UNIVERSITY OF SOUTHAMPTON

INSTITUTE OF HUMAN NUTRITION, DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

SCHOOL OF MEDICINE

Salmon In Pregnancy Study (SIPS): The effects of increased oily fish intake during pregnancy on maternal and cord blood fatty acid composition, cord blood immunity and atopy outcomes in infants at 6 months of age

by

Maria Vlachava

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

INSTITUTE OF HUMAN NUTRITION, DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE DIVISION, SCHOOL OF MEDICINE

Doctor of Philosophy

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Parallel increases in many inflammatory diseases including atopy over the last 40 years suggest that common environmental changes may be promoting inflammatory immune responses. Modern diets have become increasingly rich in n-6 polyunsaturated fatty acids (PUFAs) and relatively deficient in n-3 PUFAs. These dietary changes are believed to promote a pro-sensitisation, pro-allergic and pro-inflammatory environment. Exposure to such an environment during pregnancy and in the very early life period is considered to influence subsequent patterns of the immature and developing neonatal immune system, and this may contribute to the increase in allergic disease in early life. As allergic diseases often first manifest in infancy, prevention strategies need to be targeted early, even *in utero*. Epidemiologic and experimental data provide a plausible link between dietary changes and increased incidence of childhood atopic disease. Although there have been studies examining the potential benefits of giving n-3 PUFA-rich fish oil supplements during pregnancy, there are no studies examining the effects of increased consumption of oily fish in pregnancy on neonatal immune responses and subsequent clinical outcomes.

The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial of oily fish intervention during pregnancy. The hypotheses being investigated in SIPS is that increased intake of salmon, a source of long chain (LC) n-3 PUFAs (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), in pregnancy will a) increase maternal LC n-3 PUFA intake, b) increase maternal and infant blood LC n-3 PUFA status, c) modulate fetal/neonatal immune responses and d) lower the risk of infant atopy determined at 6 months of age. The primary outcome measures of SIPS were the clinical signs of atopy in the offspring.

Pregnant women (n=123) at high risk of having atopic offspring, and with low habitual intake of oily fish ($\leq$ 2/month) were randomised at 20 weeks of pregnancy to either consuming 2 portions/week of farmed salmon (n=62) or continuing their habitual diet (n=61) until the end of pregnancy. The woman attended a clinic at 20 (n=123), 34 (n=110) and 38 (n=91) weeks of gestation at which fasting blood was collected and a food frequency questionnaire (FFQ) was administered (at 20 and 34 weeks). At delivery umbilical cord blood was collected (n=101) for fatty acid and immunological analysis. Infants attended a clinic at 6 months of age (n=86) for assessment of allergic sensitisation by skin prick testing (SPT) using various allergen extracts and of atopic dermatitis (SCORAD index).

Maternal and cord plasma and cord blood mononuclear cell (CBMC) fatty acid compositions were determined by gas chromatography. Neonatal (cord) immune cell subsets were identified by flow cytometry. Ex-vivo cytokine production by CBMC in response to stimulants (allergen, mitogen, and toll-like receptor (TLR) ligands) was determined by cytometric bead array and flow cytometry. Ex-vivo prostaglandin E_2 production by CBMC was determined by enzyme-linked immunosorbent assay. Immunoglobin E concentration was measured in cord blood plasma and in 6 month infant blood plasma.

Eating oily fish twice a week during pregnancy resulted in a higher maternal intake of LC n-3 PUFAs (both EPA and DHA) and in higher maternal and cord blood plasma status of LC n-3 PUFAs (both EPA and DHA). LC n-3 PUFA content of CBMC was not significantly affected. CBMC production of interleukins-2, -4, -5, and -10 and tumour necrosis factor-α was lower in the salmon group. There was no effect of salmon on the atopic outcomes assessed at 6 months.

Declaration of Authorship

I, Maria Vlachava, declare that the thesis entitled:

Salmon in Pregnancy Study (SIPS): The effects of increased oily fish intake during pregnancy on maternal and cord blood fatty acid composition, cord blood immunity and atopy outcomes in infants at 6 months of age

& the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University;
- No part of this thesis has been previously been submitted for a degree or any other qualification at this University or any other institution;
- Where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help
- Where the thesis is based on work done by myselfor jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as:

Journal Papers:

Calder PC, Kremmyda LS, Vlachava M, Noakes PS, Miles EA. Is there a role for fatty acids in early life programming of the immune system? Proceedings of the Nutrition Society 2010, 69, 373-380.

Kremmyda LS, Vlachava M, Noakes PS, Diaper ND, Miles EA, Calder PC. Atopy risk in infants and children in relation to early exposure to fish, oily fish, or long-chain omega-3 fatty acids: A systematic review. Clinical Reviews in Allergy and Immunology 2009 in press (published online as Dec 9).

Miles EA, Noakes PS, Kremmyda L-S, Vlachava M, Diaper ND, Rosenlund G, Urwin H, Yaqoob P, Rossary A, Farges M-C, Vasson M-P, Liaset B, Frøyland L, Helmersson J, Basu S, Garcia E, Olza J, Mesa MD, Aguilera CM, Gil A, Calder PC. The salmon in pregnancy study - study design, subject characteristics, maternal fish and marine n-3 fatty acid intake, and marine n-3 fatty acid status in maternal and umbilical cord blood. American Journal of Clinical Nutrition 2011, in press.

Abstracts:

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Signed:

Date:

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Abbreviations

AHR	Airway hyper-responsiveness
ANOVA	Analysis of variance
APC	Antigen presenting cell
ALA	Alpha-linolenic acid
ARA	Arachidonic acid
BC	Body composition
CAPS	Childhood Asthma Prevention Study
CB	Cord blood
CBA	cytometric bead array
CBMC	Cord blood mononuclear cell
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CI	Confidence interval
COMA	Committee on medical aspects of food policy
ConA	Concanavalin A
COX	Cyclooxygenase
DAG	Diacylglycerol
DC	Dendritic cells
DDT	Dichloro diphenyl trichloroethane
Der p 1	Dermatophagoides pteronyssinus
DGLA	Digomo γ-linolenic acid
DHA	Docosahexaenoic acid
DHT	Delayed type hypersensitivity
DI	Dioxin like
DMSO	Dimethyl sulphoxide
DPA	Docosapentaenoic acid
DRI	Dietary reference intake
EDD	Expected date of delivery
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
FA	Fatty acids
FACS	Flow cytometer
FAME	Fatty acid methyl ester
FFFD	Fish fingers and fish dishes
FFQ	Food frequency questionnaire
FID	Flame ionization detector
FITC	Fluorescein isothiocyanate isomer 1
FO	Fish oil
FSC	Forward scatter
GC	Gas chromatography
HDL	High density lipoprotein
HDM	House dust mite
HEP	Histamine equivalent in skin prick testing
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HI-FBS	Heat-inactivated fetal bovine serum
HLA	Human leukocyte antigen
HPEPE	Hydroperoxyeicosapentaenoic acid

HPETE	Hydroperoxyeicosatetraenoic acid
HRP	Horseradish peroxidase
ICAM	Itracellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IHN	Institute of Human Nutrition
IL	Interleukin
ISAAC	International Study of Asthma and Allergies in Childhood
LA	Linoleic acid
LC	Long chain
LDL	Low density lipoprotein
LH	Lithium heparin
LOX	Lipoxygenase
LPL	Lipoprotein lipase
LPS	lipopolysaccharide
LRNI	lowere reference nutrient intake
LT	Leukotriene
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mo	month(s)
mRNA	Messenger ribonucleic acid
MTHF	Methyl-tetra-hydrofolic acid
MUFA	Monounsaturated fatty acids
n-3	Omega 3
n-6	Omega 6
NDNS	National Diet and Nutrition Survey
NEFA	Non-esterified fatty acids
NK	Natural killer
NPU	Nitrogen producing units
NSAID	Non-steroidal and anti-inflammatory drugs
NSB	Non-specific binding
OR	Odds ratio
Ova	Ovalbumin
p	p-value
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Phospholipids/Phosphatidylcholine
PCA	Principal component analysis
PCB	Polychlorinated biphenyls
PE	Phycoerythrin
PG	Prostaglandins
PGN	Peptidoglycan
PHA	Phytohaemaglutinin
Poly I:C	Polyinosine-polycytidylic acid
PTWI	Provisional tolerable weekly intake
PUFA	Polyunsaturated fatty acid
RCT	Randomised controlled trial
RNI	Reference nutrient intake
SACN	Scientific advisory committee on nutrition
Sal s1	Salmon paralbumin
SD	Standard deviation
Se	Selenium
SFA	Saturated fatty acid

SIPS	Salmon in Pregnancy study
SPE	Solid phase extraction
SPSS	Statistical package for social sciences
SPT	Skin Prick testing
SSC	Side scatter
SUHT	Southampton
SWS	Southampton Women's Survey
TAG	Triglyceride
TCR	T-cell receptor
TDI	Tolerable daily intake
TGF	Transforming growth factor
Th	Helper T-cell
Th0/1/2/17	Helper T-cell type 0/1/2/17
TLR	Toll-like receptor(s)
TNF	Tumour necrosis factor
Tr	Traces
T-reg	T-regulatory
TX	Thromboxane
VCAM	Vascular cell adhesion molecule
VLDL	Very low density lipoprotein
vs.	Versus
WHO TEQ	World Health Organisation toxic equivalent
χ^2	Chi-square

1 General Introduction

1.1 Fatty acids

1.1.1 Terminology of fats and lipids

Like other organic compounds, all lipids are composed of a carbon skeleton with hydrogen and oxygen substitutions. Nitrogen, sulphur and phosphorus are also present in some lipids. Water insolubility is a characteristic distinguishing most lipids from proteins and carbohydrates. There are some exceptions to this general rule, since short- to medium-chain fatty acids, soaps, and some complex lipids are soluble in water. There are four general categories of lipids: simple, complex, derived and miscellaneous (1). Simple lipids are esters of fatty acids with various alcohols. They include triglycerides, waxes, cholesteryl esters and vitamin A and D esters. Complex lipids are esters of fatty acids in combination with both alcohols and other groups. They include phospholipids, glycolipids, lipoproteins and lipopolysaccharides. Derived lipids are hydrolysis products of simple or compound lipids, including fatty acids, monoglycerides and diglycerides, straight-chain and ring-containing alcohols, sterols and steroids. Miscellaneous lipids include some wax lipids, carotenoids, vitamins E and K (1).

Table 1.1: Classification of lipids (1)

LIPID CATEGORY	EXAMPLES
Simple (Fatty acids esterified with alcohols)	• Fats (fatty acids esterified with glycerol) • Waxes (true waxes, sterol esters, vitamin A and D esters)
Complex (Fatty acids esterified with alcohols plus other groups)	• Phospholipids (contain phosphoric acid and usually a nitrogenous base) • Glycolipids (lipids containing a carbohydrate and nitrogen but no phosphate and no glycerol) • Sulpholipids (lipids containing a sulphur group) • Lipopoteins (lipids attached to plasma or other proteins) • Lipopolysaccharides (lipids attached to polysaccharides)
Derived (obtained by hydrolysis of simple or complex lipids)	• Fatty acids (saturated, monounsaturated or polyunsaturated) • Monoglycerides and diglycerides • Alcohols (include sterols, steroids, vitamin A and D)
Miscellaneous	• Straight chain hydrocarbons • Carotenoids • Squalene • Vitamins E and K

1.1.2 Structure and types of fatty acids

Saturated and unsaturated fatty acids

The main components of dietary lipids are fatty acids which may vary in length from one to greater than thirty carbons. They are carboxylic acids with the structure RCOOH, where R is hydrogen in formic acid, CH_3 in acetic acid, or else a chain of one to over thirty CH_2 groups terminated by a CH_3 group.

Fatty acids having their full complement of hydrogen atoms are termed saturated. These usually exist as straight chains. The most abundant saturated fatty acid (SFA) in the context of the human diet is palmitic acid, a 16-carbon fatty acid.

If two hydrogen atoms are removed from the chain, a carbon-to-carbon double bond or point of unsaturation is created and the molecule bends. This bending causes the molecule to occupy more space and become more fluid. The presence of one or more double bonds in a fatty acid defines it as unsaturated. Fatty acids with one double bond are called monounsaturated. The most common monounsaturated fatty acid (MUFA) is oleic acid, an 18-carbon fatty acid with its double bond nine carbons from the methyl end of the hydrocarbon chain (Table 1.2). Oleic acid is the major fatty acid in olive oil. Polyunsaturated fatty acids (PUFAs) have two or more double bonds and include both omega-6 and omega-3 families. For example, linoleic acid (LA) is an 18-carbon omega-6 fatty acid, with two double bonds. It is abundant in plants and seed oils. Its counterpart in the omega-3 family is alpha-linolenic acid (ALA), an 18-carbon fatty acid with three double bonds, also found in plant lipids and oils.

1.1.3 Physicochemical properties of fatty acids

In general, SFAs have a higher melting point than unsaturated fatty acids, and unsaturated fatty acids with a greater degree of unsaturation have a lower melting point than those with fewer double bonds. Also, fatty acids with a longer hydrocarbon chain usually have a higher melting point. Therefore, PUFAs are usually more easily liquefied than SFAs or MUFAs. Unsaturation results in structural variety in fatty acids and allows for isomerisation. A SFA occupies, in general, less space than an unsaturated fatty acid of the same length. This is because the insertion of double bonds increases the curvature of the unsaturated fatty acid causing it to occupy a greater area. When the two hydrogens at the double bond are on the same side of the molecule the bond is termed *cis*-unsaturated and when the hydrogens are on the opposite side of the double bond the bond is termed *trans*-

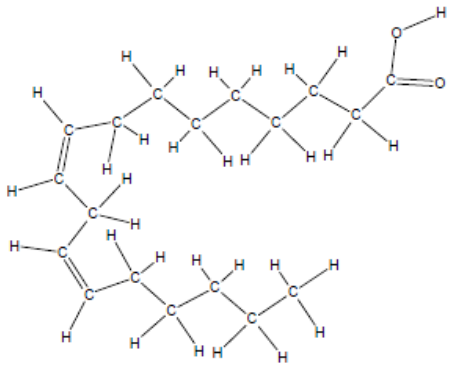
unsaturated. This leads to different orientation of the adjoining carbons across the double bond. A *cis*-fatty acid molecule has a curved structure whereas a *trans*-fatty acid does not. Thereby the physicochemical properties of a *trans*-fatty acid are closer to those of a SFA. Lastly, most naturally occurring PUFAs have a methylene (CH_2) group between the two double bonds. This methylene interruption of the double bonds can be lost when bonds move one carbon closer as a result of food processing. These isomers are termed conjugated.

1.1.4 Nomenclature of fatty acids

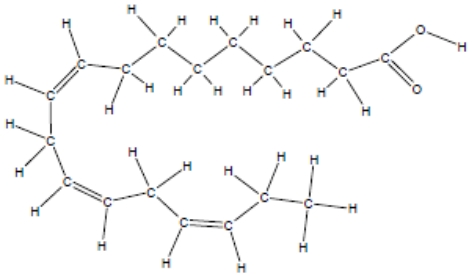
Individual fatty acids have both common and systematic names, as well as a shorthand notation that reflects the number of carbons, the number of double bonds and the position of the first double bond from the methyl-terminus. Because of the methylene interruption of the double bonds in PUFAs, the position of the rest of the double bonds is assumed and not referred to in the shorthand notation. The n- notation is also referred to as ω or omega. For example, the shorthand notation 18:1n-9 refers to the common MUFA oleic acid which has 18 carbons and one double bond at the ninth carbon from the methyl-terminus. Table 1.2 presents the nomenclature and sources of common fatty acids and Figure 1.1 shows the chemical structures of selected PUFAs.

Table 1.2: Fatty acid nomenclature and sources (2)

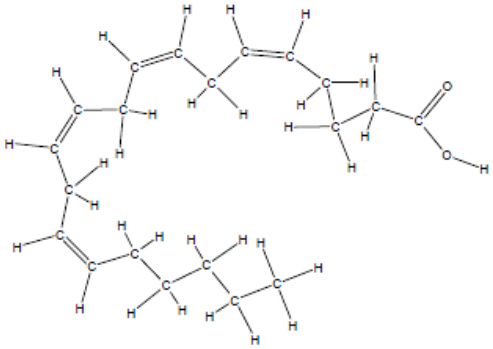
SYSTEMATIC NAME	COMMON NAME	SHORTHAND NOTATION	SOURCES
Saturated fatty acids			
. Dodecanoic	. Lauric	. 12:0	. Coconut oil, milk
. Tetradecanoic	. Myristic	. 14:0	. Coconut oil, milk
. Hexadecanoic	. Palmitic	. 16:0	. Milk, eggs, animal fats, meat, fish, some vegetable oils (e.g. palm oil)
. Octadecanoic	. Stearic	. 18:0	. Milk, eggs, animal fats, meat, cocoa butter
Monounsaturated fatty acids			
. 9-Hexadecanoic	. Palmitoleic	. 16:1n-7	. Animal fats, vegetable oils, fish, fish oils
. 9-Octadecanoic	. Oleic	. 18:1n-9	. Animal fats, olive oil
Polyunsaturated fatty acids			
. 9,12-Octadecadienoic	. Linoleic	. 18:2n-6	. Vegetable oils, seeds, nuts, green leaves
. 9,12,15-Octadecatrienoic	. α-Linolenic	. 18:3n-3	. Vegetable oils, seeds, nuts, green leaves
. 6,9,12-Octadecatrienoic	. γ-Linolenic	. 18:3n-6	. Synthesised from linoleic acid, evening primrose oil
. 5,8,11-Eicosatrienoic	. Mead	. 20:3n-9	. Synthesised from oleic acid
. 8,11,14-Eicosatrienoic	. Dihomo-γ-linolenic	. 20:3n-6	. Synthesised from linoleic acid
. 5,8,11,14-Eicosatetraenoic	. Arachidonic	. 20:4n-6	. Synthesised from linoleic acid, eggs, meat, marine algae
. 5,8,11,14,17-Eicosapentaenoic	. Eicosapentaenoic	. 20:5n-3	. Fish, fish oils, marine algae
. 4,7,10,13,16,19-Docosapentaenoic	. Docosapentaenoic	. 22:5n-3	. Fish, fish oils, marine algae
. 4,7,10,13,16,19-Docosahexaenoic	. Docosahexaenoic	. 22:6n-3	. Fish, fish oils, marine algae



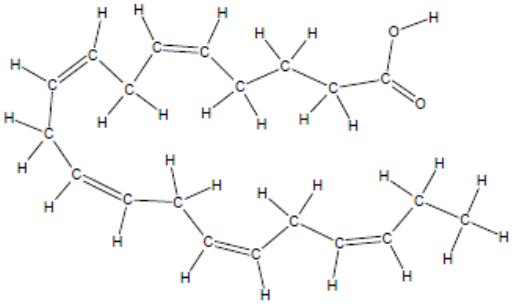
LA, linoleic acid, 18:2ω6 (18:2n-6)
(contains 18 carbon atoms and 2 double bonds
or unsaturation sites)



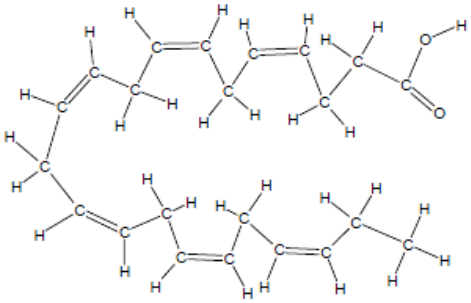
ALA, alpha-linolenic acid, 18:3ω3 (18:3n-3)
(contains 18 carbon atoms and 3 double bonds
or unsaturation sites)



AA, arachidonic acid, 20:4ω6 (20:4n-6)
(contains 20 carbon atoms and 4 double bonds
or unsaturation sites)



EPA, eicosapentaenoic acid, 20:5ω3 (20:5n-3)
(contains 20 carbon atoms and 5 double bonds
or unsaturation sites)



DHA, docosahexaenoic acid, 22:6ω3 (22:6n-3)
(contains 22 carbon atoms and 6 double bonds
or unsaturation sites)

Figure 1.1: Chemical structures of linoleic acid (LA), alpha-linolenic acid (ALA), arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (3)

1.1.5 Long-chain PUFAs - Essential fatty acids - Sources

By definition, long-chain PUFAs (LC PUFAs) are those with 20 or more carbon atoms; the precursor fatty acids, LA and ALA are not included when using this term. The major LC PUFAs of the n-6 family are arachidonic acid (ARA; 20:4n-6) and docosapentaenoic acid (22:5n-6). The latter accumulates in brain and retinal membranes when n-3 docosahexaenoic acid (DHA; 22:6n-3) is inadequate, as happens in ALA deficiency. Both LC n-6 PUFAs are synthesized from linoleic acid.

