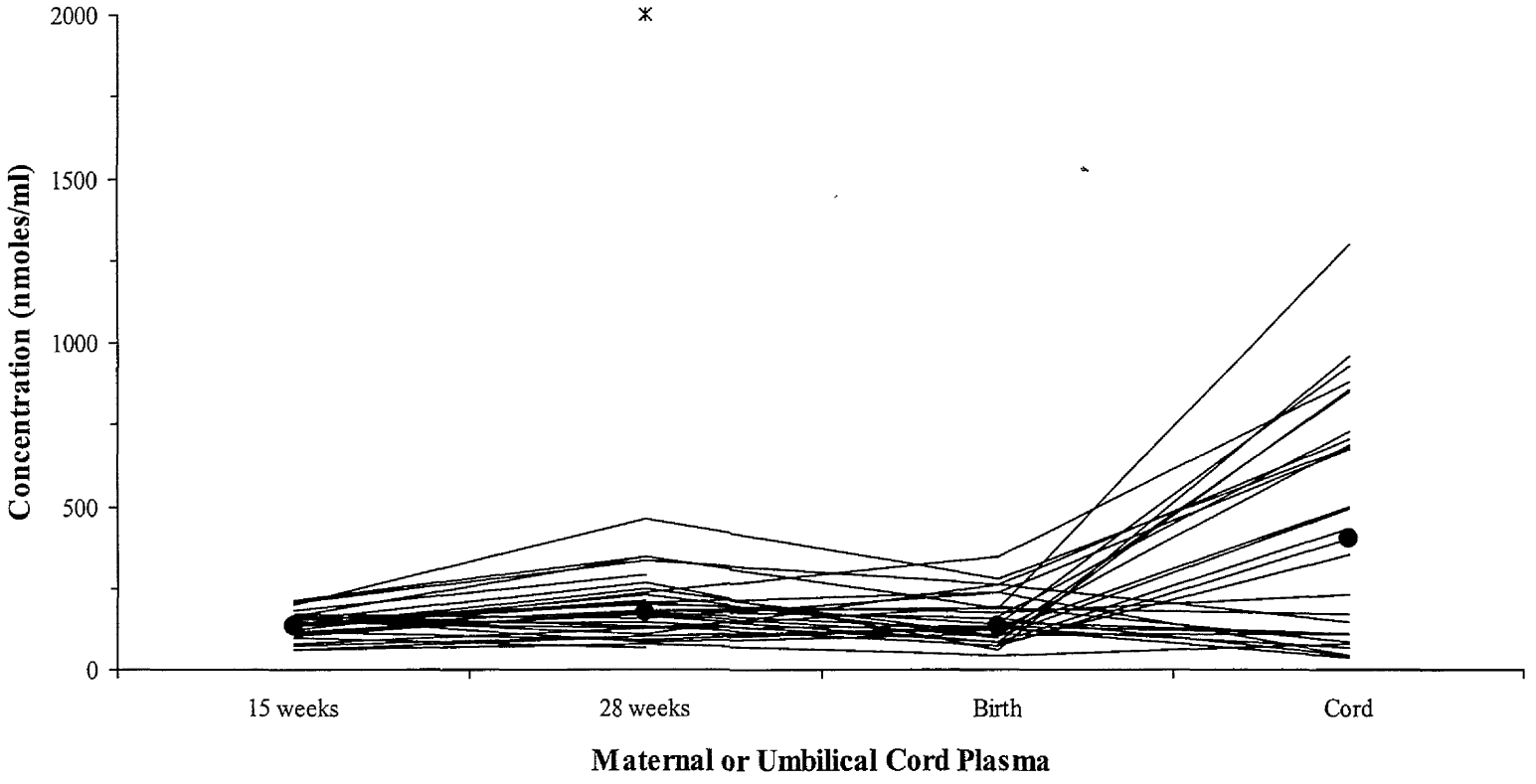



Figure 32. Longitudinal changes in DHA concentration in plasma of placebo group. Each line represents an individual mother and infant. ● Group median values; * significant difference from maternal sample at previous time point; x significant difference between maternal and umbilical cord samples at birth

## 9.4 COMPARISON OF THE FATTY ACID PATTERNS OF FISH OIL AND PLACEBO GROUPS

This section summarises the inter-group differences, the intra-group patterns of change and the relationship between maternal and fetal status. The longitudinal maternal changes within each group and their materno-fetal differences are compared in order to explain the differences arising between the supplement and placebo groups.

### 9.4.1 RBC Fatty Acids: % Total Fatty Acids (Relative Levels)

9.4.1(a) Maternal RBC at 15 and 28 weeks gestation

By 28 weeks, the mothers receiving fish oil supplementation had increased their DHA status by (median) 38% of their 15 week status, whereas the placebo group had a 25% increase above their 15 weeks % DHA. Thus, although showing the same pattern as the fish oil group, *i.e.* an increase in DHA between 15 and 28 weeks, the magnitude of change was smaller in the placebo group, both in relation to the % TFA (0.7% TFA *vs.* 1.1% TFA) and to the original level (25% *vs.* 38% increase in 15 weeks level). As a result the median % TFA of DHA in RBC at 28 weeks in the placebo group was 80% of that in the fish oil group. Moreover, total n-3 fatty acids in the placebo group amounted to 87% of total n-3 levels in the fish oil group.

An increase in both myristic (14:0) and palmitoleic (16:1n-7) acids between 15 and 28 weeks was observed in the two treatment groups, although the groups differed in the patterns of change for other fatty acids. The fish oil group decreased its % TFA of LA (18:2n-6), while the placebo group decreased in % of palmitic acid (16:0) and stearic acid (18:0). In addition, only the placebo group increased over this time in DHGLA (20:3n-6), EPA (20:5n-3) and DPA (22:5n-3). However, at 28 weeks, the groups did not differ significantly from each other in any n-3 fatty acids other than DHA. Thus, the elevated DHA observed in the fish oil group was sufficient to cause the fish oil group to have significantly higher total n-3 fatty acids, and therefore lower n-6/n-3 ratio, at 28 weeks.

The treatment groups did, however, differ in the amount of AA (20:4n-6) as a % TFA, with the placebo group significantly higher at 28 weeks. Since the sum of DHA (22:6n-3) and AA (20:4n-6) was approximately equal in both groups (14.8% in the fish oil group, 14.5% in the placebo group), it is possible that a compensatory

mechanism was occurring, with an increase in DHA counterbalancing a decrease in AA, and *vice versa.*

9.4.1(b) Maternal RBC at 28 weeks and Birth

DHA as a % TFA in both groups declined in maternal RBC between 28 weeks and birth. Although maternal RBC DHA at birth was not significantly different from that at 15 weeks in either fish oil or placebo group, the magnitude of decrease between 28 weeks and birth was greatest in the placebo group (median decrease in 28 weeks level of 41% compared to 19%). At birth, maternal RBC DHA in the fish oil group was 0.8% TFA lower than at 28 weeks, and was equivalent to 84% of its level at 28 weeks. In the placebo group, RBC DHA declined by 1.4% TFA between 28 weeks and birth, such that maternal birth DHA was 71% of its 28 week level. Thus in absolute (as % TFA) and relative (as % of previous measurement) terms, the placebo group increased by less between 15 and 28 weeks (see above), but decreased by more between 28 weeks and birth, compared to the fish oil group. The median maternal % DHA of the placebo group at birth was 77% of that of the fish oil group; the median % of total n-3 fatty acids in the placebo group was 84% of that in the fish oil group. Thus, the same magnitude of difference between the two groups, for DHA and total n-3 fatty acids, was observed at birth and at 28 weeks gestation.

There were decreases between 28 weeks and birth in both groups in DHGLA (20:3n-6), AA (20:4n-6), EPA (20:5n-3), adrenic acid (22:4n-6) and DPA (22:5n-3). However, in the placebo group, after an increase between 15 and 28 weeks, DHGLA (20:3n-6), EPA (20:5n-3), and DPA (22:5n-3) decreased between 28 weeks and birth to levels comparable to those at 15 weeks. This is in contrast to the fish oil group, where there were no changes in these fatty acids between 15 and 28 weeks, but DHGLA (20:3n-6), EPA (20:5n-3) and DPA (22:5n-3) decreased between 28 weeks and birth, such that the levels of 20:5n-3 and 22:5n-3 were lower at birth than at 15 weeks. Despite the decline in individual n-3 fatty acids as % TFA in the two groups prior to birth, the fish oil and placebo groups remained significantly different in % DHA and total n-3 at birth, with higher levels and hence a lower n-6/n-3 ratio observed in the fish oil group.