In the n-3 family, the major LC PUFAs are eicosapentaenoic acid (EPA; 20:5n-3) and DHA. Both are synthesized from ALA. EPA and DHA can be interconverted in the body via another fatty acid, docosapentaenoic acid (DPA; 22:5n-3).

By definition, essential fatty acids (EFAs) cannot be synthesized in the body, and because they are required for optimal health, must be obtained from the diet. In classical nutrition, a nutrient is considered essential if its removal from the diet leads to symptoms of deficiency, which often include impaired growth, development and immune function. LA is considered essential because its absence from the diet is associated with the loss of dermal integrity (4), as it is an essential component of complex lipids, sphingolipids, in skin (5;6). LA deficiency is associated with lower growth and reduced tissue accumulation of ARA. LA is also essential as the precursor of ARA (Figure 1.2), which is important in brain development and function and as a precursor for the production of eicosanoids, which are potent regulatory substances. LA is widespread in plants and seed oils, whereas ARA is found mainly in meats, egg yolk, and some fish.

Scientific consensus on the question of the essentiality of n-3 fatty acids was achieved recently. Initially, because ALA did not fully ameliorate the symptoms of EFA deficiency in laboratory animals, it was not considered an EFA. Studies by Bjerve *et al.* in Europe and Holman *et al.* in the U.S. provided evidence for the essentiality of n-3 fatty acids in humans, although deficiency experiments in people cannot be undertaken (7;8). ALA is considered essential as it is the precursor of DHA; a vital fatty acid in the structural lipids of brain and other nervous tissues (Figure 1.2). Cunnane (9) and Carlson (10) have suggested that DHA be considered as a "conditionally essential nutrient" in human nutrition because its production from ALA may be inadequate to meet needs for brain growth in early human development and it confers some functional benefit even when ALA is present in the diet. For this reason, provision of preformed DHA is considered

optimal, especially early in life. Both DHA and ARA are present in breast milk and in many infant formulas in developed countries. ALA is found in plants, particularly flax seed and oil, canola and soybean oils, and English walnuts. It is also abundant in perilla seeds and oils and in purslane, a vegetable used in the Middle East. DHA, while present in all fish, is found in abundance in fatty fish such as salmon, tuna, trout, sardines, and mackerel (1). The dietary sources of n-3 and n-6 PUFAs are summarised in section 1.1.6.

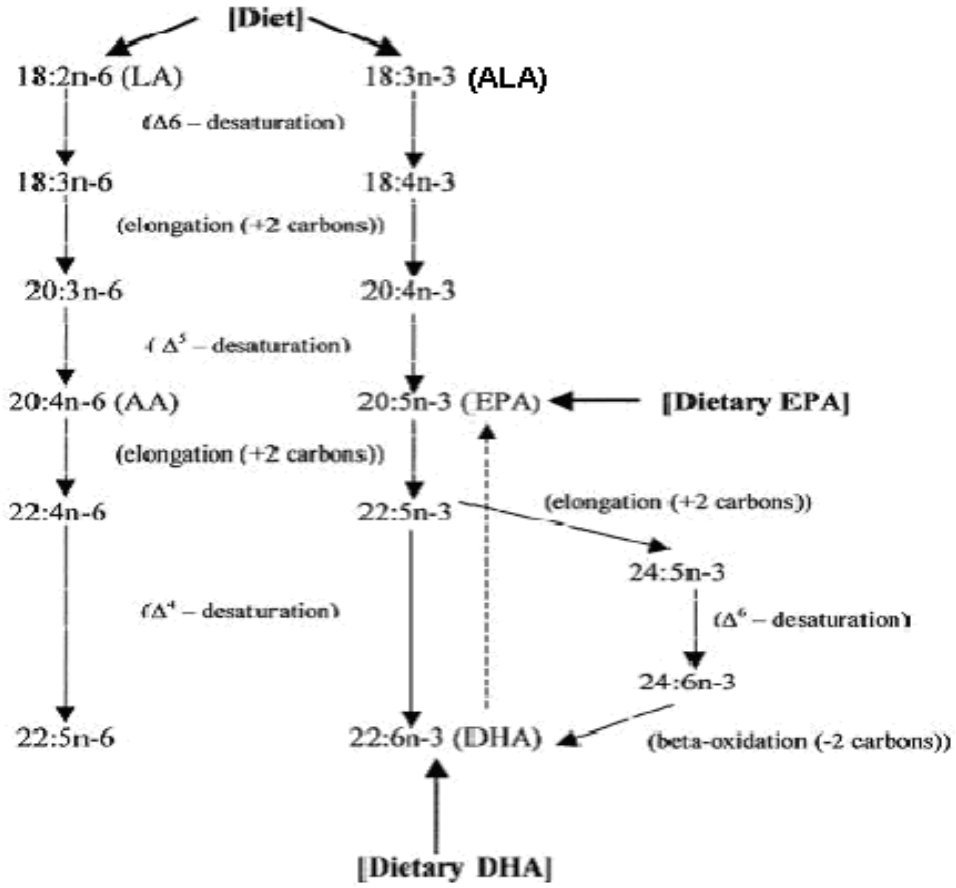


Figure 1.2: Desaturation, elongation, and retroconversion of n-6 and n-3 polyunsaturated fatty acids (11)

Figure 1.2 shows the metabolic steps (desaturation plus elongation reactions) by which LA is converted to ARA and by which ALA is converted to EPA and DHA. Further details on fatty acid metabolism are given in later sections. It is noted in Figure 1.2 that elevations of EPA and DHA in human cells and tissues can be readily provided for by the direct dietary consumption of EPA and DHA (11). Unlike LA, which is present at considerable levels in most cellular lipids (particularly membrane phospholipids) throughout various tissues and cells, ALA does not usually accumulate to particularly high concentrations in cellular/tissue lipids/phospholipids even when ingested at relatively high dietary levels. This is partly due to the fact that much of the ALA which is consumed in the diet undergoes beta oxidation in the mitochondria and only a limited amount is available for the

very limited conversion to EPA plus DHA (11). Figure 1.2 also indicates that dietary DHA has the potential to undergo some reverse metabolism (retroconversion) back to EPA.

1.1.6 Dietary Sources of n-3 and n-6 PUFAs

Table 1.3 and Table 1.4 show the contents of LA and ALA in nuts, seeds and oils and of various fatty acids in fish and fish products, respectively. LA is found in abundance vegetable oils (corn, safflower, sunflower, soybean), some which contain significant amounts of ALA (Table 1.3). The LC n-3 PUFAs EPA and DHA are found in oily fish and fish oils (12;13). White fish, like cod, have flesh that is very low in fat as these fish accumulate fat in their liver. Oily fish have fat in their flesh and its amount is related to their breeding cycle. After breeding the fat content falls significantly. Oily fish contain 5-20% fat as opposed to white fish which contain only 1-2% fat. Oily fish include sardines, salmon, pilchards, mackerel, herring, and trout, whether fresh, frozen or canned. Fresh tuna is also included, but unlike other canned oily fish, canned tuna is not considered as oily, because the processing of tuna during canning reduces its fat content to the levels of non-oily/white fish (Table 1.4).

Table 1.3: LA and ALA contents of selected nuts, seeds and oils (14)

FOOD	POLYUNSATURATED FATTY ACIDS (G PER 100 G TOTAL FATTY ACIDS)[1]	
	α-Linolenic acid (ALA; 18:3n-3)	**Linoleic acid (LA; 18:2n-6)**
Nuts and Seeds		
. Almonds	0.27	10.19
. Brazil nuts	0	25.43
. Hazelnuts	0.12	6.50
. Peanuts	0.35	12.75
. Poppy seeds	0.45	27.20
. Pumpkin seeds	0.13	21.58
. Sesame seeds	0.15	25.35
. Sunflower seeds	0.09	28.06
. Walnuts	7.47	39.29
Vegetable/Plant Oils		
. Corn oil	0.90	50.40
. Evening primrose oil	0.12	68.81
. Peanut oil	0	31.00
. Rapeseed oil	9.60	19.70
. Safflower oil	0.10	73.90
. Sesame oil	0.30	43.10
. Soya oil	7.30	51.50
. Sunflower oil	0.10	63.20
. Walnut oil	11.5	58.40

Table 1.4: Total fat, SFA, MUFA and PUFA content of fish and fish products (12;13)

	TOTAL FAT, SATURATED, MONOUNSATURATED AND POLYUNSATURATED FATTY ACIDS (G PER 100 G)										
Food	**Total Fat**	**SFAs**	**Cis MUFAs**	**Total cis PUFAs**	**n-6 PUFAs**	**n-3 PUFAs**	**LA 18:2 n-6**	**ARA 20:4 n-6**	**ALA 18:3 n-3**	**EPA 20:5n-3**	**DHA 22:6n-3**
White fish											
Cod, raw	0.7	0.13	0.08	0.28	0.02	0.26	Tr	0.02	Tr	0.08	0.16
Haddock, raw	0.6	0.12	0.09	0.20	0.03	0.17	0.01	0.01	Tr	0.05	0.10
Plaice, raw	1.4	0.23	0.39	0.35	0.01	0.32	0	0	0	0.16	0.10
Sea Bass, raw	2.5	0.4	0.6	0.6	0.1	0.5	Tr	Tr	0	0.1	0.4
Fatty fish											
Eel, jellied	7.1	1.89	3.50	0.96	0.32	0.64	0.15	0.10	0.12	0.19	0.15
Herring, raw	13.2	3.65	5.98	2.17	0.32	1.83	0.29	0.04	0.18	0.51	0.69
Kippers, raw	17.7	2.76	9.26	3.86	0.24	3.52	0.18	0.03	0.24	1.15	1.34
Mackerel, raw	16.1	3.23	7.87	3.27	0.51	2.78	0.30	0.07	0.22	0.71	1.10
Pilchards, canned in tomato	8.1	1.71	2.22	3.36	0.28	2.97	0.12	0.07	0.07	1.17	1.20
Salmon, raw	11.0	1.9	4.4	3.1	0.5	2.4	0.3	0.2	0.2	0.5	1.3
Salmon, canned in brine	7.8	1.38	3.29	2.24	0.27	1.85	0.13	0.11	0.08	0.55	0.86
Sardines, raw	9.2	2.7	2.5	2.7	0.2	2.2	0.1	0.1	0.1	0.9	1.1
Sardines, canned in tomato	9.9	2.79	2.90	3.22	1.04	2.11	0.96	0.04	0.16	0.89	0.68
Sprats, raw	11.0	2.15	4.67	3.04	0.31	2.68	0.11	0.07	0.08	0.93	1.35
Trout, rainbow, raw	5.2	1.1	1.85	1.73	0.41	1.32	0.34	0.03	0.06	0.23	0.83
Tuna, raw	4.6	1.2	1.2	1.6	0.2	1.5	0.1	0.1	0	0.3	1.1
Tuna, canned in brine	0.6	0.23	0.10	0.21	0.04	0.17	0.01	0.02	0	0.02	0.14
Crustacea and mollluscs											
Crab, boiled	5.5	0.68	1.55	1.36	0.24	1.10	0.02	0.12	0.02	0.47	0.45
Mussels, boiled	2.7	0.52	0.40	0.76	0.08	0.68	0.02	0.05	0.02	0.41	0.16
Oysters, raw	1.3	0.26	0.14	0.41	0.05	0.37	0.02	0.01	0.01	0.14	0.16
Prawns, frozen, raw	0.6	0.13	0.15	0.13	0.03	0.11	0.01	0.01	0	0.06	0.04
Squid, raw	1.7	0.35	0.22	0.61	0.16	0.45	0.12	0.01	0	0.13	0.29
Fish products and dishes											
Fish cakes, frozen, raw	3.9	0.59	1.43	0.93	0.60	0.33	0.58	0.01	0.18	0.05	0.09
Fish fingers cod, frozen, raw	7.8	1.81	1.99	1.38	1.28	0.17	1.21	0	0.10	0.04	0.03
Roe, cod, raw	1.9	0.41	0.37	0.55	0.05	0.49	0.02	0.04	0.01	0.16	0.30
Scampi, breaded, cooked	0.9	0.07	0.34	0.20	0.14	0.06	0.14	0	0.06	0	0
Taramasalata	52.9	3.90	27.71	15.14	10.81	5.00	10.52	0	4.43	0.14	0

1.1.7 Triglycerides and triglyceride metabolism

Fatty acids are rarely found "free" in the body. They are most commonly found as components of triglycerides (TAGs) also known as triacylglycerols. TAGs are three-carbon alcohols to which are attached three fatty acids as esters (Figure 1.3).

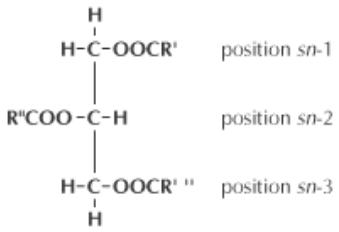


Figure 1.3: Triglyceride structure

In vegetable oils, PUFAs are most frequently found in positions one and three in the TAG. In fish oils and animal fats, the LC PUFAs are usually located in the central sn-2 position.

TAGs are the storage form of fatty acids in adipose tissue, food fats, and oils. In order to be digested in the gut they need to be emulsified or made soluble, then hydrolysed to remove the fatty acids from the glycerol backbone. Emulsification is accomplished in the small intestine by bile salts, while cleavage of the fatty acids is achieved by the action of the enzyme pancreatic lipase, also released into the intestine. Pancreatic lipase removes fatty acids from the 1 and 3 positions to generate free fatty acids and a TAG remnant with one fatty acid in the 2-position, a monoglyceride (2-acylglycerol). Monoglycerides may be further broken down by the enzyme phospholipase A_2 into free fatty acids and glycerol. Fatty acids and monoglycerides form complexes with bile salts called micelles, which are absorbed passively into the intestinal cell or enterocyte. Once inside the enterocyte, fatty acids are re-esterified into TAGs and packaged with cholesterol, phospholipods and proteins in a type of lipoprotein called chylomicrons. These large complexes are released into the lymph system and then into the blood circulation. Certain free fatty acids are also transported directly to the liver via the portal vein.

Once in the blood stream, chylomicrons acquire additional proteins and interact with the enzyme lipoprotein lipase found in endothelial cells lining the blood vessels. This enzyme removes some of the fatty acids, which are then taken up by tissues and resynthesized into TAGs for storage, export, oxidation, or further metabolism. The chylomicron remnants and

glycerol backbone are removed by the liver, while the remaining lipids and proteins are transferred to high density lipoproteins in the blood.

The liver is a primary site for fatty acid metabolism. Fatty acids are removed from the circulation, synthesized into TAGs, and packaged with proteins, cholesterol, and other lipids into very low density lipoproteins (VLDLs). Released back into the circulation, VLDLs are the primary vehicle for delivering TAGs to tissues, particularly adipose tissue and skeletal muscle. In the circulation, TAGs are removed from VLDLs through the action of the enzyme lipoprotein lipase, leaving intermediate-sized particles and low density lipoproteins (LDLs) as products. LDLs are rich in cholesterol which is made available for membrane structure and intracellular storage (1).

1.1.8 Fatty Acid Metabolism

Fatty acid metabolism is a complex combination of different pathways which act to derive energy from the fatty acids via oxidation or to convert the fatty acids into active derivatives.

B-oxidation is the process by which fatty acids are utilized for energy generation and occurs largely within mitochondria. B-oxidation of SFAs appears to be simpler than that of unsaturated fatty acids. However, abundant in vivo and in vitro research shows that unsaturated fatty acids with one to three double bonds are more readily β-oxidised than SFAs of equivalent chain length (1).

Ketogenesis and ketosis: Large amounts of fatty acids inhibit glycolysis and thereby the production of oxaloacetate in the Krebs Cycle. This situation favours the production of ketones (acetoacetate and acetone) from fatty acids. When glucose is limiting, ketones are an alternative source of energy for certain organs, particularly the brain. They are also efficient substrates for lipid synthesis during early postnatal development.

Carbon recycling and peroxidation: Carbon recycling (i.e. conversion of fatty acids to other fatty acids via simple units such as acetate) appears to be a ubiquitous feature of the metabolism of PUFAs, although its biological significance is still unclear (1). PUFAs are particularly vulnerable to peroxidation (autooxidation) at the double bonds. This process can produce free radicals which abstract further hydrogens from the same or different fatty acids and propagate the peroxidation process. Peroxidation of PUFAs is a potential hazard facing lipoproteins, membranes and dietary lipids. Antioxidants such as vitamin E are

usually present in sufficient amounts to prevent or block peroxidation in living cells and tissues. However lipid peroxidation is an important part of many necessary biological processes such as activating the immune response.

Desaturation, chain elongation and shortening: Desaturation is the insertion of a double bond into a fatty acid chain. Elongation is the extension of the carbon chain (usually by two carbon units), while shortening is the opposite.

Hydrogenation: This is opposite to the desaturation process i.e. it is the removal of unsaturated bonds in lipids.

Eicosanoid synthesis: Eicosanoids are a family of highly active substances derived from ARA, dihomo-γ-linolenic acid or EPA that affect immune and inflammatory responses, amongst other processes. They include prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). Eicosanoids are considered to be fast-acting local hormones, the presence of which in the plasma and urine is largely a spillover from localized production, in response to an injury, or a stimulus that releases the free precursor, most commonly, ARA. Those made from ARA are generally pro-inflammatory, whereas those from EPA are less inflammatory or may even be anti-inflammatory. Four important characteristics of eicosanoid action should be noted. Firstly, individual eicosanoids often have biphasic actions. Secondly, several abundant eicosanoids that arise from the same precursor fatty acid (i.e. ARA) have opposite actions to each other. Thirdly, eicosanoids derived from dihomo-γ-linolenic acid and from EPA often have effects that oppose those derived from ARA. Fourthly, varying the ratio of the precursor fatty acid in the diet is an effective way to modulate eicosanoid production (1).

1.1.9 Metabolic fate of LA, ALA, and LC n-3 PUFAs

As described earlier, LA and ALA can be elongated and desaturated to form the long-chain derivatives ARA, and EPA and DHA, respectively (Figure 1.4). Conversion of LA to ARA occurs more readily than that of ALA to DHA.

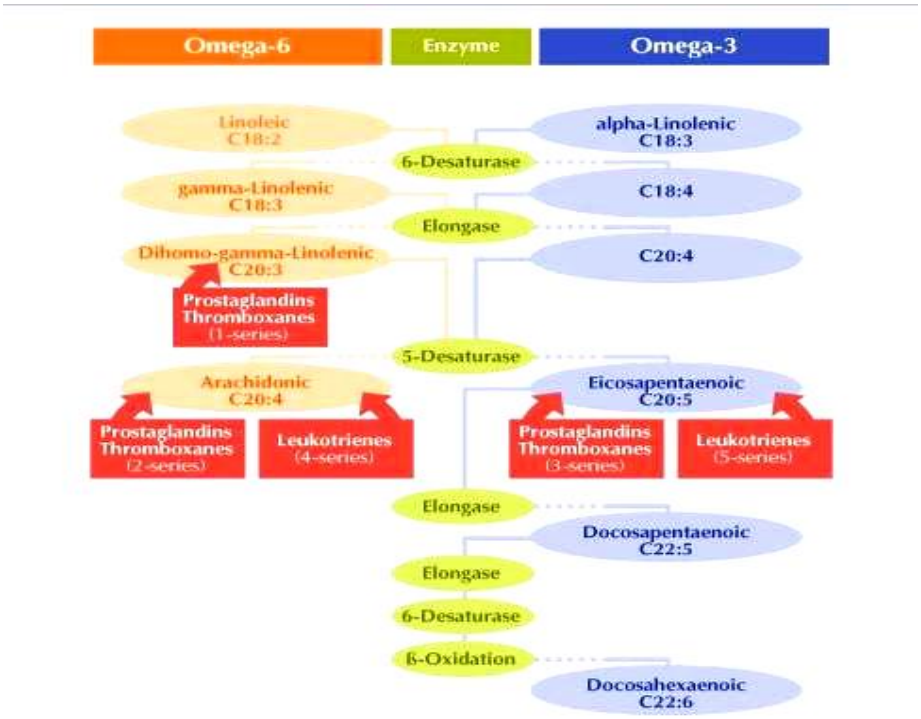


Figure 1.4: Metabolic competition between n-3 and n-6 fatty acids (15)

Most ALA and much LA is consumed in energy production via β-oxidation. More than 80% of dietary ALA may be used by this pathway (16). As a result, little ALA remains for conversion to long-chain derivatives and synthesis into other fatty acids and lipids.

LC PUFAs have several destinies in vivo:

- Incorporation into phospholipids in membranes
- Eicosanoid synthesis – conversion to prostaglandins, thromboxanes, leukotrienes
- Oxidation to provide energy
- Incorporation into TAG and tissue storage.

Using isotope tracer and other methods, Cunnane's laboratory has shown that membrane lipids in brain and liver preferentially use exogenous or dietary LC PUFAs rather than those produced endogenously (16). ARA not incorporated into membranes may be consumed by eicosanoid metabolism which requires a readily available supply at all times (16).

LC PUFAs are present in cell membrane phospholipids and are stored in small amounts in TAG in adipose tissue. LA and ALA in adipose tissue have been used as markers of dietary intake (17).

As structural components of membranes, ARA, EPA and DHA influence the functional characteristics of certain tissues, particularly neural and immune tissues. For example, DHA is highly concentrated in the outer segment of the rods in the retina of the eye. The presence of DHA is related to light activation and visual processing (18). ARA is the major precursor for the synthesis of eicosanoids involved in platelet aggregation, T-cell proliferation, lymphocyte migration, vasoconstriction and dilation, and inflammatory processes (19;20). In the presence of EPA, alternative eicosanoids can also be produced, and these attenuate or inhibit the action of the ARA-derived eicosanoids. Those derived from EPA also have weaker biological activity than those from ARA. Competition from n-3 fatty acids for the same eicosanoid synthesizing enzymes may reduce the production of ARA-derived eicosanoids. Thus, the presence of EPA can moderate the production and activity of ARA-derived eicosanoids. It is believed by some researchers that the predominance of eicosanoids derived from ARA contributes to the development of several chronic human diseases, including heart disease (21;22).

1.1.10 Nutritional regulation of long-chain fatty acid profiles

Phospholipids of all cellular and subcellular membranes contain a diverse range of long-chain fatty acids, the profile of which is subject to both dietary influence and endogenous control. Usually, the fatty acid profile of most organs is responsive to changes in dietary fatty acid composition and other nutritional variables. When changes in dietary fat change fatty acid profiles, appropriate membrane fluidity can be maintained by the addition or removal of other lipids such as cholesterol. Insufficient energy intake and the presence of disease have important consequences for fatty acid synthesis, desaturation and chain elongation and eventually tissue fatty acid profiles (1).

1.1.11 LC n-3 PUFA intakes, recommended intakes & contaminants

Typical daily intakes of EPA plus DHA in adult individuals in the UK who do not regularly consume either oily fish or fish oil capsules are quoted as less that 0.25 g (23). One portion/serving of oily fish (140 g) such as salmon, trout, pilchards, sardines, mackerel or kippers will provide between 1.5 g and 3.5 g EPA plus DHA (24;25). Data from the last National Diet and Nutrition Survey (26) of adults aged 19-64 years showed that most people in UK consume very little fish. Mean consumption of white fish per week was 103 g and of oily fish 50 g per week (23). This low intake of fish explains the low intake of EPA plus DHA. Data (27) from the Australian National Nutrition Survey in 1995 showed that the average daily intakes of EPA and DHA acids were 0.056 and 0.106 g,

respectively, and LC n-3 PUFA intake (EPA plus DPA plus DHA) was 0.189 g, similar to the above data for the UK. However, median intakes were considerably lower (0.008 g EPA, 0.015 g DHA, and 0.029 g LC n-3 PUFA) (27). Thus 50% of the adult Australian population was consuming < 0.03 g LC n-3 PUFA/day. Similar low intakes seem likely in the UK.

Recommended intakes for n-3 PUFAs are not uniform. In 2000, Simopoulos recommended a daily intake for EPA plus DHA of 650 mg, with at least 222 mg for both EPA and DHA, and 2.22 g/d for ALA (28). The American Heart Association recommends adults to eat fish (in particular fatty fish) at least two times per week (29). The guidelines proposed by the US Food and Nutrition Board established adequate intakes for ALA at 1.6 g/d and 1.1 g/d for adult men and women, respectively, and that EPA plus DHA could account for up to 10% of the total n-3 fatty acid intake as a contribution toward the adequate intake for ALA (30).

Current UK recommendations by the Scientific Advisory Committee on Nutrition (SACN) propose that people should eat at least 2 portions of fish, of which one should be oily, per week and agree that this recommendation should also apply to pregnant women (23). This was equated to a daily intake of at least 450 mg of LC n-3 PUFAs. There are several published recommendations concerning the intake of n-3 fatty acids during pregnancy and lactation listed below. Dietary Reference Intakes (DRI) suggest that the recommended daily dosage for ALA be 1.4 g/day for pregnant women and 1.3 g/day for lactating women (31) and recommend a DHA dosage of 100 mg/day (plus 50-60 mg/day in the third trimester) in pregnant women; Makrides & Gibson (32) suggest an intake of LC n-3 PUFAs of 0.2-0.4 g/day in pregnant and lactating women; European Food Safety Authority (EFSA) suggests intake of 250 mg/day DHA during pregnancy and lactation (33).

Despite fish being the best source of preformed LC n-3 PUFAs, because of concern about contaminants such as methylmercury, dioxins and dioxin like PCBs in some species of oily fish, the UK Foods Standards Agency (FSA) has advised that pregnant women, women intending to become pregnant and girls under 16 years old should avoid eating shark, marlin and swordfish (21). The Committee on Toxicology advises that women of reproductive age and girls should aim to consume within the range of one to two portions of oily fish per week, based on maintaining consumption of dioxins and dioxin like PCBs below the TDI of 2 pg WHO-TEQ/kg bodyweight per day (21). Women past reproductive age, boys and men should aim to consume within the range of one to four portions of oily

fish a week, based on maintaining consumption of dioxins and dioxin-like PCBs below the guideline value of 8 pg WHO-TEQ/kg bodyweight per day (23).

The demand for LC PUFAs for the development of the mammalian brain is high (34). Preformed LC PUFAs, particularly DHA, but also EPA, may need to be provided in the diets of infants to meet the high requirements of rapidly growing tissues and organs (23). The mother is the primary source of these fatty acids for the fetus and breast-fed infant.

According to data reviewed by the Scientific Advisory Committee on Nutrition in 2004 (23), there is some evidence that for many women there is a marginal status for LC n-3 PUFAs during pregnancy and lactation. However, there is a lack of agreed markers which are suitable for defining LC n-3 PUFA status. The markers usually reported relate to concentrations of fatty acids within the circulation. They may be expressed as a percentage of total fatty acids or as their absolute concentrations in different circulating pools. Concentrations of fatty acids in maternal or umbilical blood have been used as measures of LC n-3 PUFA status during pregnancy. These measures have been related to maternal dietary intake (35) and to pregnancy outcomes (gestation duration, infant growth) (36-39). What is more, during pregnancy a woman must also meet the additional demands related to the accretion of maternal, placental, and fetal tissues. Although, the formation of DHA and EPA appears to be tightly regulated, a marginal state for many women during pregnancy and lactation cannot be excluded. The additional demands for EPA and DHA or other fatty acids during a normal pregnancy have not been adequately defined. It was concluded that the extent of dietary dependence on increased levels of consumption of n-3 PUFAs, or specifically DHA, to improve pregnancy outcome needs to be demonstrated (23).

Since eating fish is advised, but fish are also a source of contaminants, aquaculture of fish to have low contaminant levels could be an ideal solution to enable oily fish consumption by pregnant women and possibly the intake of the recommended amounts of LC n-3 PUFAs without the concern about contaminants. In this way, consumption of tailor-made salmon by pregnant women could prevent the depletion of the mother and could improve fetal and neonatal status of EPA and DHA.

1.2 Immune system

The role of the immune system is to achieve a balance between "protection against threats" including infectious agents that exist in the environment (bacteria, viruses, fungi, parasites) and other noxious insults and maintaining "tolerance to harmless things" such as "self" proteins and cells and harmless environmental factors, including those from the diet. The following sections will give an overview of the key components of the immune system, and more specifically the immunology of the atopic disease and how the environment encountered during pregnancy and fetal life may play a role in the predisposition to atopy (40).

1.2.1 Innate immunity

The immune system has two functional divisions: the innate (or natural) immune system and the acquired (specific or adaptive) immune system. The immune response, in most cases requires the coordination of the two types of immunity. All cells of the immune system originate in the bone marrow and result from the differentiation of stem cells (41;42).

Innate immunity consists of physical barriers (skin, mucous membranes, lysozyme, stomach acid, commensal bacteria), soluble mediators (macrophage derived cytokines), circulating molecules (complement), phagocytic cells which include granulocytes (neutrophils, basophils, eosinophils), monocytes and macrophages and natural killer cells. Innate immunity has no memory and is therefore not influenced by the prior exposure to an antigen-bearing organism (43). Phagocytic cells, the main effectors of innate immunity, express surface receptors specific for bacterial surface structures. Binding of microbes to these receptors triggers phagocytosis and subsequent destruction of the pathogenic microorganism by complement or by toxic chemicals, such as superoxide radicals and hydrogen peroxide (44). Bacterial lipopolysaccaride (LPS; known also as endotoxin) is an important exogenous trigger. LPS is a component of the cell wall of gram-negative bacteria. Natural killer cells also possess surface receptors and destroy pathogens by the release of cytotoxic proteins (45). In this way, innate immunity provides a first line of defense against invading pathogens (40).

1.2.2 Acquired immunity

This type of immunity involves the specific recognition of antigens on an invading pathogen which distinguish it as being foreign to the host. The components of the acquired immune immune system also consist of physicochemical barriers (cutaneous and mucosal immune systems, antibodies in mucosal secretions), soluble mediators (lymphocyte derived cytokines), circulating molecules (antibodies) and cells (lymphocytes). Lymphocytes, which are subdivided into T- and B-lymphocytes, effect this form of immunity. All lymphocytes originate in the bone marrow. B-lymphocytes undergo further development and maturation in the bone marrow before being released into the circulation, while T-lymphocytes mature in the thymus. From the bloodstream lymphocytes can enter peripheral lymphoid organs, including lymph nodes, the spleen, mucosal lymphoid tissue, tonsils and gut-associated lymphoid tissue. Immune responses occur largely in these lymphoid organs, which are designed to promote interaction between cells and invading pathogens (43).

The characteristics of the acquired immune response are that is specific, diverse, has memory and is self-regulated (46).The acquired immune system is highly specific, since each lymphocyte carries surface receptors for a single antigen. However acquired immunity is extremely diverse, recognizing approximately 10^{11} antigens, meaning that only a small number of lymphocytes will be able to recognize any given antigen. For that reason the acquired immune system involves clonal expansion that involves the proliferation of a lymphocyte after an interaction with its specific antigen has occurred, so that a single lymphocyte gives rise to a clone of lymphocytes, all of which have the ability to recognize and destroy the source of the antigen causing the initial response (47). The acquired immune response becomes effective over several days after the initial activation, but it also persists for some time after the removal of the initiating agent, giving rise to another characteristic of acquired immunity, immunological memory (40). Figure 1.5 depicts the coordinated actions of both innate and acquired immunity. The last feature of the acquired immune system, self-regulation, reflects its ability to re-establish homeostasis through communication between cells (48).

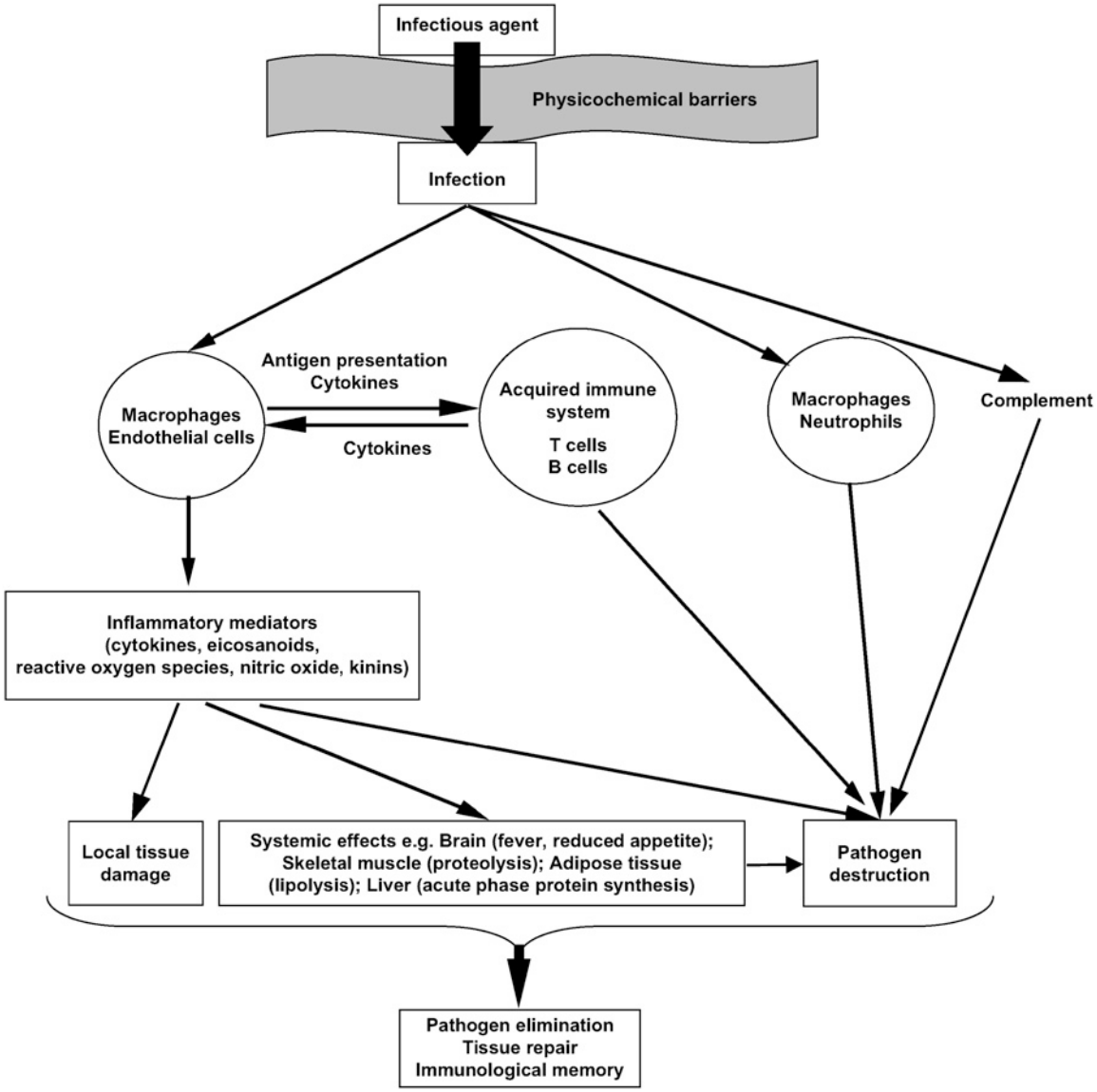


Figure 1.5: A general overview of the immune system (21)

1.2.3 B- and T-Lymphocytes

B-lymphocytes have the ability to produce antibodies (immunoglobulins which are specific for individual antigens). This form of protection is named humoral immunity and is conducted exclusively by B-lymphocytes. B-lymphocytes carry immunoglobulins that bind with an antigen on their cell surfaces. This attachment causes proliferation of B-lymphocytes and induces their transformation into plasma cells, which secrete large amounts of antibody with the same specificity as the parent cell. There are 5 major classes of immunoglobulin, IgA, IgD, IgG, IgM, and IgE, each of which elicits different components of the humoral immune response. Antibodies work in several ways to combat invading pathogens. They can neutralize toxins or microorganisms by binding to them and preventing their attachment to host cells and

they can activate complement proteins in plasma, which in turn promote the destruction of bacteria by phagocytes. Since antibodies have binding sites for both antigen and for receptors on phagocytic cells, antibodies can also promote the interaction of the two components by forming physical 'bridges' a process known as opsonisation. The antibody class determines the antibody bound on the type of phagocytic cell, for example eosinophils are specific for IgE. In this way antibodies are a form of communication between the acquired and the innate immune responses; they are elicited through highly specific mechanisms, but are ultimately translated to a form that can be interpreted by the innate immune system, enabling it to destroy the pathogen (47).

Humoral immunity deals with extracellular pathogens, but viruses and some bacteria infect individuals by entering cells. These pathogens will be dealt with cell mediated immunity, which is conferred by T-lymphocytes which express antigen-specific T-cell receptors (TCR) on their surface. T-lymphocytes are only able to recognize antigens that are presented to them on a cell surface of an antigen presenting cell (APC), which ingest the pathogen proteins and digest them into small peptides. This is the distinguishing feature of cell mediated immunity.

The peptide fragments derived from the pathogen are transported to the surface of the APC and expressed there in conjunction with proteins termed histocompatibility complex (MHC), after migration of the cells to the regional lymph nodes; in man MHC is termed human leucocyte antigen (47). The combination of the pathogen peptide fragment with the MHC is recognised by T-lymphocytes. There are two classes of MHC (MHC class I and MHC class II). MHC I binds peptides that are produced within the host cell cytosol originating from pathogen proteins (viruses, certain bacteria) whereas MHC II binds peptides that originate from pathogens that have been phagocytosed by macrophages or endocytosed by APCs (macrophages, dendritic cells, B-lymphocytes). MHC I bound peptides are recognised by T-lymphocytes expressing CD8, whereas MHC II bound peptides are recognised by T-lymphocytes expressing CD4 (49).

Activation of the TCR results in the entry of T-lymphocytes into the cell cycle and ultimately proliferation. Activated T-lymphocytes secrete interleukin (IL)-2, which promotes further proliferation and differentiation of T-lymphocytes. The expanded T-lymphocytes have the ability to migrate to sites of infection, injury or tissue damage.

Cytotoxic ($CD8^+$) T-lymphocytes are stimulated by intracellular pathogens and secrete cytotoxic enzymes which lyse the target cell, or secrete the antiviral cytokine interferon-γ (IFN-γ) or induce apoptosis of target cells (50). Helper T-lymphocytes ($CD4^+$) are stimulated by extracellular pathogens and they induce the phagocytic activity of macrophages, and also proliferation of B-lymphocytes and antibody secretion from them (51). The secretion of cytokines by antigen activated $CD4^+$ T-lymphocytes causes recruitment of neutrophils and monocytes from the blood stream to the site of infection and also activation of monocytes. This type of cell-mediated immunity is called delayed-type hypersensitivity (DTH) and is the primary defence against intracellular bacteria. DTH may be caused by contact with chemicals and environmental antigens. Intradermal injection of microbial antigens also leads to DTH and has been used as a rapid *in vivo* marker of cell-mediated immunity (46).