The increases in % of palmitic (16:0) and oleic (18:1n-9) acids between 28 weeks and birth were observed in both groups.

9.4.1(c) Maternal RBC at Birth and Fetal Umbilical Cord RBC

Fish oil group cord RBC DHA was (median) 37% higher than maternal birth RBC, with a median of 4.3% TFA compared to 3.1% TFA. Thus, the elevation of DHA in cord RBC above that in maternal birth RBC was of the same magnitude as the increase in maternal DHA between 15 weeks and 28 weeks. Indeed, the % TFA of DHA in cord RBC was not significantly different from that in maternal RBC at 28 weeks, and maternal RBC at birth was not significantly different from maternal 15 weeks RBC.

Placebo group cord DHA was 74% higher than maternal plasma % DHA. Moreover cord RBC DHA (median 4.2% TFA) was 19% higher ($p=0.003$) than maternal 28 weeks DHA (median 3.4% TFA).

The elevation of cord RBC % DHA above baseline maternal RBC levels (15 weeks) was similar in both groups. The difference between cord and maternal 15 weeks RBC was (median) 1.4% TFA or 48% of 15 weeks levels in the fish oil group, and 1.4% TFA or 51% of 15 weeks levels in the placebo group.

Although cord RBC DHA was not significantly different between treatment groups, the difference between cord and maternal birth RBC DHA was greater in the placebo group, being 1.2% TFA in the fish oil group, and 1.8% TFA in the placebo group. Thus, maternal birth DHA was 72% of cord DHA in the fish oil group, and 58% of cord DHA in the placebo group. Cord RBC DHA was significantly higher than 28 weeks maternal DHA in the placebo group, in contrast to the fish oil group where cord DHA was not significantly different from the maximal maternal DHA status observed at 28 weeks. The two groups differed further in the differences between cord and 28 weeks maternal values. In the placebo group, *all* cord RBC fatty acids significantly different to maternal birth RBC were also different to maternal 28 weeks RBC. In the fish oil group, DHA was the only fatty acid in cord RBC that was significantly different from maternal RBC at birth, which was not significantly different from maternal levels at 28 weeks; other cord fatty acids significantly different from

maternal birth levels also showed the same difference when compared to 28 weeks maternal levels.

In both groups, the relative levels of stearic acid (18:0), arachidic acid (20:0), DHGLA (20:3n-6), AA (20:4n-6) and adrenic acid (22:4n-6) were higher in cord than maternal RBC at birth. Both groups also had a conversely lower status of oleic acid (18:1n-9), LA (18:2n-6), nervonic acid (24:1n-9) and DPA (22:5n-3) in cord compared to maternal RBC.

### 9.4.2 RBC Fatty Acids: Concentration (Absolute Levels)

9.4.2(a) Maternal RBC at 15 and 28 weeks gestation

Despite non-significant increases in DHA concentration between 15 and 28 weeks in both treatment groups, the fish oil group had a significantly higher concentration of DHA at 28 weeks, compared to the placebo group. At 28 weeks, the median DHA concentration in the placebo group had a value of only 88% of that of the fish oil group.

The magnitude of the increase in palmitoleic acid (16:1n-7) concentration from 15 to 28 weeks, in both absolute (nmol/ml) and relative (% of 15 weeks level) terms, was larger in the placebo compared to the fish oil group.

9.4.2(b) Maternal RBC at 28 weeks and Birth

Maternal DHA concentration was maximal at 28 weeks in both groups, but in the fish oil group, 28 weeks concentration was not significantly different to 15 weeks or birth maternal levels. The placebo group also did not differ significantly between 15 and 28 weeks, but was, however, significantly higher at 28 weeks than at birth.

By birth, the placebo group had a median 32% decline in its 28 weeks concentration of DHA. Such a significant decrease in RBC DHA concentration between these times was observed only in the placebo group. The fish oil group did not exhibit a significant change in RBC DHA concentration between 28 weeks and birth, although concentrations at 28 weeks were slightly higher. Thus, when comparing the two groups, the significantly higher concentration of DHA observed in the fish oil group at birth was mediated by a decline in DHA in the placebo group, rather than an increase

in the fish oil group. Consequently, the placebo group achieved only 70% of the maternal DHA concentration in the fish oil group at birth.

Both groups declined in concentrations of EPA (20:5n-3) between 28 weeks and birth. The differences between these time points was similar in the two groups – a (median) reduction of 7 nmol/ml or 60% of 28 weeks level in the fish oil group, and 7 nmol/ml or 65% of 28 weeks level in the placebo group. The lower EPA concentration at birth tended to a significant difference ($p=0.04$) from 15 weeks concentration in the fish oil group, but not in the placebo group. The placebo group also declined significantly in DHGLA (20:3n-6), adrenic acid (22:4n-6) and DPA (22:5n-3) (and non-significantly in AA (20:4n-6)). The difference in total n-3 fatty acids between the fish oil and placebo groups may therefore be accounted for by the greater decline in DHA, coupled with the decrease in both EPA (20:5n-3) and DPA (22:5n-3), observed in the placebo group. The overall results were thus significantly higher maternal concentrations of DHA and total n-3 fatty acids at the time of birth in the fish oil group, compared to the placebo group, accounting for the significantly lower n-6/n-3 ratio observed in the fish oil group.

Concentrations of palmitic acid (16:0) in both groups were higher at birth but with probability greater than 0.01.

9.4.2(c) Maternal RBC at Birth and Fetal Umbilical Cord RBC

Despite a lack of significant difference between treatment groups in cord RBC DHA concentrations, the groups differed somewhat in the relationship between maternal and cord DHA concentrations.

In the fish oil group, cord DHA concentrations differed significantly from maternal birth DHA by (median) 68 nmol/ml, with median maternal concentration equal to 76% of that in cord RBC. In contrast, placebo group cord DHA concentrations differed from corresponding maternal birth concentrations by (median) 149 nmol/ml, such that median maternal DHA was only 44% of cord concentration. Fish oil group cord DHA was not significantly different to maternal 28 weeks concentrations at a significance level of $p<0.01$, with maternal 28 weeks DHA equivalent to 85% of cord DHA ($p=0.02$), or to maternal 15 weeks DHA concentration ($p=0.02$). Conversely,

placebo group cord DHA was higher than maternal 28 weeks concentrations at a significance level of $p<0.01$, with maternal 28 weeks concentrations equal to 65% of cord levels ($p<0.0001$). The higher concentration of DHA in cord RBC was also significantly different from maternal 15 weeks concentrations in the placebo group ($p=0.01$).

Both groups had higher concentrations in cord than maternal birth plasma for DHGLA (20:3n-6), AA (20:4n-6) and adrenic acid (22:4n-6). The placebo group also had elevated concentrations of arachidic acid (20:0) in cord RBC; the fish oil group had lower cord than maternal palmitic acid (16:0) concentrations. Cord LA (18:2n-6) concentrations were lower than corresponding maternal levels in both groups. Cord oleic acid (18:1n-9) concentration, significantly lower than maternal birth concentration in the fish oil group, was only lower at $p=0.02$ in the placebo group.

### 9.4.3 Plasma Fatty Acids: % Total Fatty Acids (Relative Levels)

9.4.3(a) Maternal Plasma at 15 and 28 weeks gestation

Neither treatment group exhibited significant changes in the % TFA accounted for by DHA in plasma over the period of 15 to 28 weeks. Thus, the treatment groups had similar % DHA in maternal plasma at both 15 and 28 weeks.

Both groups did increase their % palmitoleic acid (16:1n-7), although not significantly so in the placebo group. In addition, a decrease in stearic acid (18:0) as % TFA was observed in both groups. The fish oil group, however, had a decrease in lignoceric acid (24:0), while the placebo group decreased in AA (20:4n-6) and (non-significantly) in nervonic acid (24:1n-9). The placebo group also had a significant increase in % αLA (18:3n-3).

The elevated DHGLA (20:3n-6) observed in the placebo group at 28 weeks may have induced a reciprocal decrease in % total n-3 fatty acids, accounting for the higher total n-3 and lower n-6/n-3 ratio of the fish oil group.

9.4.3(b) Maternal Plasma at 28 weeks and Birth

The decline between 28 weeks and birth in maternal plasma DHA as % TFA was 28% of the 28 weeks value in the fish oil group, and 33% of the 28 weeks value in the

placebo group. The similar magnitude of change ensured that the treatment groups did not differ significantly from each other in % plasma DHA at birth.