While effective immune responses are highly desirable some aspects of immunity have undesirable consequences, such as toxicity to host tissues caused by mediators secreted by macrophages. For this reason immune responses need to be tightly controlled and the self-regulatory properties of the immune system are normally effective (52).

1.2.4 Cytokines, Th1, Th2 and T-reg cells

Communication between acquired and innate immune system as well as within the acquired immune system is achieved by adhesion molecules and by production of chemical messengers, which send signals from one cell to another. Chief amongst these messengers are proteins called cytokines which act to regulate the activity of the cell that produced the cytokine or of other cells. Cytokines act by binding to specific receptors on the cell surface and thereby induce changes in the growth, development or activity of the target cell (53). Tumour necrosis factor-α (TNF-α), IL-1 and IL-6 are among the most important cytokines produced by monocytes and macrophages. These cytokines activate neutrophils, monocytes and macrophages to initiate bacterial and tumour cell killing, increase adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulate B and T cell lymphocyte proliferation and initiate the production of other pro inflammatory cytokines (e.g. TNF induces production of IL-1 and IL-6, and IL-1 induces production of IL-6). The actions of inflammatory cytokines are opposed by anti-inflammatory cytokines such as IL-10 and by receptor antagonists such as IL-1 receptor antagonist.

Thus TNF, IL-1 and IL-6 are mediators of both natural and acquired immunity and are an important link between them (54). In addition these cytokines mediate the systemic effects of inflammation such as fever, weight loss and acute-phase protein synthesis in the liver. Production of appropriate amounts of TNF, IL-1 and IL-6 is clearly important in response to infection but overproduction can be dangerous and these cytokines, particularly TNF are implicated in causing some of the pathological responses that occur in chronic inflammatory conditions (e.g. rheumatoid arthritis, inflammatory bowel disease and psoriasis) (54).

T-cells that have not previously encountered antigen produce mainly IL-2 upon the initial encounter with an antigen. These cells may differentiate into a population, Th0 cells which differentiate further into either Th1 or Th2 cells. This differentiation is regulated by cytokines: Th1 cells produce IL-2 and IFN-γ, which activate macrophages, natural killer cells and cytotoxic T-lymphocytes and are the principal effectors of cell-mediated immunity. Interactions with bacteria, viruses and fungi tend to induce Th1 activity. Since Th1 cytokines activate monocytes and macrophages, these cytokines may also be regarded as proinflammatory (55). Inflammatory conditions are frequently associated with Th1 responses (56).

Th2 cells produce IL-4, IL-5 and IL-13. IL-4 stimulates IgE production by B-cells (antibody responses) and IL-5 activates eosinophils. IL-4 also suppresses cell-mediated immune responses by Th1 cells, and thus these cytokines may be regarded as anti-inflammatory (52). On the other hand, cytokines produced by Th1 cells, suppress Th2 cell activity. Th2 responses induce IgE-mediated activation of mast cells and basophils to eliminate helminthic parasites and are associated with allergy and asthma (57).

Cells with typical Th1 and Th2 profiles have been demonstrated in mice. In humans, although some cells might have typical responses, there is no discrete division between the two types of cells. Helper T-cells most of the time produce a mixture of Th1 and Th2 cytokines in various proportions (55). Consequently, helper T-lymphocytes can be ‘Th1 dominant’ (inflammatory conditions) or ‘Th2 dominant’ (allergy), and the dominant activity antagonises the non-dominant one, leading to polarisation towards the dominant phenotype. Figure 1.6 represents Th1 and Th2 immune responses (48;58).

More recently a novel helper T-cell subset which produces IL-17 has been identified as a different population from Th1 and Th2. These cells have been termed Th17 and are

the result of naïve T-cell differentiation through the effect of IL-1, IL-6 and IL-23 (59;60). These cells provided new insight into the molecular mechanisms involved in immune responses and/or disease development, and their discovery has led to revision of the classic Th1/Th2 paradigm in such settings. Likewise, new T cell subsets, particularly Th17 cells, may also contribute to the pathogenesis of classically recognized Th2 mediated allergic disorders. Oboki *et al.* reviewed that IL-17 production by Th17 cells may contribute to the pathogenesis of non-atopic and/or non-eosinophil/neutrophil dominant asthma, and may be a new marker for classification of such non-Th2 type asthma (60).

Regulatory T cells (T-reg cells) control peripheral immune responses and are likely to play a central role in determining the incidence and severity of several immune pathologies, including autoimmune, infectious, allergic, and asthmatic diseases (52;61). There are two major categories of T-reg cells described to date. The first is the naturally occurring, thymically derived $CD4^{+}CD25^{+}$T-reg cells that express high levels of the transcription factor FOXP3, which is essential for their development and function. The other category is the antigen-specific T-reg cells, which can be induced in vitro and in vivo under particular conditions of antigenic stimulation. These antigen-specific T-reg cells secrete anti-inflammatory cytokines such as IL-10 and/or transforming growth factor (TGF)-β, and regulate immune responses and inflammatory pathologies. Induced T-reg cells that secrete IL-10 are often referred to as IL-10–T-reg cells, or Tr1 cells; those that secrete TGF-β have been referred to as Th3 cells. However, it is likely that both categories of T-reg cells require one or both of these cytokines to evoke their suppressive functions during different stages or types of inflammatory responses (61).

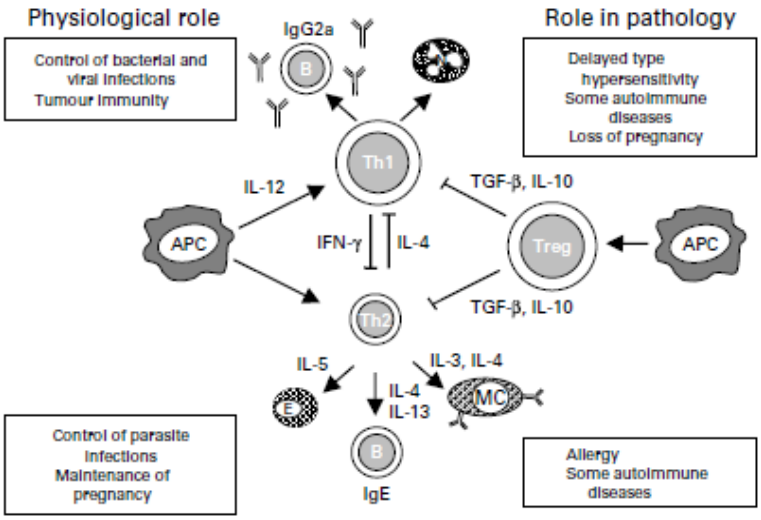


Figure 1.6: Schematic representation of the roles of helper T cells in regulating immune responses and in physiological and pathological responses (58)

APC, antigen presenting cell; B, B cell; E, eosinophil; IFN, interferon; MC, mast cell; N, neutrophil; TGF, transforming growth factor; Th1, type 1 helper T cell; Th2, type 2 helper T cell; T-reg, regulatory T cell

1.2.5 Inflammation

Inflammation is the body's immediate response to infection or injury. It is typified by redness, swelling, heat and pain. These occur as a result of increased blood flow, increased permeability across blood capillaries which permits large molecules to leave the blood stream and cross the endothelial wall and increased movement of leukocytes from the bloodstream into the surrounding tissue. Thus inflammation is an integral part of the innate immune response (62).

1.2.6 The immune system in health and disease

Some of the undesirable features of the immune response are described below:
Firstly, the immune system is responsible for the rejection of transplanted tissues, which occurs because of the ability of the immune system to recognize and eliminate foreign antigens effectively.

Secondly, the ability to discriminate between 'self' and 'non-self' is an essential requirement of the immune system and is normally achieved by the destruction of self-recognising T-and B-lymphocytes before their maturation. However, since lymphocytes are unlikely to be exposed to all possible self-antigens in this way, a second mechanism termed clonal anergy exists, which ensures that an encounter with a self-antigen induces tolerance. In some individuals there is a breakdown of the mechanisms that normally preserve tolerance; a number of factors contribute to this, including a range of immunological abnormalities and a genetic predisposition in some individuals. As a result, an inappropriate immune response to host tissues is generated and this leads to autoimmune and inflammatory diseases, which are typified by ongoing chronic inflammation and a dysregulated Th1 response. Examples of this type of disease include psoriasis, multiple sclerosis and rheumatoid arthritis (63).

Thirdly, the immune system of some individuals can become sensitized to usually 'innocent' antigens from the environment and can respond inappropriately to them. Such antigens include components of food or of so-called allergens (e.g. cat or dog fur, house dust mite, some pollens), such that this response can lead to allergies, asthma and related atopic diseases (63;64). These diseases are often termed chronic inflammatory diseases but have a different immune basis from the diseases described above.

1.2.7 Immunology of atopic disease

Atopy (a derivation of the Greek word "atopos" which means out of place) is defined by a familial and/or personal predisposition to develop IgE antibodies to environmental (e.g. inhaled) antigens (65), which has a genetic background. The sensitization process, i.e. the generation of allergen specific IgE antibodies, is driven by interactions between antigen presenting cells (APCs, particularly dendritic cells) and T-lymphocytes. It involves multiple mechanisms that are influenced by the genetic background, allergen exposure and co-factors. In genetically susceptible individuals, dendritic cells activated by the allergen in a pro-inflammatory milieu in the airways, the intestinal mucosa or the skin instruct T cells to polarize into Th2 cells, which produce a specific set of cytokines, including IL-4, IL-5, IL-9 and IL-13. These cytokines drive B cell switching to IgE production (IL-4, IL-13), basophil/mast cell development (IL-4, IL-9), eosinophil accumulation (IL-5, IL-9) as well as epithelial mucus production (IL-9, IL-13), all of which are hallmarks of allergic inflammation.

The *Th2 bias* in allergy could either relate to a defective stimulation of Th1 and/or Treg immunity. The number and/or function of Treg cells that control immune tolerance to allergens (and self-antigens), might be deficient in allergic individuals (61). This regulatory T-cell subset includes natural $FOXP3^+$ Treg cells, which exert their suppressive effects on effector T cells probably through TGF-ß signalling and membrane mechanisms, and IL-10 producing $CD4^+CD25^+$ Tr1 cells. Tissue macrophages could also play an important suppressive role, notably in the lung (66), while the role of other cells in allergy, including the recently described Th17 cells still need to be investigated.

The epithelium could also play a key primary role during sensitization. Functional dysregulation of the so-called *epithelial-mesenchymal trophic unit* has been suggested to play an important role in asthma (67) as well as atopic dermatitis. Resulting from a deficient barrier function, these changes would favour Th2-type allergic inflammation towards allergens and lead to abnormal repair processes. The epithelium can release various mediators regulating the induction and fate of the immune response, such as chemokines, cytokines and destructive proteins. Several cofactors probably influence the sensitization processes, including products of commensal (and pathogenic) microbes that may act by activating APCs and epithelial receptors of innate immunity (e.g. *Toll-like* receptors), and the involvement of the environment is increasingly recognized with a

complex role of combined exposure to allergens, pollutants and microbes which will be described further in section 1.3.

In summary, atopy is the genetic predisposition for mounting an immunoglobin (predominantly IgE) dominated response by B-lymphocytes in response to exposure to an antigen for the first time, such as components in foods, pollen or dust. Binding of IgE to specific receptors on the surfaces of the mast cells and basophils occurs and is termed sensitization. If an antigen is reintroduced it will interact with the bound IgE, leading to activation of the cells and the release of both preformed and newly synthesized inflammatory mediators, particularly histamine and Th2 cytokines, like IL-4 and IL-5. Atopy is associated with increased levels of IgE in the circulation and tissues and is believed to play a central role in the pathogenesis of the allergic diseases which include:

- Asthma
- Atopic dermatitis (eczema)
- Seasonal rhinitis (hay fever)
- Perennial rhinitis (all year round symptoms like hay fever)
- Urticaria (hives or nettle rash)
- Acute Urticaria - short lived / chronic urticaria lasting six weeks or more - not necessarily on a daily basis. The majority of chronic urticarias are not IgE mediated.

However, not everyone who has inherited the tendency to be atopic will necessarily go on to develop an allergic disorder. Nevertheless this inherited tendency or disposition could result in the development of symptoms later in life. Atopy may remain clinically silent in a good number of subjects and is only detected through a positive skin prick test (SPT) or serum IgE to specific allergens. Factors enhancing the translation of atopic status to clinically allergic syndromes remain unknown.

1.2.8 International data of allergy prevalence

Chronic inflammatory diseases (allergies/autoimmune diseases) are highly prevalent in Western and industrialised countries (Figure 1.7). Large increases in incidence and prevalence of these disorders were observed during the last 4 decades or so (68-72). Among these diseases, allergies represent an immunologic disorder strongly exemplifying the interaction between genetic and environmental conditions especially in childhood (73;73).

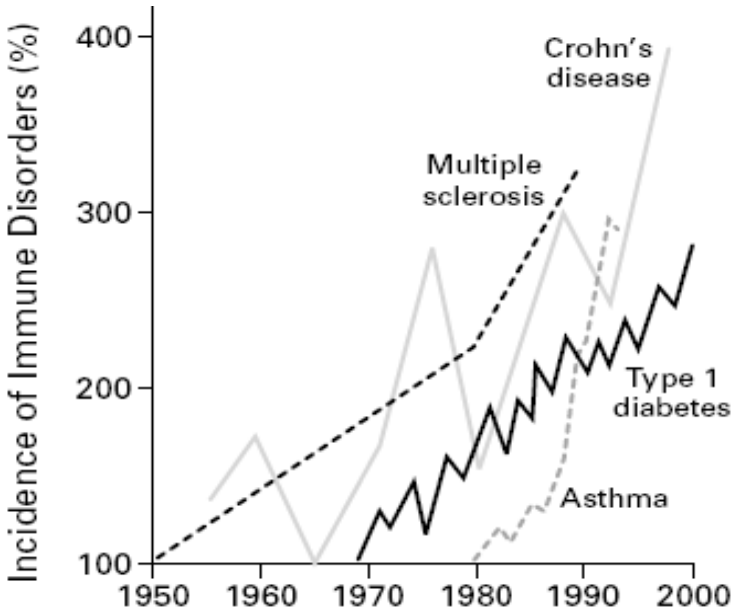


Figure 1.7: Data on changing incidene of immune disorders over time (72)

Systematic international comparisons of the prevalences of asthma and other allergic disorders in children are needed for better understanding of their global epidemiology so as to generate new hypotheses, and to assess existing hypotheses of possible causes. The International Study for Asthma and Allergy in Childhood (ISAAC) investigated worldwide prevalence of asthma, allergic rhinoconjunctivitis, and atopic dermatitis. They studied 463,801 children aged 13-14 years in 155 collaborating centres in 56 countries. Children self-reported symptoms of three atopic disorders. The researchers found differences of between 20-fold and 60-fold between centres in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema, with four-fold to 12-fold variations between the 10th and 90th percentiles for the different disorders. For asthma symptoms, the highest 12-month prevalences were from centres in the UK, Australia, New Zealand, and Republic of Ireland, followed by most centres in North, Central, and South America; the lowest prevalences were from centres in several Eastern European countries, Indonesia, Greece, China, Taiwan, Uzbekistan, India, and Ethiopia. The centres with the lowest prevalences were similar to those for asthma symptoms. For atopic dermatitis, the highest prevalences came from scattered centres, including some from Scandinavia and Africa that were not among centres with the highest asthma prevalences; the lowest prevalence rates of atopic dermatitis were similar to those for asthma symptoms. The variation in the prevalences of asthma, allergic rhinoconjunctivitis, and atopic dermatitis symptoms was striking between different centres throughout the world. These findings formed the basis of further studies to investigate factors that potentially lead to these international patterns (74).

Figure 1.8 shows the positive correlation between the gross national product and the incidence of asthma in 12 European countries, emphasising the effect of western lifestyle and better living on the prevalence of asthma (72).

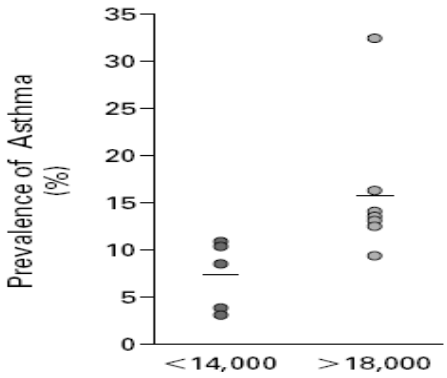


Figure 1.8: The frequency of asthma in 12 European countries according to the gross national product per capita (American dollars) (72)

The European Community Respiratory Health Survey (ECRHS) was a multi centre survey of the prevalence, determinants and management of asthma. This study presented a description of the variation in self-reported attacks of asthma and asthma symptoms across Europe. A screening questionnaire, including seven questions relating to the 12 month prevalence of symptoms of asthma, was distributed to representative samples of 20–44 year old men and women in 48 centres, predominantly in Western Europe. The prevalence of all symptoms varied widely. Although these were generally lower in northern, central and southern Europe and higher in the British Isles, New Zealand, Australia and the United States, there were wide variations even within some countries. Centres with a high prevalence of self-reported attacks of asthma also reported high prevalences of nasal allergies and of waking at night with breathlessness. The use of asthma medication was more common where wheeze and asthma attacks were more frequent. In most centres in The Netherlands, Sweden, New Zealand and the UK over 80% of those with a diagnosis of asthma were currently using asthma medication. In Italy, France and Spain the rate was generally less than 70%. These data are the best cross-sectional evidence indicating that geographical differences in asthma prevalence exist (71).

In addition, there has been a worldwide increase in allergic disease (75) with as many as 40% of children in industrialized countries developing allergic sensitization. Recent Australian figures estimated that 20% of school-aged children experienced wheezing in the previous 12 months and that prevalences of eczema and rhinitis were roughly 17% and 13%, respectively, in this age group (76). In the UK, as in most prosperous industrialized western countries, there are well documented increases in the prevalence of asthma and the

atopic diseases hay fever, eczema, and food allergy (77). Data from the Office for National Statistics published in 2004 shows that between 1995 and 1997, the prevalence of asthma symptoms and diagnosis among children aged 5 to 15 years, in England, varied little between the parental social classes. However, the prevalence was slightly higher among children of manual workers, and this was more pronounced in the pre-school age group (Figure 1.9).

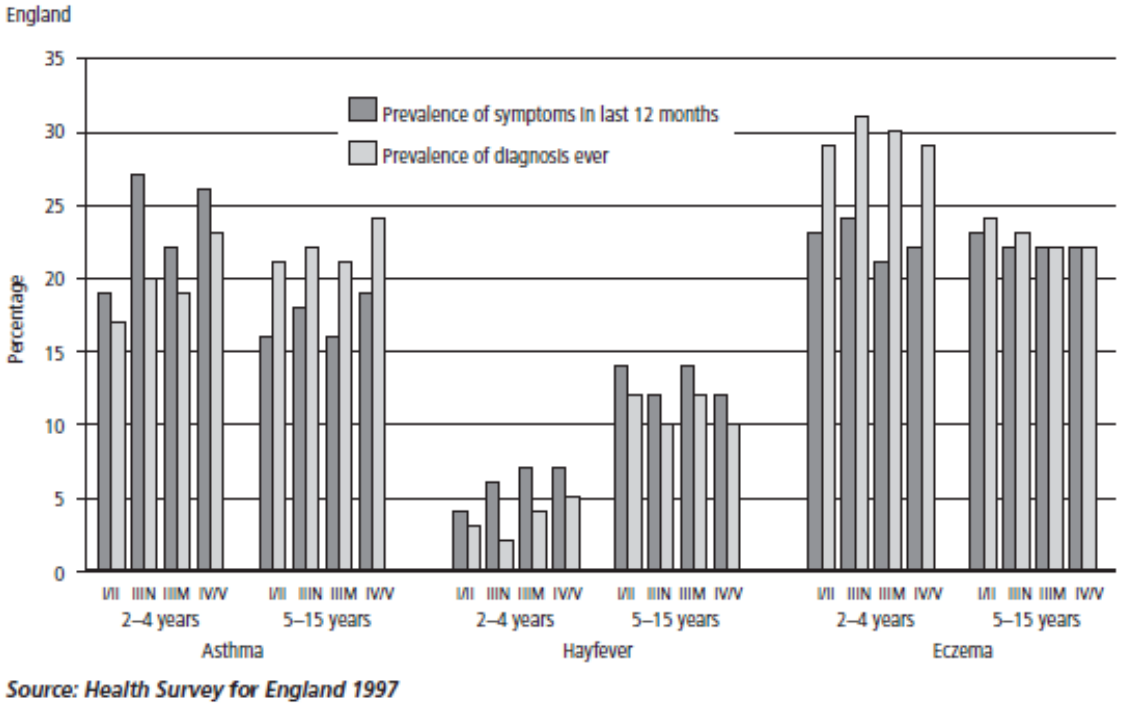


Figure 1.9: Prevalence of symptoms and diagnosis of asthma, hayfever and eczema, by age and social class of head-of-household, 1995-1997 in England (78)

The rapid rate of this rise suggests that environmental factors, perhaps including alterations in diet, may be responsible (79;80). Those environmental factors and especially their effect during the critical period of pregnancy and infancy will be explored in section 1.3.

1.3 Atopy in infancy: Maternal influence, infant immune origins and responses

1.3.1 Infant immune system

Up until recently, it was considered that the neonate was immunologically naive and that the development of specific immune responses was restricted to the postnatal period. However, it is now recognised that infants are born with the capacity to mount a specific immune response that can only have developed *in utero*, often to common environmental antigens. A number of studies have shown cord blood mononuclear cell (CBMC) sensitivity to allergens exists at birth (81;82) and, by studying the CBMC responses from fetuses through gestation, it has been possible to establish that specific allergen-induced responses can occur from as early as 22 weeks gestation (83). These responses are modified by events after birth and allergens, infections, diet, and microbial gut flora have all been implicated in the development, or not, of subsequent allergy (58). However, the mechanisms put in place during gestation may well be the starting point for eventual disease. This is consistent with the "early life origins of health and disease" hypothesis.

Stem cells are present in the human yolk sac at 21 days of gestation with the first lymphocytes seen in the thymus at the end of the 9th week of gestation. B lymphocytes can be seen in a range of organs including the lungs and gut from 14 weeks and by 19-20 weeks circulating B cells have detectable surface IgM. This implies that the full sensitization process must have occurred from antigen presentation through T-cell proliferation to B-cell stimulation and antibody production (84).

The culture of fetal blood collected by fetoscopy at 12-19 weeks of gestation yields high levels of both erythroid and granulocytic/monocytic progenitor cells; monocytes comprising 42-68%, neutrophils 27-41%, and eosinophils 5-30%. Despite this high number of granulocyte progenitors in the circulation at this time, granulocytes are not formed in large numbers until after birth, neutrophils being the last population to appear in the blood during early life. What follows is a summary of the development of the cell populations which play different roles in the allergic response (85).

1.3.1.1 Macrophages and dendritic cells

Macrophages and dendritic cells, which have a central role in the generation of an antigen-specific immune response, process and present antigens to T cells. Although dendritic cells are considered professional antigen presenting cells because they can prime naive T cells, very little is known about them in the fetal period. The major population of yolk sac macrophages is MHC class II-negative, and there is a minor population that is MHC class II-positive (85).

1.3.1.2 T cells

Putative prothymocytes can be identifed in the fetal liver from 7 weeks of gestation as highly proliferative cells that are positive for CD7, CD45, and cytoplasmic CD3. From 18-24 weeks of gestation, the mesenteric lymph nodes have a high percentage of $CD45RA^+$ T cells but very few B cells or monocytes. The fetal spleen at this time has equal numbers of T cells, B cells, and monocytes/macrophages. The spleen is considered already fully immunocompetent by 18 weeks of gestation, having sufficient accessory cells to ensure T-cell activation, whereas the mesenteric lymph nodes are deficient in accessory cells numerically or functionally. There are few memory T cells ($CD45RO^+$) in the blood and spleen of the newborn, whereas half the T cells in adult tissues have this phenotype. This low memory cell number is consistent with limited antigen exposure in early life. $CD3^+$ T cells are detectable in the fetal circulation at about 15-16 weeks of gestation, at which time they also express CD2 and CD5. Proliferation in response to the mitogen phytohaemaggluttinin (PHA) is first seen at 17 weeks of gestation (85).

The question is how early antigen-specific responses occur. Umbilical CBMC collected at full term exhibit antigen-specific reactivity to allergens, including those of house-dust mite and cows' milk (81;82). One of the frequently observed properties of neonatal T cells is their poor cytokine production in comparison to the adult (86), particularly in relation to Th1 cytokines. The underlying mechanisms that account for this deficiency are incompletely understood, but appear to derive in part from the secretory functions of the placenta, which are discussed below. The relatively poor capacity of neonatal T cells to produce cytokines is thought to contribute to the impaired responses of other neonatal cell populations that rely on these factors for their functions.

1.3.1.3 B cells

The percentage of pre-B cells in the fetal omentum and liver is similar over 8-12 weeks gestation, but the percentage of these cells decreases during weeks 13-23 in the omentum, remaining the same in the liver. The proportion of immature B cells in the bone marrow decreases with age, and cells expressing maturity markers increase. B cells in the spleen are diffusely distributed at 22 weeks, and then form primary nodules around 24 weeks. The liver, spleen and lymph nodes are important sites of B-cell differentiation in mammals. B cells emerge into the peripheral circulation at 12 weeks of gestation, and they are positive for CD19, CD20, CD21, CD22, HLA-DR, IgM, and IgD. The percentage of $CD5^+$ B cells is higher in the fetal circulation than the adult, and declines with increasing gestational age, yet even at birth most cord-blood B cells are $CD5^+$ (B-1 B cells), in contrast to the adult, where few peripheral blood B cells express this molecule.

1.3.1.4 Immunoglobulin production

Early IgG and IgM synthesis occurs primarily in the spleen, large amounts of both being produced by the spleen as early as 10 weeks of gestation, although levels are maximal at 17-18 weeks of gestation. IgG traverses the placenta throughout gestation with a marked upregulation in the transfer rate occurring from 20 weeks, and this upregulation is maximal from 32 weeks of gestation. IgE synthesis was observed at 11 weeks of gestation in fetal liver and lung, and by 21 weeks in the spleen. Despite this early burst of production in fetal life, the production of Ig isotypes at birth is impaired. Neonates have very low serum IgM and even lower IgA and IgE levels, and the IgG present is essentially of maternal origin. Neonatal B cells are mature in their capacity to switch to IgE-producing cells if they are given exogenous IL-4, although they require higher levels of IL-4 than required by adult B cells to switch to IgE production. Thus, the minimal production of IgE is not due to the immaturity of the B cells but to the lack of IL-4 produced by fetal cells, i.e., to the immature helper T-cell function. As IgE has a central role in the allergic response, it is worth noting that despite the low levels of total IgE detectable in the circulation, specific IgE (either allergen or parasite) is detectable in cord plasma from some neonates (87).

1.3.1.5 Mucosal immunity

A functioning mucosal immune system is essential for survival in infancy and beyond. IgA and IgM are important in the first line of defence. In the fetal parotid gland (20-40 weeks),

occasional IgM and IgA-producing cells were observed, but no cells producing IgD, IgG, or IgE isotypes were seen.

1.3.1.6 Eosinophils

Eosinophil granulopoiesis occurs in the fetal liver, and eosinophilic granulocytes are evident for the first time at 5 weeks in the hepatic laminae. Numbers at this site increase gradually over gestation, and then, after 20 weeks of gestation, they appear in the portal areas. The eosinophil population in the portal areas comprises a greater number of mature cells than is seen in the hepatic laminae. This was postulated to reflect increasing activity in the portal areas by the component cells that are also developing and beginning to provide growth factors. Although eosinophilia at 3 months of age has been associated with a greater risk of the development of atopic disease at 18 months of age, there are no studies of eosinophil numbers and/or function at birth with regard to the development of allergic disease (85).

1.3.2 Events after birth

The areas currently most discussed in relation to the influences on the developing immune response in early life are: (i) the hygiene hypothesis; (ii) gut microbial flora; and (iii) allergen exposure. It seems likely that they may all have a role to play in determining whether the events set up during pregnancy result in the development of allergic disease and they will be considered in the following section.

1.3.2.1 The hygiene hypothesis

In 1989, Strachan demonstrated an inverse relationship between birth order in families and the prevalence of hay fever. He proposed that infections in early infancy brought home by older siblings might prevent sensitization (88). There is clearly a quite convincing biological explanation to support this hypothesis. Early infection, whether with viruses or bacteria, will tend to stimulate a Th1 immune response, which, if early enough postnatally, will switch any Th2 biased allergic immune responses to common allergens to a Th1 immunising pattern (89). It must, however, be noted that there are some anomalies to this hypothesis, in that some infections, for example respiratory syncytial virus, are actually associated with more, rather than less, allergy, and immunisation with altered organisms does not have the same effect as active infection (84).

1.3.2.2 Gut microbiota

It has been reported that allergic children are more likely to have a low colonisation of lactobacilli in their gut than non-allergic children. Also, children in Estonia, where the prevalence of allergies is low, have a very different gut flora to those in Sweden where the prevalence of allergy is higher (90). Allergic children tended to have higher counts of aerobic micro-organisms such as coliforms and *Staphylococcus aureus.* A number of groups have questioned whether this might explain the remarkable observation that there is a much lower risk of asthma amongst children of farmers who have been born on farms. Ingestion of higher quantities of raw and sometimes unpasteurised milk containing a higher microbial load, particularly of lactobacilli, may well have been protective (91). This observation may, however, also be explained by a greater exposure to infecting organisms and particularly their products such as LPS which would be consistent with the earlier hygiene hypothesis (92). LPS-induced immune responses are primarily mediated via the receptor CD14. It is likely that the early switch from a Th2 to a Th1 biased response postnatally is a consequence of postnatal microbial exposure as described above. Those infants with an intact response to microbial antigens such as LPS will have a very rapid switch orchestrated through the CD14 molecule once the gut becomes colonised with organisms. Those infants with abnormalities in this response and those who have already had significant over commitment to a Th2 response as a result of antenatal factors, will not be so easily switched and have a higher probability of having a persistent response going on to atopic disease.

1.3.2.3 Allergen exposure

There is a good correlation between early high level exposure to house dust mite and the subsequent increase in prevalence and severity of asthma. Indeed, high level exposure to a number of indoor allergens is strongly associated with sensitisation in the first 3 years (93) and early sensitisation is associated with greater probability of persistence of bronchial hyper-responsiveness and symptoms of asthma in late childhood and adolescence (94). The main difficulty in employing allergen avoidance strategies is that techniques to reduce exposure to the commonest allergen, the house dust mite, are far from satisfactory. Indeed, meta-analysis of trials published in 1998 suggested that it was not likely to be effective (95). The studies that have attempted allergen avoidance in high risk infants with or without pre-existing evidence of sensitisation but not yet disease have yielded very

disappointing outcomes. Some interventions have even commenced antenatally. The attitude to antenatal dietary avoidance has been formed on the basis of one study where elimination of egg, milk, fish and nuts from the maternal diet in the last trimester of pregnancy had no impact on outcome in relation to disease. Furthermore, the mothers gained less weight during pregnancy as a consequence of the diet (96). However, perhaps the intervention was started too late, as it is now known that sensitisation might have occurred at an earlier stage in the pregnancy.

Postnatal avoidance has again tended to focus on diet with promotion of breast feeding. Many studies have been performed with very diverse results extending from reduced prevalence of food associated atopic disease, through no effect, to some studies showing a higher prevalence of atopy in intervention groups. Importantly, however, only one of many studies has demonstrated any long-term effects of early dietary manipulation on the prevalence of asthma (97). Where studies have demonstrated benefits these have been to reduce prevalence of food-associated atopic disease in infancy but with no long-term impact on any atopic problems and no effect on asthma (98).

1.3.3 Factors affecting the development of atopic disease

As said before, global increases in the incidence and prevalence of many chronic inflammatory conditions, including autoimmune diseases, allergies, asthma and atopy, and inflammatory bowel diseases, have been observed over the last sixty years or so, particularly in Westernized and industrialized countries (72). Genetic studies have documented the risk of developing atopy based on family history. Development of atopic disease has a strong familial component and as a result much research has focused on interventions in infants and children with an increased risk of atopy. Children born into atopic families have a 50-80% risk of developing atopic diseases; children from families with no history of atopy have approximately a 20% risk. Risk of allergy appears to be higher if both parents are allergic (versus only one parent) and if the mother (versus the father) has allergic disease (99). Although there are genetic predispositions to these diseases (100), the presence of certain gene polymorphisms alone is unlikely to explain the shift in disease prevalence. Thus, in addition to genetics, environmental factors must be taken into consideration; these are listed for atopic disease in Table 1.5. Epidemiological investigations have identified that early exposure to microbes may be an important protective factor for allergy and asthma, perhaps acting as an ‘immune educator’. This is the so called ‘hygiene hypothesis’ (101), which suggests that the absence of early exposure

to certain types of microbes as a result of modern hygienic and medical practices removes the drive to mature Th1 cell effector responses and so allows the dominant early Th2 cell responses to continue unchecked. The role of microbial exposure in educating the immune system away from Th2 predominance may explain why certain probiotic strains of bacteria appear to have a protective role in atopic diseases (102). It is also speculated that the different gut flora present in infants born by caesarean section, or other factors, like antibiotics given to the mother, may put the child at risk for allergic sensitization (103).

Epidemiology has also identified that early exposure to certain nutrients, including LC n-3 PUFAs may be protective (25;104). LC n-3 PUFA status in relation to developing atopic disease is discussed in section 1.4.

Table 1.5: Potential determinants of atopic disease (58)

POTENTIAL FACTORS AFFECTING ATOPY
Genetics
Family history
Low birth weight
Exposure to pets
Exposure to allergens
Infections/Microbial stimulation/Gut microflora
Exposure to parasites
Obesity
Dietary fatty acids
Dietary antioxidants
Exposure to tobacco smoke
Exposure to air pollutants-indoor/outdoor

1.3.4 Early origins of atopic disease-summary

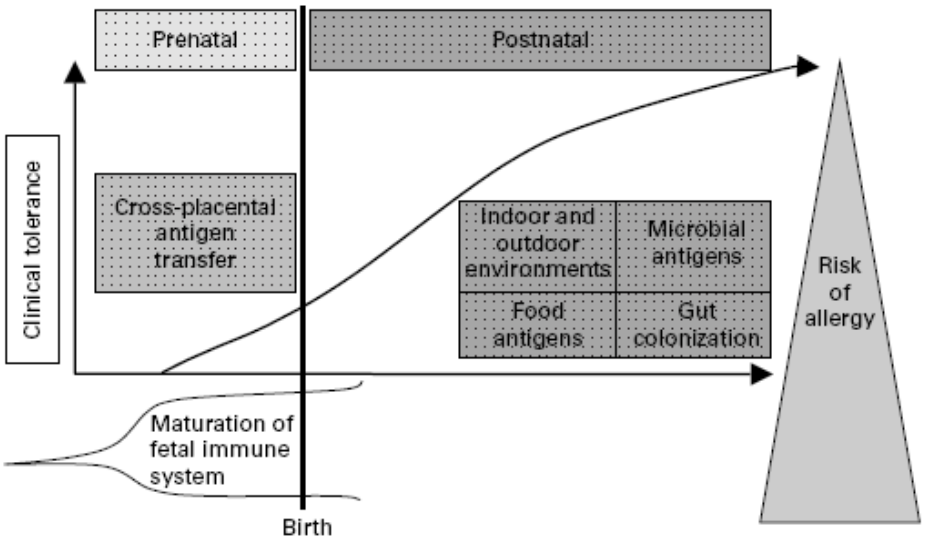


Figure 1.10: Schematic representation of the development of clinical tolerance through the pre- and postnatal periods and of how this relates to the risk of allergy (58)

A characteristic action of immune system is tolerance to self, to food, to environmental components and to commensal bacteria. The development of tolerance is the result of active immune mechanisms requiring antigen-contact and acting in a T cell dependent fashion (105). However, as described in section 1.3.1, both development and maintenance of tolerance are lifelong processes which start very early in life, even prenatally. In this regard early 'education' of the immune system seems to begin in utero (106;107) and continues after birth, particularly during the first 2 years of life (Figure 1.10). Moreover, it is now recognized that profound immunologic changes occur in the mother during pregnancy. These changes involve a polarization of T helper (Th) cells towards a dominance of Th2 and T-reg cell effector responses (108). The fetal immune response shows the same polarization and this phenomenon has been considered as a Th2/Treg default pathway early in life (82). The default pathway is important to maintain pregnancy, through suppression and avoidance of the rejection of the immunologically incompatible fetus (108). Th2-driven IgE and IgA responses allow the recognition of ultra-low antigen doses (109). Since there is significant uptake and transfer of food-derived and environmental antigens via the placental barrier into amniotic fluid (106), the ability to recognize low antigen doses may be important in order to develop early specific T cell responses against such antigens. Thus, the formation of antigen-specific IgE (and IgA) starts prenatally (110), and this pattern of Ig production is heavily driven through the Th2-biased environment. Mature $CD4^+$ and $CD8^+$ T cells can be detected in the fetus as early as around week 20 in human pregnancy (107). Therefore, particularly during the third

trimester of human pregnancy, the fetal T cell compartment is ready and able to mount antigen-specific T cell responses to environmental and food-derived antigens. Indeed, such T cells are detectable in cord blood in virtually all newborns indicating in utero sensitization (83). This seems to be a normal phenomenon although its functional consequences are not clear. One idea is that this is designed as a route to the early development of antigen-specific tolerance, particularly to food-derived antigens. However, if the neonatal immune system is not able to effectively down-regulate the pre-existing Th2 dominance and overcome the presence of low levels of allergen-specific IgE antibodies then an allergic phenotype may develop.

Although the fetus at term may be sensitized to certain antigens, it does lack a fully functional immune system and has a sterile gastrointestinal tract. Changes occur at, and soon after, birth in order that the immune system of the neonate becomes competent and functional and that the gut becomes colonized with bacteria (84). Exposure to bacteria during birth and from the mother's skin and the provision of immunologic factors in breast milk are amongst the key events that promote maturation of the infant's gut and gut-associated and systemic immune systems. The introduction of formula and of solid foods exposes the infant to novel food antigens and also affects the gut flora. The nature of the infant's environment (e.g. the presence of older siblings or of pets, the use of nurseries) will determine exposure to novel bacterial and environmental antigens to which it must respond in an appropriate manner (111;112). Thus, pregnancy, the suckling period, and the periods during which formula and solid foods are introduced offer windows during which nutrition might affect the immunologic development of the fetus and young infant. In this way, nutrition may:

- be the source of antigens to which the immune system must become tolerant;
- provide factors, including nutrients, that themselves might modulate immune maturation and responses;
- provide factors that influence intestinal flora, which in turn will affect antigen exposure, immune maturation and immune responses.

Through these mechanisms it is possible that nutrition early in life might affect later immune competence, the ability to mount an appropriate immune response upon infection, the ability to develop a tolerogenic response to 'self' and to benign environmental antigens, and the development of atopic disease (58).