The fatty acids 14:0 (myristic), 20:0 (arachidic), 20:3n-6 (DHGLA), 22:4n-6 (adrenic), 24:0 (lignoceric) and 22:5n-3 (DPA) all decreased in both groups between 28 weeks and birth, but in the fish oil group, the values for all of these at birth were also lower than those at 15 weeks. In contrast, only 20:0, 20:3n-6, 24:0 and 22:5n-3 accounted for lower % TFA at birth than 15 weeks in the placebo group.

Nervonic acid (24:1n-9) in the fish oil group decreased non-significantly between both 15 and 28 weeks, and then 28 weeks and birth, such that % 24:1n-9 was significantly lower at birth than 15 weeks. Maternal αLA (18:3n-3) and EPA (20:5n-3) also decreased between 28 weeks and birth, but remained similar to their % at 15 weeks.

AA (20:4n-6) decreased in the placebo group over the entire period from 15 weeks to birth, such that both subsequent levels were lower than at 15 weeks. The placebo group also exhibited increased % of palmitate (16:0), oleate (18:1n-9), LA (18:2n-6) and adrenic acid (22:4n-6), accounting for the significantly higher % 16:0 observed in the placebo compared to fish oil group at birth.

9.4.3(c) Maternal Plasma at Birth and Fetal Umbilical Cord Plasma

Maternal plasma in the fish oil group had a % DHA level significantly lower than that of cord plasma by (median) 49% of the cord level. Maximal maternal DHA, observed at 28 weeks, was also significantly lower than cord DHA but to a lesser extent, by a median of 30% of the cord level (1.8% TFA *vs.* 3.0% TFA). Thus, maternal plasma DHA at birth was equivalent to 47% of the cord level, while maternal DHA at 28 weeks was 61% of that of cord plasma.

In the placebo group, the difference between maternal and cord plasma % TFA for DHA was a median of 58% of the cord level, with maternal levels significantly below that of cord. The difference between 28 weeks and cord levels was also significant and equivalent to 31% of the cord level.

The median differences between maternal birth and cord plasma DHA in the fish oil and placebo groups was 1.5% TFA and 1.7% TFA respectively. In relative terms, these differences were 49% and 58% of their respective cord levels, such that maternal birth levels were 47% of those in the cord in the fish oil group, and 39% of those in the cord in the placebo group. At 28 weeks, maternal levels were 61% of those in cord for the fish oil group, and 58% of those in cord in the placebo group. Thus although the differences are of the same pattern (*i.e.* higher in cord plasma), the magnitude of difference between maternal and cord plasma DHA is smaller in the fish oil group. The two groups did not, however, differ significantly from each other in their respective % DHA in cord plasma.

Both groups had higher levels in cord than maternal birth plasma for: palmitoleic acid (16:1n-9), stearic acid (18:0), arachidic acid (20:0), DHGLA (20:3n-6), AA (20:4n-6), adrenic acid (22:4n-6), lignoceric acid (24:0) and nervonic acid (24:1n-9). In both groups, only cord 24:0 and 24:1n-9 were not also significantly higher than maternal 28 weeks values, although the difference in 24:1n-9 tended to significance (p=0.02) in the fish oil group.

The magnitude of these changes, for fatty acids other than DHA, were similar in the treatment groups in both absolute (% TFA) and relative (as % of maternal levels) terms, accounting for the lack of significant differences on comparison of the two groups. For example, αLA (18:3n-3) was higher in maternal birth plasma than cord plasma by 0.4% TFA, with cord plasma only 20% of the maternal birth value, in the fish oil group. In the placebo group, maternal plasma 18:3n-3 was 0.3% TFA higher than cord 18:3n-3, with cord 18:3n-3 equivalent to 20% of the maternal level.

### 9.4.4 Plasma Fatty Acids: Concentration (Absolute Levels)

9.4.4(a) Maternal Plasma at 15 and 28 weeks gestation

The fish oil group had a higher plasma concentration of 18:1n-9 (oleic acid) at 15 weeks *prior* to supplementation. This was the only time the two groups differed in their levels of oleic acid, despite the expectation that the placebo group would accrue higher amounts.

By increasing a median of 107 nmol/ml between 15 and 28 weeks, fish oil supplemented mothers significantly increased their baseline DHA by 79%. The placebo group had a significant 42% increase in their baseline DHA concentration by 28 weeks, increasing by a median 59 nmol/ml. At 28 weeks, maternal DHA concentration in the placebo group was only 82% of that in the fish oil group, *i.e.* significantly lower.

The increases in concentrations of myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n-7), oleic acid (18:1n-9), arachidic acid (20:0) and DHGLA (20:3n-6) were observed in both groups. However, 28 weeks plasma of the fish oil group had increased stearic acid (18:0) and EPA (20:5n-3), while that of the placebo group had increased AA (20:4n-6).

The larger increase in DHA, coupled with the increase in EPA (20:5n-3), observed in the fish oil group account for the significantly higher concentrations of plasma DHA, EPA and total n-3 fatty acids present in the fish oil group at 28 weeks, when compared to the placebo group.

9.4.4(b) Maternal Plasma at 28 weeks and Birth

There were no significant differences between 28 weeks and birth in DHA concentration for either group, although both groups had highest concentrations at 28 weeks, with the change in the fish oil group tending ($p=0.02$) to significance. As a result, there was no difference in maternal DHA concentration between the two groups at birth. However, in the fish oil group at birth, the concentration of DHA in maternal plasma tended ($p=0.02$) to be higher than at baseline (15 weeks), while baseline and birth plasma DHA concentrations were almost identical (both median 137 nmol/ml) in the placebo group.

Also similar in both groups were the patterns of change in the concentrations of myristic (14:0) and arachidic (20:0) acids. Having increased between 15 and 28 weeks, both fatty acids decreased between 28 weeks and birth, to levels not significantly different to those as 15 weeks.

Concentrations of oleic acid (18:1n-9) increased between 15 and 28 weeks in both groups, but did not change significantly between 28 weeks and birth in the fish oil group. The placebo group, however, continued to increase in 18:1n-9 concentration, such that by birth, maternal 18:1n-9 concentration was elevated significantly above 28 weeks concentration. Nonetheless, there was no significant difference between the fish oil and placebo groups in plasma 18:1n-9 concentration at birth.

9.4.4(c) Maternal Plasma at Birth and Fetal Umbilical Cord Plasma

There were no significant differences between concentrations of DHA in maternal birth plasma and cord plasma for either group, although the higher concentration in cord plasma was almost significant for the fish oil group ($p=0.03$). There was no significant difference between treatment groups in cord plasma DHA concentrations.

Thus, the two groups followed the same pattern in plasma DHA concentration: increase from 15 to 28 weeks, with no significant differences between 28 weeks and birth, or mother at birth and cord. However, the fish oil group had a median 107 nmol/ml or 79% increase in 15 weeks DHA concentration by 28 weeks, while the placebo group only had a 59 nmol/ml or 42% increase. Thus, the magnitude of increase was greater in the fish oil group.

Concentrations of myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1n-7), stearic acid (18:0), oleic acid (18:1n-9), LA (18:2n-6), αLA (18:3n-3), DHGLA (20:3n-6), AA (20:4n-6) and EPA (20:5n-3) were all lower in cord plasma than maternal plasma at birth in both treatment groups. These cord fatty acids were also significantly lower than maternal 15 and 28 weeks concentrations, except 16:1n-7 which was only lower than the 28 weeks levels, a characteristic noted in both groups.

In both groups, the concentration of arachidic acid (20:0) decreased in maternal plasma between 28 weeks and birth. Although there were no differences between cord plasma and maternal plasma at birth, cord plasma was, as therefore expected, significantly lower in this fatty acid than maternal 28 weeks plasma in both groups.

Cord plasma of the placebo group had a higher concentration of adrenic acid (22:4n-6) than maternal birth plasma.

### 9.4.5 Placental Tissue Fatty Acids: % Total Fatty Acids (Relative Levels)

The only placental tissue fatty acid which differed in its % TFA between the two groups was myristic acid (14:0), which was lower in the fish oil group.

Although there were no differences in individual or total fatty acids from the n-3 or n-6 families when measured as % TFA in placental tissue, the ratio of n-6/n-3 fatty acids was lower in the fish oil group.