1.4 Long-chain n-3 PUFAs and atopy in early life

This section will describe and discuss the current evidence linking LC n-3 PUFA intake or status to the immune response, especially as it relates with atopic disease in early life. There have been many studies conducted, both human and animal, in vivo and in vitro, looking at the effect of long chain n-3 PUFAs on production of inflammatory mediators of allergy, eicosanoids, T cell proliferation, cytokine production and IgE production.

1.4.1 Eicosanoids

As described in the section 1.1, eicosanoids are a group of mediators whose precursors are 20-carbon PUFAs (ARA and EPA). These PUFAs are contained in the phospholipid bilayer of cell membranes, with ARA being present in most cell membranes in relatively large amounts. Eicosanoid synthesis involves PUFA mobilisation from the cell membrane by various phospholipase enzymes, most notably phospholipase A_2. The free PUFA is converted by cyclooxygenase (COX) enzymes into PGs, TXs and related compounds, or by lipoxygenase (LOX) enzymes into LTs and related compounds. Figure 1.11shows the synthesis of eicosanoids from ARA (48).

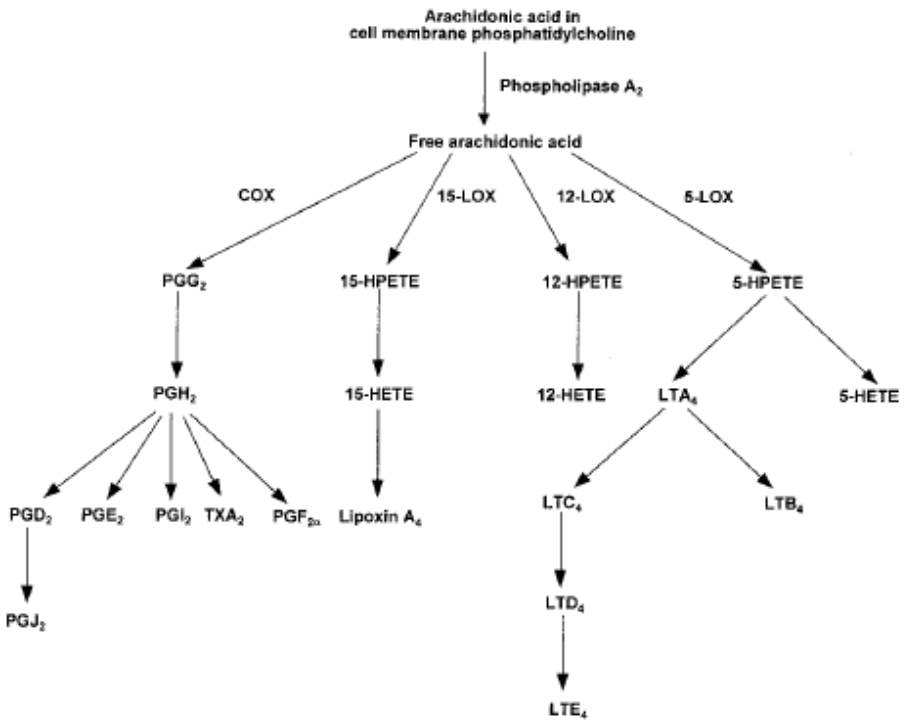


Figure 1.11: Generalized pathway for the conversion of arachidonic acid to eicosanoids (104)

COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane

ARA is the principal precursor of eicosanoids because it is present in greater amounts than other precursors. However, different cell types produce different compounds belonging to each class of eicosanoids. Monocytes and macrophages produce large amounts of PGE_2 and PGF_2, neutrophils produce moderate amounts of PGE_2 and mast cells produce PGD_2. The LOX enzymes have a tissue-specific distribution. The 5-LOX enzyme is mainly found in mast cells, monocytes, macrophages, and granulocytes, and 12- and 15-LOX in epithelial cells.

Eicosanoids are the key link between fatty acids and the immune system. They are involved in modulating the intensity and duration of inflammatory and immune responses (113). The effects of eicosanoids may vary even if these have the same precursor, and in addition, a single eicosanoid may have opposing effects. The overall physiological effect of eicosanoids will be determined by the nature of cells producing them, the nature of the stimulus, their concentrations, the timing of their production, and the sensitivities of target cells. For example, ARA is the precursor of different mediators (PGE_2, LTB_4) which may have opposing effects to one another. PGE_2 has various proinflammatory effects (increases vascular permeability, vasodilatation, pain, oedema), but at the same time exhibits immunosuppressive and anti-inflammatory effects (suppresses lymphocyte proliferation, inhibits IFN-γ, TNF-α and IL-1 production). Recent studies have shown that PGE_2 inhibits 5-LOX leading to decreased production of 4-series LTs, and induces 15-LOX promoting the formation of lipoxins (114) which have been shown to have anti-inflammatory effects. LTB_4 also has proinflammatory effects such as increasing vascular permeability and enhancing the production of inflammatory cytokines, but it also inhibits lymphocyte proliferation. Table 1.6 lists the anti- and pro-inflammatory effects of these two different eicosanoids (48).

Table 1.6: Pro- and anti-inflammatory effects of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). (Adapted from (48))

EICOSANOID	PRO-INFLAMMATORY EFFECTS	ANTI-INFLAMMATORY EFFECTS
PGE_2	Induction of fever Increase of vascular permeability Increase of vasodilatation Cause of pain Enhancement of pain caused by other agents Increased production of IL-6	Inhibition of production of TNF and IL-1 Inhibition of 5-LOX leading to decreased 4-series LT production Induction of 15-LOX leading to increased lipoxin production
LTB_4	Increase of vascular permeability Enhancement of local blood flow Chemotactic agent for leukocytes Induction of release of lysosomal enzymes Induction of release of reactive oxygen species by granulocytes Increased production of TNF, IL-1, IL-6	

LOX, lipoxygenase; LT, leukotriene; IL, interleukin; TNF, tumour necrosis factor

1.4.2 Arachidonic acid and eicosanoids

The amount of ARA in immune cells can be increased by including ARA in the diet of rats (115), or by increasing its intake from the diet in humans (116). Increasing the dietary intake of linoleic acid (18:2n-6) may also increase the amount of ARA in immune cells although this is not well defined in humans and studies are contradictory (48;117).

Animal feeding studies have shown that increasing the amount of ARA in immune cells is related to increased production of eicosanoids, such as PGE_2, by these cells (115). The effects of increased dietary ARA intake on immune processes have been little investigated in humans. It has been shown that modest increase of ARA intake in humans results in increased production of proinflammatory eicosanoids (PGE_2 and LTB_4) by LPS-stimulated PBMCs (118). However, it was shown that the production of inflammatory cytokines (TNF-α, IL-1β, IL-6) by these cells was not affected (118;119). Furthermore ARA intake did not affect T-cell responses, generation of reactive oxygen species (superoxide) by neutrophils and monocytes, or the concentrations of adhesion molecules in plasma (119).

1.4.3 Long-chain n-3 PUFAs and eicosanoid production

Increased dietary intake of EPA and DHA results in increased proportions of these fatty acids in immune cell membrane phospholipids (117). The incorporation of LC n-3 PUFAs occurs in a dose-response fashion and leads to decreased levels of ARA in immune cell membranes (120). Consequently, there is less substrate available for the synthesis of ARA-derived eicosanoids (121;122)). Furthermore, EPA can be converted to 3-series PGs and TXs, and 5-series LTs, by COX and 5-LOX respectively (Figure 1.12). Fish oil supplementation of the human diet has been shown to result in increased production of LTB_5, LTE_5 and 5-hydroxyeicosapentaenoic acid (5-HEPE) by inflammatory cells (123). EPA-derived eicosanoids have a slightly different structure and are less biologically potent than their ARA-derived analogues, and some of them exhibit anti-inflammatory effects (PGE_3, LTB_5). The decreased production of ARA-derived eicosanoids, followed by an increase in EPA-derived eicosanoids as a result of fish oil consumption, has led to the idea that fish oil may have anti-inflammatory effects and may influence the function of the immune system.

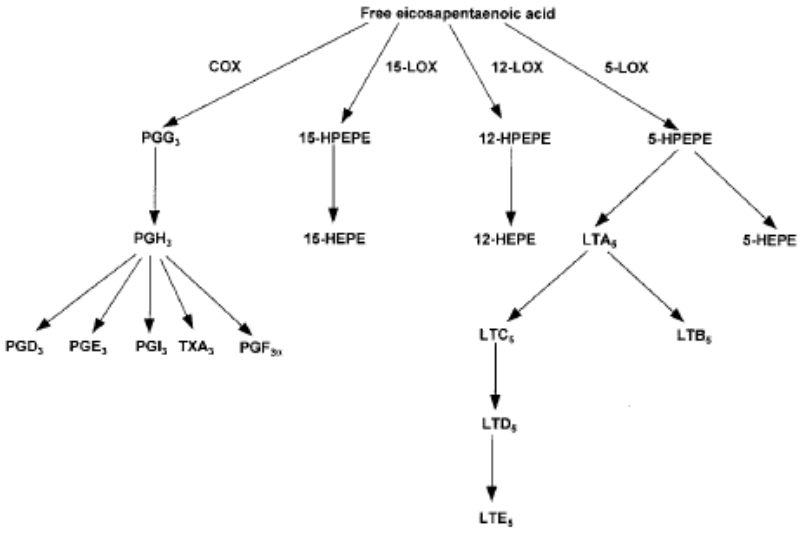


Figure 1.12: Generalized pathway for the conversion of eicosapentaenoic acid (EPA) to eicosanoids (104)

COX, cyclooxygenase; HEPE, hydroxyeicosapentaenoic acid; HPEPE, hydroperoxyeicosapentaenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane

Recent studies have demonstrated the production of a novel group of mediators, termed E-series resolvins. These compounds are produced when EPA acts as a substrate for COX-2 and they have anti-inflammatory actions (124-126). Moreover, DHA also competitively inhibits ARA metabolism acting as a substrate for COX-2, giving rise to DHA-derived mediators termed D-series resolvins, docosatrienes and neuroprotectins, that appear to be anti-inflammatory. These novel inflammatory mediators may be of great importance for a variety of conditions (127;128).

1.4.4 Clinical applications

LC n-3 PUFAs from oily fish and fish oils decrease the production of inflammatory eicosanoids, cytokines and adhesion molecules (19;21;48). They act both directly, by replacing ARA as an eicosanoid substrate, by inhibiting ARA metabolism and by giving rise to anti-inflammatory resolvins, and indirectly, by altering the expression of inflammatory genes through effects on transcription factor activation. Thus, LC n-3 PUFAs are potentially useful anti-inflammatory agents (Figure 1.13). The recognition that the LC n-3 PUFAs have anti-inflammatory actions has led to the ideas that an absolute or relative lack of these fatty acids might contribute causally to inflammatory conditions and that supplementation of the diet of patients with inflammatory diseases may bring about clinical benefit. Possible therapeutic targets for LC n-3 PUFAs include rheumatoid arthritis, Crohn's disease, ulcerative colitis, lupus, type-1 diabetes, cystic fibrosis, asthma, allergic disease, chronic obstructive pulmonary disease, psoriasis, multiple sclerosis, atherosclerosis, acute cardiovascular events, neurodegeneration and the systemic inflammatory response to surgery, trauma and critical illness (25). Supplementation trials with long chain n-3 PUFAs have been conducted in most of these conditions. In most cases these demonstrate anti-inflammatory effects, as indicated by changes in circulating concentrations or ex vivo production of inflammatory mediators.

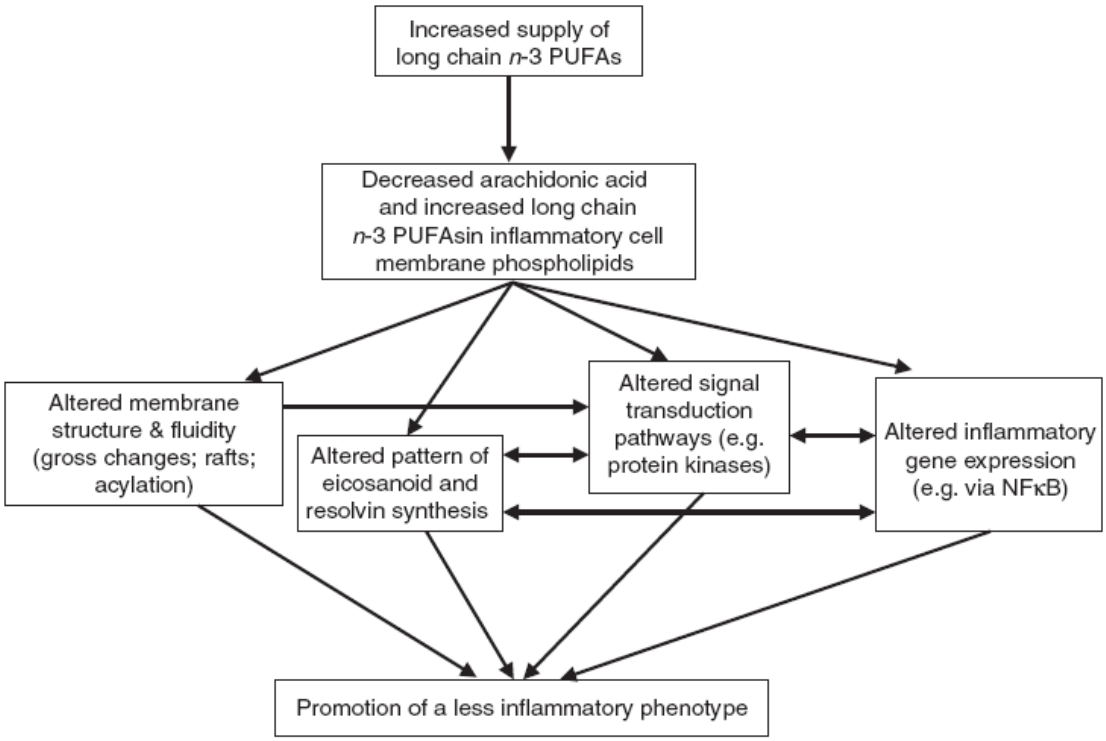


Figure 1.13: Representation of the cellular mechanisms by which long chain n-3 PUFAs result in decreased inflammation (48)

Effects of LC PUFA intake in pregnancy

Prescott (112) has described that newborns who subsequently develop allergic diseases have a number of changes in immune function at birth such as allergen-specific immune responses. Jones *et al.* have noted that neonates at high risk of atopy already had differences in vitro T-cell responses at birth (83). Evidence coming from several studies suggests that there are differences in LC PUFA profiles in neonates at high risk of allergic disease compared with low risk newborns, although the findings are not consistent (129;130). Despite speculation about biochemical alterations or metabolic abnormalities which could contribute to the development of atopy, specific relationships have not been confirmed.

1.4.5 Potential pathways during early immune programming

Although there is debate about when immune programming first occurs, it is clear that this occurs in the neonatal period, if not before. For this reason, the capacity for LC PUFAs to influence immune function during this period is of central interest. A complex series of events occur during the programming of antigen-specific immune responses (as illustrated in Figure 1.14). This typically begins in local tissues where antigen (allergen) proteins are first encountered by surveying antigen presenting cells (APCs), such as tissue dendritic cells (DCs), which ingest the proteins and digest them into small peptides. These are expressed on the cell surface with MHC class II receptors to effector T cells, after migration to the regional lymph nodes. The local tissue conditions play an important part in determining the maturation and activity of APCs. This in turn has a significant effect on down-stream T cell programming (below). Local inflammation has a direct effect on increasing the expression of MHC class II, cytokines (such as IL-12) and co-stimulatory molecules on APCs, which all act to promote an effector response. In contrast, the absence of inflammation is more likely to lead to T cell apoptosis, anergy and tolerance. Once activated, the pattern of APC cytokine production also determines the pattern of T helper cell differentiation. Th1 cells develop under the influence of IL-12, whereas Th2 cytokine responses develop in the relative absence of IL-12 (or the presence of pro-Th2 factors such as PGE_2). These differences in T cell cytokine production determine the pattern of B-cell antibody production, with Th2 cytokines (IL-4, IL-5 and IL-13) promoting IgE production and allergic inflammation while Th1 cytokines (IFNγ) largely inhibit this in favour of low level IgG production (Figure 1.14). Tissue factors are again important in determining

whether these immune responses result in clinically relevant disease (such as asthma, allergic rhinitis or atopic dermatitis) although this is still not well understood. More recently there has been growing recognition of the role of specialized ‘‘regulatory’’ cells in immune programming and subsequent immune regulation. This encompasses a broad range of cells that regulate effector T cell responses through direct cell contact and/or production of regulatory cytokines such as IL-10 and TGF-β (Figure 1.14). Variations in LC PUFA composition of neonatal cell membranes have the potential to influence immune programming at many levels during this complex process (131).

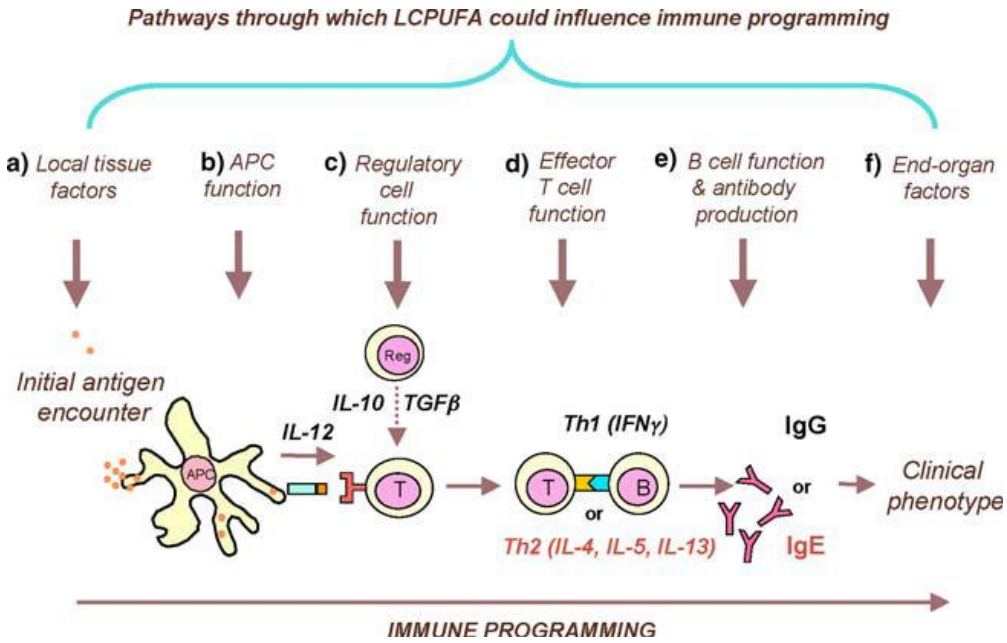


Figure 1.14: Potential pathways of influence of long-chain (LC) PUFA on immune development (131)

1.4.5.1 Effects on Local Tissue Inflammation in Neonates

Higher levels of the LC n-6 PUFA ARA lead to preferential production of the pro-inflammatory 2-series PGs (such as PGE_2) and the 4-series LTs (LTB_4). In contrast eicosanoids (PGE_3 and LTB_5) produced from the LC n-3 PUFA EPA are less potent. In the pregnancy intervention trial conducted in Australia by Dunstan *et al*. (132;133) neutrophils isolated from umbilical cord blood were used to test the hypothesis that maternal fish oil supplementation would alter the pattern of neonatal LTB_4 and LTB_5 production. Neutrophil samples were available from 30 neonates whose mothers had received a daily dose of 4 g of fish oil (containing 3.7 g LC n-3 PUFAs as 56% DHA and 27% EPA) and 34 neonates whose mothers received the same amount of olive oil for the last 20 weeks of pregnancy. Following stimulation with calcium ionophore, production of pro-inflammatory LTB_4 was significantly lower in the fish oil group ($p=0.03$). This confirms that the maternal diet can have effects on the propensity of neonatal neutrophils to generate inflammatory products. Secondly, they examined the effects of maternal fish oil supplementation on neonatal oxidative stress. Excessive oxidative stress may play a role in inflammation and tissue damage. It has been demonstrated that changes in oxidative status can influence APCs during T cell programming by altering cytokine production. Improved oxidative balance can promote pro-Th1 signalling by APCs, by enhancing IL-12 (134) (135). In contrast, adverse oxidative balance (oxidative stress and reduced glutathione) promotes $CD4^+$ T cell Th2 differentiation (134) and the production of IL-4 and IL-5 which contribute to allergic inflammation. So, they assessed the effects of fish oil supplementation on oxidative stress, using plasma F2-isoprostanes as a measure of lipid peroxidation. Maternal fish oil supplementation resulted in significantly lowered plasma ($p<0.001$) F2-isoprostanes (136). Of note, there was a significant relationship between F2-isoprostanes and APC (monocyte) MHC Class II (HLA-DR) expression ($p=0.007$), although HLA-DR expression was not different between the groups. This shows that maternal supplementation with fish oil can attenuate neonatal lipid peroxidation, and suggests potential effects on APC function. Thus, neonatal LC PUFA status has the potential to influence both the oxidative balance and the production of inflammatory mediators in local tissues.