### 9.4.6 Placental Tissue Fatty Acids: Concentration (Absolute Levels)

DHA was present in higher concentrations in the placental tissue of the fish oil group, although the inter-group difference was not significant. The concentration of myristic acid (14:0) was lower in the placental tissue of the fish oil group. The concentrations of individual and total n-3 and n-6 fatty acids, as well as the ratio of n-6/n-3 fatty acid concentrations, did not differ between the groups.

### 9.4.7 Umbilical Cord Tissue Fatty Acids: % Total Fatty Acids (Relative Levels)

Umbilical cord tissue did not differ between groups in the relative amounts of any fatty acids, although the % TFA accounted for by DHA was slightly higher in the fish oil group.

### 9.4.8 Breast Milk Fatty Acids: % Total Fatty Acids (Relative Levels)

The only significant difference between the fish oil and placebo groups was the % palmitic acid (16:0), with the highest relative amount observed in the breast milk of the fish oil group.

### 9.4.9 Breast Milk Fatty Acids: Concentration (Absolute Levels)

Despite higher breast milk % AA (20:4n-6) in the placebo group, the totals and ratio of n-6 and n-3 fatty acids were similar in both groups.

Chapter 10

# Discussion

## 10.1 OVERVIEW

This study was undertaken because of strong evidence that the accumulation of DHA in fetal and neonatal tissue is important for optimal infant development, and that the DHA status of the fetus/neonate is affected by several factors, including maternal diet and nutritional status.

In examining the relationship between diet and maternal and/or fetal fatty acid status, previous studies have evaluated either habitual diet (Hornstra *et al* 1992, Lakin *et al* 1998, Olsen *et al* 1991, Reddy *et al* 1994, Sanjurjo *et al* 1995) or maternal supplementation during the last trimester of pregnancy (Connor *et al* 1996, van Houwelingen *et al* 1995). Similarly, the fatty acid composition of breast milk has been studied in relation to diet (Finley *et al* 1985, Innis & Kuhnlein 1988, Sanders & Reddy 1992) or supplementation (Fidler *et al* 2000b, Harris *et al* 1984, Helland *et al* 1998, Henderson *et al* 1992, Makrides *et al* 1996a) during lactation. While maternal supplementation (with LA) from mid-trimester has been undertaken (Al *et al* 1995b), there is a deficit of studies investigating the effects of earlier maternal supplementation with DHA on both mother and fetus.

The dose of DHA provided by supplements during late pregnancy or lactation has differed between studies, ranging from 200mg/d (Fidler *et al* 2000b, Makrides *et al* 1996a) to 1.3g/d (Makrides *et al* 1996a) of DHA. Supplementation with approximately 200mg/d DHA was effective in modulating maternal and/or infant PUFA status in previous studies (Fidler *et al* 2000b, Makrides *et al* 1996a, Jensen *et al* 1999 & 2000).

The effects of neonatal diet and infant formula LCPUFA supplementation on indices of infant development have been studied, with no long-term benefits discernible (Simmer 2001a & b). Fetal nutrition, and its dependence on maternal nutrition, has not been considered in relation to development. The effect of maternal supplementation during pregnancy on infant development has not been studied.

This study was therefore designed to provide expectant mothers, from a population with habitually low fish consumption, with a dose of DHA (200mg/d) previously shown to be beneficial and which could easily be achieved by compliance with

dietary recommendations, in a palatable and convenient form. The effect of such a supplement on the biochemical status of both mother and fetus was subsequently assessed in this randomized, placebo controlled trial.

At 15 weeks gestation, 100 mothers were randomly assigned to receive either a fish oil supplement rich in DHA (n=50) or a high oleic sunflower oil placebo (n=50) until term. Maternal fatty acid status was assessed in RBC and plasma at 15 weeks, 28 weeks and term. Fetal status was assessed in umbilical cord RBC, umbilical cord plasma, placental tissue and umbilical cord tissue. Breast milk fatty acid status was assessed in lactating mothers within the first week following delivery. The relative (% TFA) and absolute (concentration) values of fatty acids were reported.

The groups were matched in terms of maternal dietary and lifestyle habits, age, parity, anthropometry and socio-economic status; their neonates were of comparable gestational age and anthropometry.

### 10.2 DHA IN RELATION TO PARITY AND DIET

The effect of previous pregnancies and fish consumption on maternal/fetal DHA status was determined prior to analysis between treatment groups, to ascertain whether the distribution of DHA status in the study population varied with these or other underlying factors as previously reported.

There have been conflicting reports as to whether maternal plasma and RBC status declines with parity and subsequent pregnancies (Al *et al* 1997, van Houwelingen *et al* 1999). Within the Glasgow population, no difference in plasma or RBC relative (% TFA) fatty acid status was observed between parous and non-parous women when not pregnant (Berry *et al* 2001). "Normalisation" of maternal status apparently occurs within one year of parturition (van Houwelingen *et al* 1999). Neonatal DHA status has been reported to be compromised in the successive pregnancies of multigravida mothers (Al *et al* 1997). In the current study population, the reports of similar maternal fatty acid status regardless of parity and the interval between pregnancies (more or less than one year), and the lower umbilical cord plasma in the neonates of parous women, thus concur with previous findings.

The observed elevation of % DHA in baseline RBC and plasma on regular consumption of fish, even if only once per week, has been noted previously (Bønaa *et al* 1992). After randomization, the two groups were matched in terms of fish consumption; within each dietary group, approximately half received the fish oil supplement and half the placebo. Supplementation with 200mg/d DHA, which effectively doubled the baseline intake of DHA, would therefore provide those not consuming fish but receiving fish oil capsules with the same amount of DHA as those consuming dietary fish and placebo capsules. It is therefore unsurprising that no effect of fish consumption *per se* was noted following supplementation. The elevation of baseline DHA (% TFA and concentration) in plasma lipids following fish consumption in the previous 24 hours is evidence of the suitability of plasma as a reliable indicator of dietary intake in the short-term (Bjerve *et al* 1993, Dougherty *et al* 1987, Pauletto *et al* 1996). Conversely, the lack of an association between recent fish consumption and RBC composition is in keeping with use of RBC as an index of longer-term dietary fatty acid intake.

The study population was therefore considered to be a representatively "normal" sample, demonstrating typical variation in their fatty acid status.

## 10.3 EFFECT OF SUPPLEMENTATION ON MATERNAL AND FETAL STATUS

### 10.3.1. Summary

Elevated status of DHA and total n-3 fatty acids was observed in the fish oil supplemented group, creating a more favourable ratio of n-6 to n-3 PUFA (Table 64). However, the most interesting findings were observed when comparing the longitudinal maternal changes within each group, which served to explain the differences arising between the groups, and when considering the differences between mother and fetus. The results illustrate the mobilization of maternal DHA in early pregnancy and the subsequent accretion by the fetus at the expense of mother, and suggest that this physiological process may be modulated by maternal DHA supplementation.

| | AA | EPA | DHA | Total n-3 | n-6/n-3 |
|---|---|---|---|---|---|
| RBC: % TFA | | | | | |
| 15 weeks | | | | | |
| 28 weeks | # | | * | * | * |
| Birth | | | * | * | * |
| Cord | | | | | |
| RBC: Concentration | | | | | |
| 15 weeks | | | | | |
| 28 weeks | | | * | * | * |
| Birth | | | * | | * |
| Cord | | | | | |
| Plasma: % TFA | | | | | |
| 15 weeks | | | | | |
| 28 weeks | | | | * | * |
| Birth | | | | | |
| Cord | | | | | |
| Plasma: Concentration | | | | | |
| 15 weeks | | | | | |
| 28 weeks | | * | * | * | * |
| Birth | | | | | |
| Cord | | | | | |
| Placenta: % TFA | | | | | * |
| Placenta: Concentration | | | | | |
| Cord: % TFA | | | | | |
| Breast Milk: %TFA | | | | | |
| Breast Milk: Concentration | # | | | | |

Table 64. Summary of indices measured and the statistically significant ($p<0.05$) differences between groups. # Placebo group significantly higher in AA. * Fish oil group significantly higher in EPA, DHA or total n-3 fatty acids, or lower in n-6/n-3 ratio.