1.4.5.2 Effects on neonatal antigen presenting cells

APC function is impaired in the neonatal period (137;138), and neonatal APCs appear to lack the capacity to deliver important Th1 polarising signals to T-cells (138). This may

contribute to an increased susceptibility to infection and tolerance induction, and a predisposition to Th2 responses in early life. There are differences in APC function between atopic and non-atopic adults (139). However, it is not clear whether altered APC function preceeds sensitisation and the onset of allergic disease, or whether functional differences arise secondary to allergic inflammation. This can only be addressed by examining APC function in early life, prior to allergic sensitisation. Increasing the content of LC n-3 PUFAs in APC cell membranes reduces the capacity of APCs to present antigen to T cells by inhibiting the upregulation of MHC class II receptors, cytokine production and expression of co-stimulatory molecules (140). A study in mice showed that DHA inhibited the IFN-γ-induced MHC class II expression on neonatal APCs (141).

1.4.5.3 Effects on Regulatory Pathways

This is another potential pathway through which LC PUFAs could mediate effects on immune function. Because the role of specialised Treg cells has only been recognised recently there are still no studies examining the effects of PUFAs on these pathways. However, there are a number of potential pathways through which PUFAs could exert an immune effect through actions on these cells. Firstly, studies have shown that PGE_2 induces Treg cell function (and FOXP3 gene expression) (142) and so it is possible that LC n-3 PUFAs could work by altering PGE_2 associated pathways. However, if this is the case it might be expected that n-3 PUFAs (which inhibit PGE_2 production) may also inhibit Treg function, which would not explain the immunomodulatory effects of these fatty acids. Alternatively, PUFAs could have effects through microbial recognition molecules (Toll-like receptors (TLRs)) that are found on both Treg cells and APCs, and are important for activation of these cells. One study (140) has shown that the immunomodulatory actions of LC n-3 PUFAs are mediated through TLR pathways on APCs. It is possible, but not yet explored if these effects are also relevant on other TLR expressing cells, such as Treg cells.

1.4.5.4 Effects on Neonatal Effector T Cell Function

Effects of modifying PUFA composition on T cell function have been extensively studied in adults. Many studies have demonstrated that dietary LC n-3 PUFAs reduce T lymphocyte proliferation to mitogens in both humans (116;118) and rodents (143). In vitro studies in which PBMCs were incubated with fatty acids, have also generally shown a decrease in T cell responses to mitogens (144). There are only two studies to examine this phenomenon in human neonates: the Australian fish oil supplementation study by Dunstan *et al.* (35;132) and an observational study which examined the relationship between cord blood fatty acid levels and immune function in a Boston cohort by Gold *et al*. (145).

In the Dunstan *et al*. study, they looked at (a) the effects of maternal fish oil supplementation on T cell cytokine levels in cord blood, (b) the effects on in vitro T cell responses to specific stimuli, and (c) the relationship between fatty acid levels and T cell cytokine responses (in vivo and in vitro). Firstly, there were significantly (65%) lower circulating Th2 IL-13 levels in the fish oil group (35). There were no differences in the levels of other cytokines (including Th1 IFN-γ which was present only at very low levels). Secondly, there was a consistent trend for all cytokine responses studied (IL-5, IL-13, IL-10, IFN-γ) to be lower in the fish oil group (although this was statistically significant only for IL-10 responses to cat allergen) (35). Thirdly, there were statistically significant inverse relationships between the the n-3 PUFA content of neonatal erythrocytes and cytokine responses (132). The Boston observation study examined the relationship between LC n-3 PUFA levels in cell membranes and in vitro immune responses. Consistent with the Dunstan *et al*. findings, they found that EPA levels were associated with reduced lymphocyte proliferation and reduced IFN-γ production (145) They also noted that LA levels were associated with higher Th2 IL-13 responses (to house dust mite and cockroach). The findings suggest that LC PUFA exposures in pregnancy affect neonatal immune function. The MUFA oleic acid (18:1n-9) is also recognised to have anti-inflammatory properties although these are generally thought to be less potent than those of n-3 PUFAs. In the Australian fish oil supplementation study described above, there was a significant inverse association between oleic acid in neonatal erythrocytes and allergen-specific (IL-5, IL-10, IL-13, IFN-γ) cytokine responses. Mitogen or bacterial LPS stimulated responses were not associated with oleic acid levels. In a previous study in adults, olive oil supplementation had little effect on immune responses (146). However in animals, high-level oleic acid supplementation results in inhibition of lymphocyte

proliferation, IL-2 responses, and NK cell function (147). No previous studies have examined oleic acid and neonatal immune response or allergen specific responses in adults.

1.4.5.5 Effects on Infant B Cell Function and Subsequent IgE Production

There have not been any studies to directly assess the relationship between neonatal PUFA status and B cell function directly. However, there are several logical pathways of influence. Firstly, variations in PGE_2 production could have a direct effect on B cell maturation as PGE_2 is known to synergise with IL-4 to promote IgE switching in B cells. Secondly, the documented effects on neonatal T cell cytokine production are likely to have secondary effects on B cell function during T cell B cell interaction (Figure 1.14). In the Dunstan *et al*. study, children in the fish oil group were three times less likely to have egg-specific IgE (a positive skin prick test to egg) at 1 year of age (132), although this could result from an effect at one or more steps that ultimately lead to IgE production and response to IgE.

1.4.5.6 Observational Studies

A number of early observational studies suggested a link between cord blood n-6: n-3 fatty acid ratios and allergy risk (129;148). However, these studies were too small (less than 60 subjects) to look at clinical outcomes definitively. They also only examined cord blood PUFAs in relation to family allergy (129) or very early outcomes (149), which are also not definitive. There has been one published study to address this in a large population with long-term follow up (150). This UK-based observational study (n=1238) examined the relationship between cord red blood cell fatty acids and clinical allergy outcomes at 3.5 years of age (150). Although they found that a higher cord red cell n-3:n-6 fatty acid ratio was associated with an increased risk of eczema (p=0.04) and late-onset wheeze (p =0.019), none of these relationships was significant after adjusting for multiple comparisons (150). They concluded that PUFAs are unlikely to be a major determinant of allergy and asthma risk.

1.4.6 Relationship between early long-chain n-3 PUFA status and subsequent atopic disease - conclusion

It is now recognized that sensitization to allergens occurs early in life and it is considered that PUFAs might affect aspects of sensitization. Thus, the LC n-3 PUFA status of the mother and the newborn may be very important in determining predisposition to atopy and studies addressing this question need to be performed in pregnant women. There is limited

data on the relationship between neonatal LC n-3 PUFA status and subsequent risk of allergic disease. Existing data derived from both observational and intervention studies are limited (largely because of small sample sizes) and not definitive at this stage. In the study described in this thesis, the effect of early consumption of oily fish as a source of LC n-3 PUFAs on factors related to offspring allergic disease will be investigated. Thus, the next two sections will review the available literature based on epidemiological studies and intervention trials that relate n-3 fatty acids during pregnancy and infancy with atopic outcomes in infancy and early childhood.

1.5 Review of epidemiological and supplementation studies

In section 1.4 the potential for PUFAs, especially LC n-3 PUFAs to influence aspects of immune function related to allergic sensitisation was described. This understanding has lead to epidemiological studies looking at the association between early fish exposure, as a source of long-chain n-3 PUFAs, and later atopy outcomes and to intervention studies with fish oil again looking at later atopy outcomes. This section reviews the published studies on the effects of fish intake and fish oil supplementation during pregnancy and lactation on immune and atopy outcomes on the offspring of those mothers, as well as the effects of fish intake and fish oil supplementation during infancy and childhood on immune and atopy outcomes on those infants/children.

1.5.1 Epidemiological studies

The aim of this section is to review the epidemiological evidence available on the association of maternal fish intake during pregnancy/lactation with atopic/allergic outcomes in the offspring of those mothers, as well as the association of fish intake during infancy/childhood with atopic/allergic outcomes in those infants/children.

1.5.1.1 Studies investigating the association of maternal fish intake during perinatal life with atopic/allergic outcomes in infants or children of those pregnancies

Table 1.7 includes all studies published to the time of writing that investigate the association between maternal fish intake in perinatal life and atopic/allergic outcomes in the offspring of those mothers. The studies were identified through Ovid Medline (1950-2008) and Embase (1980-2008) databases performing and combining searches with the following keywords: fish intake, oily fish intake, maternal, pregnancy, atopy, allergy, asthma, eczema, childhood, infancy. Five studies were identified investigating maternal fish intake during pregnancy and one study investigating maternal fish intake during lactation in relation to atopic outcomes in infants or children.

1.5.1.2 Studies investigating the association of maternal fish intake during pregnancy with atopic/allergic outcomes in infants or children of those pregnancies

Study design

The studies included in Table 1.7 had different study designs; three of them are prospective longitudinal cohort studies (151-153), one is retrospective cohort study (154) and one is nested case control (155). The prospective study design is superior to a retrospective or a cross sectional/case-control design for studying cause-effect relations. In general, all the studies were well powered, with the largest being the two prospective birth cohort studies conducted by Sausenthaler *et al*. (152) and Willers *et al*. (153). The age range of the offspring that were followed up or participated in the cohort studies varied from 2 to 6.5 years. In Table 1.7, only the statistically significant results which relate maternal fish intake with atopic outcomes on the infants/children of those mothers are presented.

Exposure measures

Regarding the quality and the method of assessing fish intake during pregnancy, three of the five studies used a food frequency questionnaire (FFQ) (151;154;155) and two used a semi-quantitative FFQ (152;153). FFQs varied in frequency categories and also in time point of administration [during pregnancy (153), shortly after birth (151;152) or retrospectively a long time after birth (154;155)] and way of administration [self-administered (153) or interviewer-administered (151;152;155)].

Two of the studies examined only the effect of total fish consumption during pregnancy on childhood/infancy atopic outcomes (152;154;155). Three studies included different fish categories in their questionnaires (151;153;155), and two of them found statistically significant results for oily fish (153;155). It has to be pointed out that although Romieu *et al.* (151) found a statistically significant lower allergy risk of total fish intake, they did not assess the impact of different types of fish because of the small number of participants reporting intake of oily fish only. Analysing different types of fish and consumption frequencies could possibly explain the heterogeneity in the extent of the suggested protective effect between the studies. For example, Salam *et al.* (155) observed statistically significant results when comparing the effects of ‘monthly intake’ versus ‘never’ whereas the other four studies (151-154) observed statistically significant results when comparing the effects of weekly consumption frequencies. Also, the latter four studies reported results only on total fish consumption, with the exception of Willers *et al.* (153) who reported the effect of oily fish consumption.

The smallest decreases in childhood/infancy atopic risk were found in those studies comparing the effect of two different weekly consumption frequencies of total fish (151;152). On the contrary, studies observing a greater decrease in risk made comparisons between the effect of weekly versus 'less than monthly' or 'never' consumption frequencies (153;154).

Moreover, in some of the studies oily fish were better defined that in other studies. For example, Salam *et al.* (155) defined oily fish as those with > 2% fat specifying which types of fish are included in this category. Other studies which gave a definition of oily fish were those of Romieu *et al.* (151) and Willers *et al.* (153). A major issue in epidemiological studies is misclassification of dietary exposure and this should always be taken into account when interpreting data.

Using a FFQ is an effective method of assessing total or oily fish intake in the diet, although it is not always possible to have a complete picture of the rest of the diet (depending on the food items included in the questionnaire) in order to identify other food categories/items related to the outcome measure or that could be possible confounding factors. A FFQ reflecting total and oily fish intake during the second and third trimester of pregnancy and which is not administered retrospectively (in order to reduce bias resulting from poor memory) nor is self-administered would be ideal. Also, apart from including fish categories it is important to include other major food categories so as to enable adjustment for other components of the diet, such as antioxidants, total energy intake and dietary fat composition.

Confounding factors

Romieu *et al.* (151) included type of fish as a confounding factor and Sausenthaler *et al.* (152) adjusted for all dietary variables included in their FFQ. Three of the studies controlled for breastfeeding (152;153;155). All five studies controlled for maternal atopy/asthma and all, apart from Salam *et al.* (155), controlled for maternal smoking. It is worth mentioning that fish consumption might be a marker of a specific lifestyle, so it is important to adjust for socio-economic factors such as social class and parental education, as well as smoking. Each of the studies in Table 1.7 adjusted for most of these confounding factors.

Outcome measures

Referring to the outcome measures and their assessment, there seems to be a great heterogeneity between the five studies. Salam *et al.* (155) focused on asthma, Calvani *et al.* (154) focused on allergic sensitisation, while Sausenthaler *et al.* (152), Romieu *et al.* (151) and Willers *et al.* (153) included various outcome measures such as eczema, atopic wheeze allergic sensitisation, or hay-fever. Also, all of the studies assessing clinical outcomes used a parental questionnaire which most of the time asked for doctor-diagnosis and gave clear definitions of each outcome. Although doctor-diagnosed atopic diseases may be more valid, limited access to health care may result in under-diagnosis of atopic disease especially when these are in mild or primary stages (155).

Results

There is consistency between the findings of the five studies of maternal diet since all of them identified beneficial associations between maternal fish intake during pregnancy and atopic or allergic outcomes in children. However, in the study of Salam *et al.* (155) the association between maternal oily fish intake and children's risk of developing asthma was greater in children whose mothers were asthmatic compared to children of non-asthmatic mothers (p-interaction = 0.02). Also, for children born to mothers with asthma, the allergy risk reduction after oily fish intake was greater for early persistent and late-onset asthma (p-trend = 0.06 and 0.01, respectively). On the contrary, Calvani *et al.* (154) observed stronger, and with higher significance, beneficial associations of increased oily fish intake during pregnancy for children of non-allergic mothers compared to those of allergic mothers. It is not clear why these findings are different.

Moreover, in the prospective cohort study conducted by Romieu *et al.* (151), although a beneficial association was observed initially for the whole population, after stratifying by breast-feeding, the risk of persistent wheeze at 6 years of age decreased among the non-breastfed infants whereas no decreased risk was observed among the breastfed infants.

The large cohort studies conducted by Sausenthaler *et al.* (152) and Willers *et al.* (153) concluded similar associations. High (≥ 1 time/week) vs. low maternal fish intake during pregnancy was associated with decreased doctor-diagnosed eczema. However the decrease in the study of Willers *et al.* (153) was greater than that in the study of Sausenthaler *et al.* (152) (43% vs. 25% respectively). This might be related to the fact that the study of Willers *et al.* (153) followed-up children for a longer period, allowing for signs of atopy to

develop and enabling doctor diagnosis. Willers *et al.* (153) also showed that there was a 72% decrease in doctor-diagnosed hay-fever in children born to mothers with higher oily fish intake during pregnancy, an association which was not significant for total fish intake.

In summary

Fish consumption in pregnancy was associated with decreased risk of atopy and its manifestations in the infants born of those pregnancies; this was seen in all five studies. However, the extent of the reduction of atopy risk was highly heterogeneous. The decrease in childhood or infant atopy ranged between 25-95% in relation to fish intake. However, most decreases in atopic risk ranged between 40-80%. Although these five studies provide strong evidence of a beneficial effect of maternal fish intake during pregnancy, they are characterised by inconsistency in the extent of the decrease in atopy risk observed, which may be due to differences in their study design, such as differences in: confounding factors controlled for, statistical analysis, definition of atopic outcome in infants/children and their mothers, method of assessing atopy, method of collecting dietary information, oily and/or total fish definition as well as categories of consumption frequencies used to make comparisons.

1.5.1.3 Studies investigating the association of maternal fish intake during lactation with atopic outcomes in the offspring of those pregnancies

The last study of Table 1.7 conducted by Hoppu *et al.* (156) assessed maternal dietary intake one month after birth (during lactation). This was not the primary exposure measure of this study, which was set up to examine the effect of breast milk fatty acid composition on atopic dermatitis during the first year of life. Although a high percentage of EPA in breast milk was related to lower risk of atopic dermatitis, fish consumption frequency during lactation was not associated with breast milk EPA content. This may be attributed to the fact that breast milk fatty acid composition is determined by fatty acids accumulated in the maternal adipose tissue during pregnancy [(157); (158) cited in (156)]. Maternal fish intake during pregnancy in relation to breast milk composition would have been more appropriate to investigate (156).

Conclusion

Epidemiological studies investigating the effect of maternal fish intake during pregnancy have differences in exposure and outcome measure definition and assessment. However,

all of them identified beneficial associations between maternal fish intake during pregnancy and atopic or allergic outcomes in infants/children of those pregnancies. The extent of lowering the atopy risk ranged between 25-95%. The one study looking at the effects of maternal fish intake during lactation did not observe any significant associations, suggesting that altered exposure during pregnancy is more likely to alter atopic outcomes in the offspring, as this is more likely to determine fatty acid composition of breast milk and thus provision of n-3 PUFAs to the infant.