### 10.3.2 RBC Fatty Acids: % Total Fatty Acids (Relative Levels)

DHA, expressed as % TFA, increased in maternal RBC between 15 and 28 weeks gestation in both groups, but the greater increase was observed in the fish oil group. The fish oil group decreased in % LA while the placebo group increased in other LCPUFA (DHGLA, 20:3n-6; EPA, 20:5n-3; DPA 22:5n-3). However, the relative levels of these LCPUFA were not significantly different between groups, except for a higher % AA (20:4n-6) observed in the placebo group. This suggests that the elevation of these LCPUFA in the placebo group between 15 and 28 weeks was in part compensatory for the lower maternal accretion of DHA. Despite this, and as a result of the enhanced increase in maternal DHA in the fish oil group, the relative amounts of DHA and total n-3 fatty acids were significantly higher, and n-6/n-3 ratio lower, in the fish oil compared to placebo group at 28 weeks.

RBC % DHA decreased in both groups between 28 weeks and birth but the greater decline was observed in the placebo group. The magnitude of the difference in %

DHA and total n-3 fatty acids observed between groups at 28 weeks was thus sustained at birth. Other LCPUFA decreased in both groups; indeed, DHGLA (20:3n-6), EPA (20:5n-3) and DPA (22:5n-3) returned to baseline levels in the placebo group, suggesting that the compensatory increase in their levels in the absence of an adequate DHA source noted between 15 and 28 weeks (see above), was transient and unsustainable.

The relative amount of DHA in cord RBC did not differ between groups. In both groups, cord RBC had a higher % DHA than maternal RBC at birth, but the materno-fetal difference was smaller in the fish oil group. In addition, cord RBC in the placebo group was significantly higher than maternal RBC at 28 weeks; cord RBC in the fish oil group was not significantly different from maternal 28 weeks levels. The difference between cord and maternal baseline RBC % DHA was however, similar in both groups. These findings suggest that fish oil supplementation changed maternal status to a greater extent than fetal status.

Both groups also had higher % TFA for stearic acid (18:0), arachidic acid (20:0), DHGLA (20:3n-6), AA (20:4n-6) and adrenic acid (22:4n-6) in cord than maternal RBC, but lower cord than maternal % oleic acid (18:1n-9), LA (18:2n-6), nervonic acid (24:1n-9), and DPA (22:5n-3).

### 10.3.3 RBC Fatty Acids: Concentration (Absolute Levels)

Maternal RBC DHA concentration increased between 15 and 28 weeks, then decreased between 28 weeks and birth, in both groups. The fish oil group attained a higher DHA concentration at 28 weeks than the placebo group, but the former did not then significantly decline in DHA between 28 weeks and birth. In contrast, the placebo group had a higher DHA concentration at 28 weeks than birth, *i.e.* the placebo group significantly decreased in maternal RBC DHA concentration in the last trimester. In addition to the decline in EPA (20:5n-3) observed in both groups, the placebo group also showed a decrease in other LCPUFA (DHGLA, 20:3n-6; adrenic acid, 22:4n-6; DPA, 22:5n-3). As a result, the maternal concentrations of RBC DHA and total n-3 fatty acids were higher, and the n-6/n-3 fatty acid ratio lower, at birth in the fish oil compared to placebo group.

The concentration of DHA in umbilical cord RBC was similar in the supplemented and placebo groups. In both groups, cord RBC had a higher concentration of DHA than did maternal RBC at birth. The materno-fetal difference was, however, less in the fish oil group. In addition, cord RBC DHA concentrations were not significantly different from maternal 15 or 28 weeks concentrations in the fish oil group. DHA concentrations in cord RBC of the placebo group were significantly higher than in maternal RBC at all time points. Thus, when maternal status is enhanced, the elevation of cord DHA above maternal DHA is less pronounced.

Both groups also had higher concentrations of RBC DHGLA (20:3n-6), AA (20:4n-6), and adrenic acid (22:4n-6), and lower LA (18:2n-6), in cord compared to maternal RBC at birth.

**10.3.4 Plasma Fatty Acids: % Total Fatty Acids (Relative Levels)**

With neither group varying in their respective % DHA between 15 and 28 weeks, no effect of supplementation on plasma % DHA was observed at 28 weeks. The increase in αLA (18:3n-3) observed in the placebo group between 15 and 28 weeks may have compensated for a lower preformed DHA intake.

Both groups declined in % DHA between 28 weeks and birth, and by comparable amounts, thus precluding a difference in % DHA between them. The patterns for most fatty acids between 28 weeks and birth were similar in each group. Although the fish oil group declined in % αLA (18:3n-3) and EPA (20:5n-3), while the placebo group increased in % LA (18:2n-6) and adrenic acid (22:4n-6), EFA and LCPUFA levels at birth did not differ from baseline within each group, or between groups. The elevation in n-6 acids and the stability of n-3 fatty acids in the placebo group may have arisen in response to a lower DHA intake.

DHA contributed a similar % TFA in umbilical cord plasma of the two groups. The % of DHA in umbilical cord plasma was significantly higher than in maternal plasma at both birth and 28 weeks in each group; the materno-fetal difference, was however, less in the fish oil group. Compared to maternal plasma at birth, cord plasma of both groups had higher % of LCPUFA - DHGLA (20:3n-6), AA (20:4n-6) and adrenic

acid (22:4n-6) - but lower αLA (18:3n-3). Moreover, the magnitude of the differences between maternal and cord plasma in these fatty acids were similar in the two groups. The similarities between the groups in the % DHA and in other fatty acids, suggest that the attenuation of the materno-fetal difference in plasma DHA was indeed a physiological response to maternal fish oil supplementation.

### 10.3.5 Plasma Fatty Acids: Concentration (Absolute Levels)

In both groups, there was an increase in maternal concentration of DHA between 15 and 28 weeks, but this was greater in the fish oil group. Similar changes in other fatty acids were observed in both groups, but the increase in EPA (20:5n-3) was restricted to the fish oil group. Consequently, the fish oil group had a more favourable n-3 status at 28 weeks, with significantly higher concentrations of DHA, EPA and total n-3 fatty acids compared to the placebo group. The increase in maternal AA (20:4n-6) concentration noted in the placebo group between 15 and 28 weeks suggests compensation for a lower DHA status.

Both groups declined (non-significantly) in the concentration of plasma DHA between 28 weeks and birth. The placebo group declined to levels similar to those at 15 weeks; the fish oil group declined to a lesser extent, such that maternal plasma at birth tended to a significantly higher concentration than at 15 weeks. Thus, although not significantly different from the maternal concentration at birth in the placebo group, plasma DHA in the fish oil group remained elevated above baseline concentrations. Other maternal fatty acids exhibited similar patterns of change in both groups.

There was no difference in the concentration of DHA in umbilical cord plasma between the two groups. In each group, the DHA concentration of cord plasma was not significantly higher than in maternal plasma at birth. Both groups had lower concentrations of LA (18:2n-6), αLA (18:3n-6), DHGLA (20:3n-6), AA (20:4n-6) and EPA (20:5n-3) in cord compared to maternal plasma at birth.

### 10.3.6 Placental Tissue

The fatty acid composition of placental tissue was similar in the fish oil and placebo groups, with the fish oil group exhibiting a lower n-6/n-3 ratio of fatty acids

measured as relative values. Since circulating levels of DHA were higher in the fish oil supplemented mothers, maternal differences obviously arose prior to uptake by the placenta. Fatty acids can be metabolised via oxidation and/or *de novo* synthesis by placental cells. It is therefore possible that the placenta regulates the fatty acid supply to the fetus. When the circulating maternal DHA status is low, such as in the placebo group, the placenta could putatively modulate the composition of maternal blood to ensure appropriate supply to and accretion by the fetus. Such compensation would explain the difference in maternal status and similarity in placental and fetal status of the treatment groups.

Few studies have included placental tissue analyses. Placental tissue fatty acid status did not reflect the differences observed in either maternal and umbilical cord blood between pregnancies delivered preterm and at term (Reece *et al* 1997). Relatively consistent differences between omnivorous, vegetarian and diabetic mothers have been observed in the fatty acid composition of maternal RBC, cord tissue and placenta (Lakin *et al* 1998). Thus, with so few reports of placental tissue composition, it is not possible to determine whether its fatty acid composition varies with maternal circulating status or remains constant, and whether this putative constancy is physiological or related to its regulation of the supply to the fetus.

### 10.3.7 Umbilical Cord Tissue

Analyses of whole cord tissue (Lakin *et al* 1998) and of the separate vessels (Al *et al* 1990, Hornstra *et al* 1989) have been reported to reflect the differential status of the fetus. As with cord blood and placental tissue, no differences in the current study population were detected in whole cord tissue following supplementation with fish oil. Given that umbilical veins reflect the blood supply from the placenta to the fetus and that the arteries reflect the returning blood, it is not surprising that cord tissue composition was similar between groups.

### 10.3.8 Breast Milk

The relative levels of individual fatty acids observed in the breast milk of study participants were similar to those published previously (Koletzko *et al* 1992). Although previous studies have shown an elevation in breast milk DHA following maternal supplementation with 120-250mg/d DHA (Fidler *et al* 2000b, Helland *et al*

1998, Henderson *et al* 1992, Makrides *et al* 1996a), supplementation did not begin until lactation was established. The current study provided mothers with a similar dose, but supplementation began at 15 weeks and continued until term. No difference in breast milk DHA was observed between groups, although the *range* of DHA as both % TFA and a concentration was greater in the fish oil group.

A proportion of breast milk LCPUFA are derived from maternal diet (Demmelmair *et al* 1989, Hachey *et al* 1987), to which breast milk composition responds rapidly (Emken *et al* 1989, Fidler *et al* 2000b, Helland *et al* 1998). The supplemented mothers consumed their final dose of DHA prior to labour and did not express the milk samples subsequently collected until at least 48 hours after their last capsules. It is therefore possible that this time delay accounts for a lack of an effect of supplementation on breast milk fatty acid composition from being detected. However, supplemented mothers presumably accumulated more DHA in their adipose tissue, which could have been mobilized and incorporated into breast milk. It is therefore possible that supplementation at this dose *prior* to lactation had no effect on breast milk. Alternatively, since breast milk DHA levels are relatively well conserved (Innis 1992, Jensen 1989a & b & 1999, Koletzko *et al* 1992) compensatory mechanisms may have ensured that the placebo group had adequate breast milk DHA.

For whatever reason a difference in breast milk composition between groups was not seen, it is reassuring that the median % DHA was 0.2-0.3% TFA in both groups. This is a "normal" % of DHA in breast milk (Innis 1992, Jensen 1989a & b & 1999, Koletzko *et al* 1992); it is thought to be adequate for postnatal tissue accretion and has been suggested as the level to which infant formulae should be supplemented (Cunnane 1999, Cunnane *et al* 1999, Cunnane *et al* 2000a & b, Farquharson *et al* 1993).

## 10.4 STUDY LIMITATIONS AND FUTURE STUDIES

### 10.4.1 Adipose Tissue

The enhanced maternal status observed in the fish oil group could be accounted for by either/both short- and long-term adaptations. Elevation of DHA in the maternal circulation probably arose, at least in part, as a result of a greater DHA intake. In

addition, supplemented mothers may also have accumulated more DHA in their adipose tissue, sustaining their superior DHA status. In the present study, it was not possible to differentiate between these two mechanisms, or to evaluate their relative contribution.

Analysis of adipose tissue would have helped to determine whether a greater quantity of DHA accumulated in the adipose stores of fish oil supplemented mothers. Collection and analysis of adipose tissue was planned for those participants undergoing a caesarean section at delivery. Several participants (13% in total) intending an elective caesarean section were identified at enrolment; the number undergoing elective or emergency caesareans at delivery (26%) was similar to that originally calculated as sufficient to perform an appropriate statistical analyses (25%). However, adipose tissue was obtained from only 31% of those participants undergoing a caesarean section, amounting to only 8% of the whole study population. Because this rate was both poor in itself and relative to the collection of other samples obtained at birth, specific factors may have been responsible.

All participants were aware of and agreed to adipose tissue sampling both at enrolment and prior to birth; only one mother undergoing an elective caesarean declined the removal of adipose while allowing placenta and cord tissue to be obtained, as adipose sampling was considered invasive at a sensitive time. At some planned procedures, the reluctance of medical staff to obtain adipose, despite ethical approval and general awareness of the study, was a contributing factor. In the case of emergency caesarean sections, the event was often sudden and medical care and attention were understandably a priority. As a result, notification of the delivery did not occur until slightly later, at which time placenta and cord tissue could be obtained but adipose tissue could not. The requirement for a caesarean section thus limited the success of adipose tissue sampling.

Since the analysis of adipose tissue could be used to determine the composition of maternal stores, and therefore help to evaluate whether the observed differences in maternal blood and similarities in breast milk were in part attributable to maternal fatty acid accretion and mobilization, future studies should consider the sampling of adipose tissue. The practical difficulties encountered in the current study may be

eliminated by the use of aspiration to obtain a buttock adipose tissue biopsy. This technique has been established, without discomfort and complications (Berry *et al* 1986, Beynen & Katan 1985) and has been performed on lactating mothers (Pugo-Gunsam *et al* 1999). Such a procedure would enable a whole study population to be sampled, including expectant mothers regardless of delivery mode. In addition, it could be performed at various times and prevent reliance on staff external to the study. Indeed, the need for tissue analysis has been highlighted (Cunnane & Francescutti 1999) and future studies should aim to incorporate such analyses in the overall study design.

### 10.4.2 Analysis of Total Lipids

This study reports the fatty acid composition of total lipids and therefore does not differentiate between the various lipid fractions of triacylglycerols, phospholipids and cholesterol esters. Previous studies also differ in the lipid fraction(s) analysed, which may account for some of the apparent discrepancies reported in the literature. Analysis of total lipids determines overall fatty acid status but does not identify any changes or differences in a class specific manner. It is therefore possible that the analysis of total lipids in this study obscured differences between supplemented and placebo groups, which may have been more evident on analysis of the distinct lipid fractions. Practical considerations, however, necessitated the analysis of total lipids. In RBC, phospholipids are the main lipid species, while triacylglycerols are dominant in breast milk (and adipose tissue). Thus, if only the major class of lipid was analysed in each sample type, the various samples would have differed in the fraction analysed, which would have limited comparison between sample types. Alternatively, the analysis of each lipid fraction in all samples would not have been feasible in the time permitted and would have incurred serious statistical concern regarding multiple testing. On considering the results of total lipid analyses, analyses of the lipid fractions may be of further interest and could be performed on the remaining samples in storage. Total lipid analysis remains, in a study with several sample types and time points, an appropriate index of overall fatty acid status with which to detect gross differences between treatment groups.

## 10.5 CONCLUSION

In all measures of circulating DHA status (relative and absolute levels in both RBC and plasma), the same pattern for DHA was noted: an increase in maternal status between 15 and 28 weeks, followed by a decline between 28 weeks and birth, and a subsequently higher cord than maternal DHA status. Although these changes and differences did not always attain statistical significance either within or between groups, differences were noted in the magnitude of change and in the materno-fetal difference between the groups. The fish oil group exhibited either an enhanced maternal DHA status relative to baseline, a less pronounced decline in maternal status during the last trimester, and/or a less compromised maternal status relative to fetal status.

Fluctuations in maternal DHA and other PUFA during pregnancy have been noted previously and shown to be consistent (Otto *et al* 1997). In particular, the elevation of DHA and LCPUFA in the early and mid trimesters (Al *et al* 1995c, Ashby *et al* 1997, Otto *et al* 2001), and subsequent decrease in the last trimester (Al *et al* 1995a) reported previously accord with the longitudinal maternal changes observed in both supplement and placebo groups of this study. The early increase in maternal DHA has been suggested as indicative of mobilization of maternal stores to facilitate preferential transfer to and accumulation by the fetus (Al *et al* 1995c).

The increase in maternal DHA observed between 15 and 28 weeks suggests that maternal mobilization of DHA inevitably occurs to ensure adequate accretion by the fetus, resulting in an elevation of fetal relative to maternal DHA status. The larger increase noted in the fish oil group may be attributable to both a greater circulating status and a larger store in adipose tissue, from which to mobilize DHA. The subsequent decline from 28 weeks to birth indicates that elevated maternal levels cannot be sustained and that accretion by the fetus probably occurs at the expense of maternal status. Maternal fish oil supplementation, however, reduces the inevitable last trimester decline in maternal DHA status, and attenuates the difference between maternal and fetal status. Maternal DHA supplementation, therefore, may not increase fetal RBC status *per se,* but by increasing the overall maternal RBC status during pregnancy, it makes available to the fetus a greater "pool" of DHA from which it can be supplied.

The fish oil supplemented mothers had concentrations of DHA that were 22% higher in plasma (p=0.02) and 13% higher in RBC (p=0.02) at 28 weeks, and 42% higher in RBC at term (p=0.02) compared to the placebo group. DHA accounted for a similarly higher % TFA in RBC of fish oil supplemented mothers at 28 weeks (p=0.003) and at term (p=0.01).

A differential elevation of circulating DHA and total n-3 fatty acids, with concomitant lowering of the n-6/n-3 ratio, was observed in mothers receiving fish oil compared to placebo supplements. The enrichment of maternal n-3 PUFA status has been noted previously in mothers with a habitually higher amount of fish in their diet throughout pregnancy (Olsen *et al* 1991, Sanjurjo et al 1995), and in mothers supplemented during the last trimester of pregnancy (Connor *et al* 1996, van Houwelingen *et al* 1995).

It is perhaps surprising that no differences were observed between groups in the circulating fatty acids of umbilical cord blood, as other studies have reported variations in cord plasma and RBC consistent with maternal diet (Al *et al* 1995b Connor *et al* 1996, Reddy *et al* 1994, Sanjurjo *et al* 1995, van Houwelingen *et al* 1995). However, habitually high intakes of marine foods in the maternal diet are not consistently associated with elevated n-3 PUFA in cord blood (Hornstra *et al* 1992), suggesting that other factors may be involved when the maternal-to-fetal supply is adequate over prolonged periods.

The relative and absolute amount of DHA in cord RBC and plasma was consistently higher than in the corresponding maternal sample at birth. Moreover, the materno-fetal difference was generally characterised by lower EFA and higher LCPUFA, including AA, in cord than maternal blood samples. These findings are in agreement with previous comparisons of maternal and fetal fatty acids (Al *et al* 1990, Al *et al* 1995c, Berghaus *et al* 2000, Crawford *et al* 1989, Hoving *et al* 1994, Otto *et al* 1997, Reece *et al* 1997, van der Schouw *et al* 1991).

Conservation of the differences between mother and fetus (in both the current and previous studies) suggests a physiological requirement by the fetus to accrue greater

amounts of and to attain a higher status of DHA and LCPUFA, relative to mother. Moreover, the requirement appears specific for preformed LCPUFA as the fetus accumulates less EFA compared to mother. Indeed, the lack of difference in cord blood samples between the supplemented and placebo groups suggests that the differential EFA and LPCUFA status of mother and infant is a natural physiological response. That manipulation of the maternal diet and DHA status did not influence fetal DHA status is further evidence that DHA accretion by the fetus is a pre-requisite. Moreover, there may be a threshold level (relative or absolute amount) which the fetus must attain; this would explain the current and previous (Otto *et al* 1997) observation that the materno-fetal difference is greater when maternal status is lower. Fetal requirements appear to be met at the expense of maternal status, which becomes relatively depleted by term. Maternal DHA supplementation apparently limits the extent to which maternal status is compromised by fetal accretion. This may have implications for the maternal and fetal status in subsequent pregnancies (Al *et al* 1997).

In conclusion, DHA supplementation enhances maternal plasma and RBC status (% TFA and concentration) during pregnancy. Supplementation may not directly increase fetal DHA status. The biomagnification (Crawford *et al* 1976) of DHA from mother to fetus appears to be physiologically pre-determined. The gradient between mother and fetus may however be enhanced on the maternal side by fatty acid supplementation.

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# *Appendices*

*Appendix 1*

# Introductory Leaflet

*This study will be undertaken by the*

Department of Child Health
University of Glasgow

Department of Fetal Medicine
The Queen Mother's Hospital

Department of Vision Sciences
Glasgow Caledonian University

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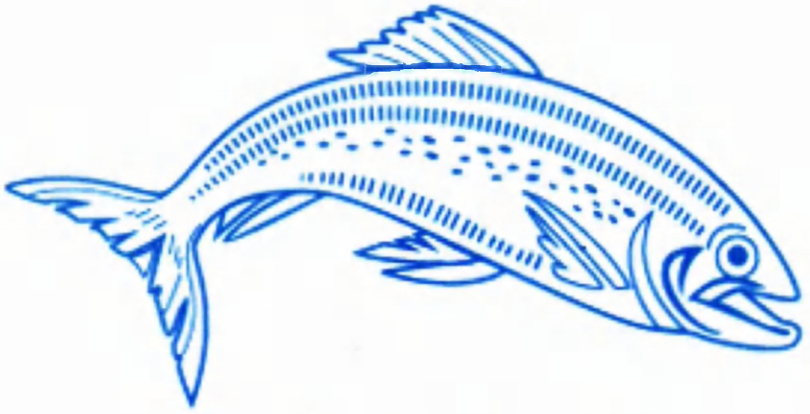

# FOMI

FISH • OILS • MOTHERS • INFANTS

## Invitation to take part in a Study of Mothers and Infants

You may have heard people say that "fish is good for you." This is because fish oils contain fats called essential fatty acids.

Babies need these fatty acids for their brains and eyes to develop normally. Babies who get adequate fatty acids may develop their vision more quickly than those babies who do not.

Babies get these fatty acids from their mother; while they are in the womb, and from milk after they are born.

We would like to find out if giving pregnant women extra fish oils will increase the amount their baby gets, and helps to develop their eyesight.

To do so, we will give expectant mothers capsules with fish oils. We will then test both mum's and baby's blood. We would also like to test how quickly the baby's vision is developing after birth.

The fish oil capsules are safe and the same as you can buy in a chemist shop. The eye tests are often done on babies.

We would like you to think about whether you would be willing to take part in this study.

We will talk about this with you at your next clinic appointment (in about 3-5 weeks time, when you come to see the midwife).

We will not ask you to take part if you are not happy to do so, and if you do take part, the study will not affect in any way the care you and your baby receive.

Please bring this leaflet with you at your next antenatal visit.

If you would like more information before your next appointment, please feel free to contact **Colette Montgomery** in the Department of Child Health, Yorkhill Hospitals, Tel: 0141-201 0785.

*Appendix 2*

# Consent Form

# FOMI

FISH • OILS • MOTHERS • INFANTS

University Department of Child Health • Yorkhill Hospitals Tel: 0141-201 0785

Certain fats (called essential fatty acids) are vital for the normal development of the brains of babies. It has been found that babies who do not receive enough of one of these fatty acids (called docosahexaenoic acid or DHA) may develop vision more slowly than those babies who do. Babies receive fatty acids in the womb from mother via the placenta, and from milk after birth. Mothers obtain fatty acids from their diet, including the oils naturally present in fish (fish oils).

We want to find out whether, if we give pregnant mothers supplements containing the fatty acid DHA, it will increase the amount of DHA in the mother's blood, her fat stores which supply the baby, breast milk and the baby's blood. We plan to give pregnant mothers capsules which do or do not contain DHA.

We will then measure the amount of DHA present in the mother's blood during pregnancy and after birth. We will also test the placenta, umbilical cord and breast milk, and the baby's blood after birth. If the mother has a caesarean section, we can obtain a piece of fat tissue at the time of operation and measure its DHA. If the mother does not breast feed, we would like to obtain a sample of the formula milk used to feed her baby.

To test whether DHA improves babies' eye sight, we would like to test the baby's vision and how his/her eyes adapt to light and dark. Vision is tested by using flashing lights while measuring from small discs attached to the head with a soft paste. At the postnatal visits, we will also do a test with the baby looking at patterns on a computer screen. Adapting to the dark is done with black eye patches over the eyes for about half an hour and then measurements are made from a contact lens. (The baby can be held, fed and changed as normal with the patches on).

The capsules are safe, and the same as those available in a chemist or health food shop. The vision tests are often done on infants and are safe. Eye drops to make the pupils larger and to prevent any discomfort will be given only for the dark adaptation test. The blood samples will be collected from the mother as part of routine tests at ante-natal clinics and at the birth. We would like to take a small sample of blood from the baby at birth, at the same time that routine blood tests are done. At the postnatal visits, we would like to take a blood sample from the mother. The vision tests will be done before the baby goes home from hospital and again at postnatal visits when the baby is 8 weeks old and 6 months old.

We very much hope that you will be able to take part in this study. In no way will this research affect the care you or your baby receive, and you will be free to withdraw from the study at any time. This research may not be of any direct benefit to you and your baby, but we will be checking your baby's health and vision and will be sure to take care of any problems.

If you have any questions or concerns, please feel free to contact Colette Montgomery, in the University Department of Child Health, Yorkhill Hospitals - Tel: 0141 201 0785.

Colette Montgomery
Department of Child Health

Professor LT Weaver
Department of Child Health

Dr. Alan Cameron
Department of Obstetrics & Gynaecology

Dr. Daphne McCulloch
Department of Vision Sciences

# Maternal DHA Supplementation, Fetal and Infant DHA Accretion, and Infant Visual Development - The FOMI Study

*Mother:* I am happy to take part in this study, and to provide

a) from myself:

- size measurements at 15 weeks, mid-pregnancy, birth, 2 months and 6 months after birth
- a blood sample at 15 weeks, mid-pregnancy, birth, 2 months and 6 months after birth
- a sample of subcutaneous fat if I have a caesarean section
- a sample of placental tissue, umbilical cord tissue and umbilical cord blood at birth
- a sample of breast milk and/or formula milk at birth, 2 months and 6 months after birth

b) from my baby:

- size measurements at birth, 2 months and 6 months after birth
- vision tests at birth, 2 months and 6 months after birth

Name.....

Signed.....Date.....

*Investigator:* I have explained the nature of the study to the above patient.

Name.....

Signed.....Date.....

*Witness:* I am satisfied that the patient has given informed and voluntary consent.

Name.....

Signed.....Date.....

*Appendix 3*

# Advice Leaflet

FOMI

FISH • OILS • MOTHERS • INFANTS

Department of Child Health • Yorkhill Hospitals Tel: 0141-201 0785

## Thank you for taking part in the FOMI trial

**Please take two capsules per day**. Take the capsules with liquid, at whatever time of day it suits you best. Please keep the capsules out of the reach of children.

You have been given enough capsules to last until your 28 week antenatal clinic appointment, plus extra capsules in case you drop or misplace any. **Do not take any more than two capsules per day**, just because there are more in the bottle - these extra capsules are just to ensure you have enough. If you miss one day, do not take extra capsules the next day - just take two capsules as normal.

Please bring the bottle, with remaining capsules, to your 28 week antenatal clinic appointment. You will be given a second bottle of capsules at this visit.

You have also been given an empty bottle, which is for your ease and convenience. It can be used to carry or store capsules if for example, you are not at home, you wish to take your capsules while you are at work, or you are on a journey.

We look forward to seeing you at your next clinic appointment.

If you have any queries or concerns, please feel free to contact

*Colette Montgomery in the*
*University Department of Child Health, Tel: 0141 201 0785.*

FOMI
FISH • OILS • MOTHERS • INFANTS
University Department of Child Health • Yorkhill Hospitals Tel: 0141-201 0785

This patient has agreed to take part in a clinical trial on the effect of fish oil supplementation on mothers and infants.

Samples will be collected as follows:

**At antenatal clinics**
Venous blood sample at 15 weeks and 28 weeks (5 ml)
*Sample tubes will be provided. A study co-ordinator will be present at the clinics to supervise and collect samples.*

**At delivery**
Venous blood sample (maternal, 5 ml)
Maternal subcutaneous fat if the mother has a caesarean section
Placental tissue
Umbilical cord tissue
Umbilical cord blood (5 ml)
*Please contact Colette Montgomery on BT page 07669104916* at delivery, (24 hours, including weekends). * - requires an outside line for dialing.*

**Before discharge**
Breast milk and/or formula milk (5 ml)
Infant vision tests
*A study co-ordinator will visit the ward after delivery to complete samples and tests.*

If you have any queries or concerns or require further information, please contact Colette Montgomery in the Department of Child Health on extn. 0785, or BT page 07669104916*. * - requires an outside line for dialing.

*Appendix 5*

# Questionnaire

# FOMI Study - Questionnaire

How often do you eat fish?.....

Have you eaten fish in the last 24-36 hours?.....

When was the last time you ate fish?.....

Please read through the list of fish, and the different ways they can be prepared. Tick the column marked (✓) if you eat that food, and then mark the appropriate column for how often you eat it.

| | ✓ | Less than once per month | Once per month | Twice per month | Number of times per week |
|---|---|---|---|---|---|
| **Anchovies** | | | | | |
| **Caviare**, bottled in brine, drained | | | | | |
| **Cod** | | | | | |
| - baked | | | | | |
| - coated in batter, frozen, baked | | | | | |
| - coated in crumbs, frozen, fried | | | | | |
| - in parsley sauce, frozen, boiled | | | | | |
| - poached | | | | | |
| - smoked, poached | | | | | |
| - steamed | | | | | |
| **Crab**, boiled | | | | | |
| **Crabsticks** | | | | | |
| **Fish cakes** | | | | | |
| - homemade | | | | | |
| - fried | | | | | |
| - grilled | | | | | |
| **Fish fingers** | | | | | |
| - fried | | | | | |
| - grilled | | | | | |
| **Fish paste** | | | | | |
| **Haddock** | | | | | |
| - coated in crumbs, frozen, fried | | | | | |
| - grilled | | | | | |
| - in batter, fried | | | | | |
| - poached | | | | | |
| - smoked, poached | | | | | |
| - smoked, steamed | | | | | |
| - steamed | | | | | |
| **Herring** | | | | | |
| - canned in tomato sauce | | | | | |
| - grilled | | | | | |
| - in oatmeal, fried | | | | | |
| - pickled | | | | | |
| **Kipper**, grilled | | | | | |

| | ✓ | Less than once per month | Once per month | Twice per month | Number of times per week |
|---|---|---|---|---|---|
| **Lemon sole** | | | | | |
| - grilled | | | | | |
| - steamed | | | | | |
| **Lemon sole goujons** | | | | | |
| - baked | | | | | |
| - fried | | | | | |
| **Lobster**, boiled | | | | | |
| **Mackerel** | | | | | |
| - pâté | | | | | |
| - canned | | | | | |
| - fried | | | | | |
| - grilled | | | | | |
| - smoked | | | | | |
| **Mussels** | | | | | |
| - boiled | | | | | |
| - canned and bottled, drained | | | | | |
| **Pilchards**, canned in tomato sauce | | | | | |
| **Plaice** | | | | | |
| - frozen, grilled | | | | | |
| - frozen, steamed | | | | | |
| - grilled | | | | | |
| - in batter, fried | | | | | |
| - in crumbs, fried | | | | | |
| **Prawns**, boiled | | | | | |
| **Salmon** | | | | | |
| - grilled | | | | | |
| - pink, canned in brine, flesh only, drained | | | | | |
| - red, canned in brine, flesh only, drained | | | | | |
| - smoked | | | | | |
| **Sardines** | | | | | |
| - canned in brine, drained | | | | | |
| - canned in oil, drained | | | | | |
| - canned in tomato sauce | | | | | |
| - grilled | | | | | |
| **Scampi**, in breadcrumbs, frozen, fried | | | | | |
| **Shrimps** | | | | | |
| - boiled | | | | | |
| - canned in brine, drained | | | | | |
| **Taramasalata** | | | | | |
| **Trout**, rainbow, grilled | | | | | |
| **Tuna** | | | | | |
| - pâté | | | | | |
| - canned in brine, drained | | | | | |
| - canned in oil, drained | | | | | |
| **Whiting** | | | | | |
| - in crumbs, fried | | | | | |
| - steamed | | | | | |

Are there any other fish or fish dishes you eat? Please specify .....

.....

.....

.....

.....

.....

.....

Please look at the photographs of food portion sizes. State the photograph number which corresponds to the portion size you would eat.

| | Photo No |
|---|---|
| Fish fillet | 42 |
| Fish in batter | 43 |
| Tuna | 44 |

Please look at the photographs of tinned fish. Specify the letter corresponding to the tin size you would use, and the amount you would consume in one portion (e.g. ¼, ½ or full tin).

| | Photo No (G14) | Amount |
|---|---|---|
| Pilchards | H / G | |
| Tuna | M / N | |
| Sardines | O | |
| Salmon | P / Q | |

Do you use butter or margarine?.....

If you use margarine, please specify the exact brand.....

.....

Please look at the photograph of margarine spread on bread. Which photograph corresponds to the amount of margarine you would have? 30.....

How many times per day would you eat this amount of margarine?.....

Which type of cooking oil or spread do you use? Please specify the exact brand.....

.....

How often do you use cooking oil or spread?.....

How much oil do you use in cooking (teaspoon or tablespoon)? Please look at the photograph of spoons (G20) to help you.....

Do you use any dietary supplements, e.g. vitamins (except folic acid), evening primrose oil, etc?.....

.....

Do you drink alcohol? If yes, please specify the number of units per week (1 unit = 1 glass of wine, 1 measure of spirit, ½ pint of beer, etc.).....

Do you smoke? If yes, please specify the number of cigarettes per day.....

Do you exercise? If yes, please specify the number of hours per week?.....

What is your occupation?.....

*Thank you for participating in the FOMI study and for completing this questionnaire.*