Table 1.7 Maternal fish intake during perinatal life and atopic/allergic outcomes in infants and children of those mothers

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Salam *et al* 2005 (155)	279 asthmatic and 412 non-asthmatic children (4^{th}, 7^{th}, 10^{th} grade) and their mothers/ guardians Nested case-control study in the Children's Health Study Public schools in southern California, USA	Maternal fish consumption during pregnancy-retrospective assessment (1999-2001) oily fish (> 2% fat) non-oily fish (≤ 2% fat) fish fingers ('fish sticks') canned fish Frequency questionnaire-telephone interviews never rarely at least 1/month	Parental report of physician-diagnosed by age 5 years: any asthma, early transient asthma, early persistent asthma, late-onset asthma (1993-1995) Confounding factors: maternal asthma, race/ethnicity, maternal age, maternal education, gestational age, number of siblings, exclusive breast feeding for four months, other fish categories	• Asthmatic mothers: at least monthly oily fish consumption during pregnancy vs. never decreased risk of any asthma in children (OR 0.20; 95% CI 0.06-0.65, p-trend 0.006). • Asthmatic and non-asthmatic mothers together: maternal oily fish consumption at least monthly was protective for early persistent asthma in children (OR 0.45; 95% CI 0.23-0.91, p-trend 0.04) Fish fingers consumption increased risk of any asthma (OR 2.04; 95% CI 1.18-3.51, p-trend 0.01) • Children of non-asthmatic mothers were not benefited from maternal oily fish consumption during pregnancy. No associations were found for the rest of the fish categories
Calvani *et al* 2006 (154)	295 allergic and 693 non-allergic mothers and their children (median age 5 years, range 17 years) Retrospective cohort General Hospitals in Rome, Italy	Maternal intake of fish, butter, margarine during pregnancy Retrospectively assessed by parental report via standardised questionnaire 1 time/month or less 1 time/week 2-3 times/week or more	Atopy in children: SPT to inhalant and food allergens (at hospital) Confounding factors: age, gender, oculorhinitis, eczema, age of gestation, maternal smoking, paternal atopy, maternal occupation, butter and margarine intake	• Non-allergic mother group: -There was a reduction in risk of food sensitisations in offspring to mothers with fish intake during pregnancy '1 time/week' (OR 0.22; 95% CI 0.08-0.55, p-trend 0.002) and '2-3 times/week or more'(OR 0.23; 95% CI: 0.08-0.69, p-trend 0.002) compared to 1 time/month or less -Intake of fish '1 time/week' and '2-3 times/week or more' reduced milk sensitisation (OR 0.15; 95% CI 0.04-0.59 and OR 0.05; 95% CI 0.00-0.54 respectively) and egg sensitisation (OR 0.26; 95% CI 0.09-0.76 and OR 0.33; 95% CI 0.10-1.07 respectively) -No associations with inhalant sensitisation • Allergic mother group: no associations between maternal intake of fish during pregnancy and children food or inhalant sensitisation • Whole study population (adjusted also for maternal atopy): -Trend between increased consumption of fish and decreased prevalence of positive SPT for foods (p-trend= 0.008) -Fish intake '1 time/week' vs ≤1 time/month decreased risk of food sensitisation (OR 0.34; 95% CI 0.15-0.75, p= 0.007)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Romieu *et al* 2007 (151)	462 pregnant women and their offspring (follow up at age 1 and 6 years) Prospective cohort Antenatal Clinics in Manorca, Spain	Maternal dietary intake during pregnancy including fish total fish oily fish non-oily fish Interviewer administered FFQ; 3 months after delivery weekly intake	Parental report: doctor-diagnosed eczema (at age 1 year), atopic wheeze and persistent wheeze (at age 6 year) SPT (at age 6 years) IgE (at age 4 years) Confounding factors: maternal asthma, type of fish, smoking during pregnancy, maternal atopy, gender, maternal social class, gestational age	For an increase in maternal fish intake during pregnancy from once per week to 2.5 times per week the risk of having the followings decreased: eczema at age 1 year (OR 0.73; 95% CI 0.55-0.98, p= 0.036) , positive SPT for HDM at 6 years (OR 0.68; 95% CI 0.46-1.01, p= 0.058) and atopic wheeze at age 6 years (OR 0.55; 95% CI 0.31-0.96, p= 0.034) Weekly maternal fish intake during pregnancy was lower for infants with: eczema at 1 year (p= 0.050), positive SPT for HDM at 6 yr (p= 0.048), atopic wheeze at 6 years (p= 0.028) Stratification by breastfeeding: for an increase in fish intake during pregnancy from 1 time/week to 2.5 times/week the risk of persistent wheeze at 6 years decreased by 90% (OR 0.10; 95% CI 0.02-0.69, p<0.05) only among the non-breastfed infants.
Sausenthaler *et al* 2007 (152)	2641 mothers and their infants followed up to age 2 years Prospective cohort in the Influences of Lifestyle Factors on the Immune System and the Development of Allergies in Childhood Study (LISA) Newborns from 4 German cities	Maternal diet during the last 4 weeks of pregnancy including fish Semi-quantitative FFQ administered shortly after delivery high (1-2 times/week) low (<1 time/week)	Parental report of lifetime doctor-diagnosed eczema at age 2 years Allergic sensitisation (IgE) Confounding factors: study area, sex, maternal age at delivery, smoking during 2nd or 3rd trimester of pregnancy, parental education, exclusive breastfeeding for ≥4 months, family history of atopy, season of birth, all dietary variables from FFQ	High maternal fish intake vs low was associated with decreased doctor-diagnosed eczema risk in children at 2 years (OR 0.75; 95% CI 0.57-0.98, p<0.05)
Willers *et al* 2007 (153)	1212 pregnant women and their children followed up to age 5 years Prospective cohort Aberdeen Maternity Hospital, Scotland	Maternal diet during pregnancy including fish; reflecting intake 2-3 months prior to 32 weeks gestation total fish oily fish Semi-quantitative FFQ: Version 5.4 of the Scottish Collaborative Group FFQ; sent by post and self-administered at 32 weeks gestation never 1/week ≥1/week <1/week	Parental report of asthma, atopic eczema, wheezing, hay-fever in children at 5 years (ISAAC core questions) Spirometry and SPT (only on small number of children) Confounding factors: maternal age of leaving full-time education, paternal social class, maternal age, maternal smoking during pregnancy, smoking in the home during childhood, energy intake, maternal asthma, maternal atopy, birth weight, presence of older siblings, child's gender, breastfeeding.	Beneficial associations (p-trend): Maternal total fish intake ≥1/week vs never decreased risk of: doctor-diagnosed eczema (OR 0.57; 95% CI 0.35-0.92, p-trend= 0.008), current treated eczema (OR 0.58; 95% CI 0.32-1.06, p-trend= 0.028), and ever having eczema (OR 0.68; 95% CI 0.43-1.10, p-trend= 0.050) Maternal oily fish intake ≥1/week vs never decreased risk of doctor-diagnosed hay-fever (OR 0.28; 95% CI 0.06-1.19, p-trend= 0.043)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Hoppu *et al* 2005 (156)	34 atopic mothers and their infants followed up to age 1 year Finland	Breast milk fatty acids; samples taken 1 month postpartum when infants were exclusively or predominantly breastfed Maternal atopic disease (asthma, allergic rhinitis, atopic dermatitis) assessed with questionnaire and SPT; 35-36 weeks of gestation Maternal dietary intake (4-consequative-day food records) and questionnaire on maternal dietary habits including fish consumption frequency; 1 month postpartum reflecting intake during lactation <1 time/week 1 time/week > 1 time/week	Clinical examination at ages 1, 3, 6, 12 months and SPT at 12 months Atopic dermatitis during the 1st year of life (Hanifin criteria)	Maternal frequency of fish consumption during pregnancy was not related to breast milk EPA content. The ratio SFA/PUFA was higher in breast milk consumed by infants developing atopic dermatitis compared to those remaining healthy (4.3 vs 3.1; p=0.05) Total n-3 PUFA (% of total fatty acids) was lower in the breast milk of mothers whose infants developed atopic dermatitis than of those whose infants remained healthy (1.61% vs 2.17%; p=0.05) EPA (% of total fatty acids) was lower in breast milk consumed by infants who developed atopic dermatitis during the 1st yr of life compared to those who did not (0.10 vs 0.15; p=0.02)

OR, odds ratio; CI, confidence interval; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; HDM, house dust mite; FFQ, food frequency questionnaire; ISAAC; International Study of Asthma and Allergies in Childhood; SPT, skin prick testing; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; Ig, immunoglobulin; vs, versus.

1.5.1.4 Studies investigating the association of fish intake during infancy or childhood with atopic/allergic outcomes in those infants/children

Table 1.8 summarises the characteristics and results of published observational studies that investigate the association between fish intake during infancy or childhood and atopic or allergic outcomes in those infants or children. Studies were identified through Ovid Medline (1950-2008) and Embase (1980-2008) databases performing and combining searches with the following keywords: fish intake, oily fish intake, childhood, infancy, atopy, allergy, asthma, eczema. Fourteen epidemiological studies were identified. The evidence provided by these studies is less consistent and there is a greater heterogeneity in study designs, exposure, outcome measures and results compared to the epidemiological studies of Table 1.7.

A total of 14 studies were identified. Eight studies observed a beneficial association between fish intake during childhood/infancy and atopic outcomes in those children/infants (159-166). Three of the studies observed an adverse effect of fish intake on childhood atopy (167-169), and three studies observed no associations (170-172). All studies were well powered, with large enough sample sizes, although was a wide range of sample size amongst the studies.

The studies of Table 1.8 will be discussed according to whether they observed a protective, adverse or a null effect.

Table 1.8: Fish intake during infancy/ childhood and atopic or allergic outcomes in those infants/ children

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Hodge *et al* 1996 (163)	468 children aged 8-11 years Case-control Sydney, Australia	Dietary intake of children including fish (reflecting intake over the last year) total fish (all categories) fish fingers canned fish total fresh fish fresh oily fish (>2% fat) fresh non-oily fish (≤2% fat) FFQ completed by parents (Commonwealth Scientific and industrial Research Organisation) Yes No	Assessed 6 months before dietary information was collected: AHR (by exercise), SPT, recent wheeze (parental questionnaire) Current asthma: presence of both recent wheeze and AHR Confounding factors: Sex, ethnicity, country of birth, atopy, respiratory infection in the first 2 years of life, parental smoking or asthma history	Children who ate any fresh (OR 0.52; 95% CI 0.24-1.15) or oily fresh fish vs none had reduced risk of current asthma (OR 0.26; 95% CI 0.09-0.72, p< 0.01)
Hijazi *et al* 2000 (171)	114 asthmatic and 202 non-asthmatic children; aged 12 years (from a population of 1444 Saudi-Arabian children) Case-Control Saudi Arabia (rural and urban areas)	Dietary intake including fish; at age 12 years -Maternal completion of semi-quantitative FFQ -Questionnaire on food types and dietary habits often sometimes rarely never	Asthma ever, wheeze in the last 12 months (ISAAC questions answered by the children) SPT Cases: both asthma and wheeze Confounding factors: social class, place of residence, nationality, sex, maternal education, family history of asthma or allergy, positive SPT	Univariate analysis: The frequency of eating fish was not significantly related to ever having asthma or wheezing in the last 12 months There was no association between PUFA intake and asthma or wheeze

Dunder *et al* 2001 (162)	1. Retrospective case-control nested in the 9-year follow-up (1980-1989): 60 atopic and 1293 non-atopic children aged 3, 6, 9, 12, 15, 18 years. 2. Case-control: 231 atopic and 231 non-atopic children (1980); mean age 10.3 years 154 atopic and 154 non-atopic children (1986) (pairs matched for age, sex and place of residence) Finland (rural and urban areas)	Dietary intake including fish intake (standardised to energy intake); in 1980 and 1986 48-h recall administered by nutritionists gr/1000 Kcal energy Serum fatty acids	Physician diagnosed: allergic rhinitis, allergic dermatitis, asthma (parental questionnaires in 1980, 1986, 1989) Atopic disease: one or more of the above diseases Confounding factors: age, sex, region, maternal education	• Follow-up: children who developed atopic diseases in 1989 had consumed less fish in 1980 compared to those who remained healthy (3.2 vs 6.6 g/1000 Kcal; p< 0.001) • Cross-sectional data in 1980: fish consumption was not associated with atopic disease, atopic dermatitis, allergic rhinitis, or asthma • Serum EPA and DHA were lower in children with atopic dermatitis in 1980 (1.11 vs 1.22; p=0.01 and 0.64 vs 0.69; p=0.01 respectively) and 1986 (0.91 vs 1.02; p=0.02 and 0.55 vs 0.61; p=0.01 respectively)
Huang *et al* 2001 (168)	1166 adolescents aged 13-17 years Cross-sectional The 1st Nutrition and Health Survey in Taiwan	Dietary intake including fish during the past month total fish oily fish shellfish other seafood 24-h recall and FFQ; administered to children Quartiles of intake	Physician-diagnosed: allergic rhinitis, asthma; (questionnaire to children) Confounding factors: levels of urbanisation	• Univariate analysis: Higher intake of oily fish (1st vs 4th quartile of intake) was associated with higher prevalence (1.5% vs 4.9%) of doctor diagnosed asthma (p=0.01) • Multivariate analysis: total and oily fish intake were not associated with asthma or allergic rhinitis • There was no association between any fish category intake and allergic rhinitis • No associations found for shellfish and other seafood and outcome measures
Takemura *et al* 2002 (169)	Children aged 6-15 years: 1673 currently asthmatic and 22109 non-asthmatic Cross-sectional The Tokorozawa Childhood Asthma and Pollinosis Study, Japan	Questions on consumption frequency of foods, including fish almost none 1-2 times/month 1-2 times/week ≥3-4 times/week Parental FFQ (Japanese Ministry of Health and Welfare)	Current asthma (parental questionnaire of the American Thoracic Society and Division of Lung Diseases adopted by the Japan Environment Agency) Current asthma: doctor-diagnosed asthma with symptoms and treatment during the past 2 years Confounding factors: age, gender, parental history of asthma, vegetable and fruit intake	Higher prevalence of asthma among subjects who ate fish 1-2 times/wk compared to those who ate fish 1-2 times/month (OR 1.117; 95% CI 1.005-1.241, p= 0.041). The risk of current asthma increased gradually with increasing frequency of fish intake (p-trend= 0.0349)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Antova *et al* 2003 (167)	20271 children aged 7-11 years Cross-sectional 25 areas in 6 Central and Eastern European Countries (Bulgaria, Czech Republic, Hungary, Poland, Romania, Slovakia)	Dietary intake of fish, fresh fruit and fresh vegetable (parental FFQ; redesigned Adult British Survey FFQ) <1/month ≥1/month	Winter cough , persistent cough, wheeze ever, current wheeze (parental questionnaires, ISAAC) Confounding factors: age, sex, area, pets, indoor moisture, gas oven for heating, additional gas heating, passive smoking, maternal education, paternal occupation, parental allergy, respondent, overcrowding, all tested nutritional factors	Low fish intake (<1/month) compared with higher (≥1/month) was associated with increased risk of persistent cough (OR 1.18; 95% CI 1.04-1.34, p=0.01), wheeze ever (OR 1.14; 95% CI 1.03-1.34, p=0.01), and current wheeze (OR 1.21; 95% CI 1.06-1.39, p=0.01)
Farchi *et al* 2003 (170)	4104 children aged 6-7 years, followed-up for one year Prospective cohort Italian Studies on Respiratory Disorders in Children and the Environment (SIDRIA), part of the ISAAC	Dietary intake including fish (at 1 year follow-up) pasta with fish: tuna, mackerel, sardines, salmon, anchovies oily ('blue') fish: tuna, mackerel, sardines, salmon, anchovies Parental semi-quantitative FFQ never <1 time/week 1-2 times/week ≥3 times/week	12 month occurrence of: wheeze, shortness of breath with wheeze, allergic rhinitis symptoms (parental completion; ISAAC questions at baseline and questionnaire at 1 year follow-up) Confounding factors: sex, study area, paternal education, household crowding, maternal or paternal smoking, dampness or mould, parental asthma	In the univariate analysis: neither oily fish nor 'pasta with fish' were associated with 12 month occurrence of wheeze, shortness of breath with wheeze or allergic rhinitis (p-trend not significant)

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Nafstad *et al* 2003 (166)	2531 infants followed up to age 4 years Prospective cohort Oslo Birth Cohort Study, Norway	Introduction of various kinds of food including fish into diet during the year 1 of life Parental questionnaires at age 1 year (no quantitative information) Yes (and month of introduction) No	Doctor-diagnosed current asthma and allergic rhinitis (parental questionnaire administered at 4 years) Confounding factors: parental atopy, atopic eczema at 0-6 months of age, gender, parity, birth weight, maternal age at delivery, birth order, uterus-related pregnancy complications, keeping pets at home when child was born, episode of lower respiratory tract infections during year 1 of life, maternal education, family income per year, maternal smoking at the end of pregnancy, length of breastfeeding.	The risk of allergic rhinitis was lower in children who had fish during the first year of life compared to children who had fish later in life (OR 0.45; 95% CI 0.28-0.74) Among children who were breastfed for >6 months, those who had fish during year 1 of age had lower asthma risk (OR 0.56; 95% CI 0.36-0.87) and allergic rhinitis risk (OR 0.28; 95% CI 0.15-0.52) compared to those who had none (respective risks 0.073 and 0.058). Among children without parental hay fever or asthma, those who had fish during year 1 of age had lower asthma risk (OR 0.50; 95% CI 0.30-0.83) and allergic rhinitis risk (OR 0.47; 95% CI 0.25-0.86) compared to those who had none Among children with early life atopic eczema, there was a decreased risk of asthma (OR 0.47; 95% CI 0.23-0.97) and allergic rhinitis (OR 0.32; 95% CI 0.15-0.69) with any fish consumption during year 1 Among children without an episode of lower respiratory tract infection during the first year of life, those who had fish during year 1 of age had lower allergic rhinitis risk (OR 0.39; 95% CI 0.24-0.66) compared to those who had none The risk of having doctor-diagnosed atopic eczema with symptoms during the 4th year of age was reduced in children who consumed fish during year 1 of life compared to those who did not (OR 0.66; 95% CI 0.52-0.84, p=0.001)
Wijga *et al* 2003 (172)	2978 children, aged 2 years; follow-up to 3 years old Prospective Cohort The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study, Netherlands	Dietary intake including fish during the previous month; at age 2 years Parental FFQ Rarely: <1 time/week Regularly: 1-5 times/week Daily: 6-7 times/week	Doctor-diagnosed ever asthma, doctor-diagnosed recent asthma (last 12 months), recent wheeze (parental questionnaire based on ISAAC); posted at age 3 years Confounding factors: sex, birth weight, presence of older siblings, parental allergy, maternal level of education, breastfeeding for at least 8 weeks, smoking in the home and during pregnancy, region, parental asthma	In the univariate analysis eating fish at least once a week was not associated with ever asthma, recent asthma or recent wheeze

Andreasyan *et al* 2005 (160)	499 children aged 8 years Cross-sectional Childhood Allergy and Respiratory Health Study Tasmania, Australia	Children's total fish intake at age 8 years (parental report) Yes No	SPT, asthma, hay-fever, wheeze, eczema (ISAAC questionnaire to parents) Confounding factors: sheepskin and plastic mattress use during infancy, sex, number of siblings at 8 years, bottle feeding at 1 month, any maternal smoking during pregnancy	Fish intake was associated with decreased risk of ryegrass-pure sensitisation (OR 0.37; 95% CI 0.15-0.90, p= 0.03). Fish intake was associated with decreased risk of asthma linked to ryegrass-pure sensitisation (OR 0.20; 95% CI 0.04-0.90, p= 0.04), and the risk of hey-fever linked to ryegrass-pure sensitisation (OR 0.25; 95% CI 0.08-0.78, p= 0.02). Fish consumption was associated with a greater reduction in risk for ryegrass-pure sensitisation in comparison to the risk reduction for HDM-pure sensitisation (OR 0.20; 95% CI 0.05-0.79)
Kim *et al* 2005 (164)	1014 children aged 5-14 years Cross-sectional All primary schools in Knivsta, Sweden	Dietary intake of specific food categories including fish (parental completion in co-operation with the child) Frequency questionnaire never <1 time/week 1 time/week >1 time/week daily Assessment of allergens in the school environment	Doctor-diagnosed asthma, current asthma (last 12 months), wheeze, daytime breathlessness, night-time breathlessness, self-reported atopic sensitisation to cat, dog, pollen, food Questions obtained from the European Community Respiratory Health Survey; parental completion in co-operation with the child Confounding factors: age, gender, and all other dietary factors	For an increase of fish intake by one frequency category there was a decreased risk of doctor-diagnosed asthma (OR 0.54; 95% CI 0.35-0.84, p<0.01), current asthma (OR 0.51; 95% CI 0.31-0.84, p<0.01), and night-time breathlessness (OR 0.36; 95% CI 0.17-0.78, p<0.05)
Kull *et al* 2006 (165)	3619 infants followed up to age 4 years Prospective cohort Stockholm, Sweden	Infant's consumption frequency and time of introducing fish during the 1st year of life Parental questionnaires (at age 1 year) never 1 time/month 2-3 times/month 1 time/week >1 time/ week Regular fish consumption defined as ≥2-3 times/week	Assessed at age 4 years (occurrence during last 1-2 years): asthma, eczema, allergic rhinitis, persistent allergic disease, sensitisation (IgE) Any allergic disease: at least one of asthma, eczema, allergic rhinitis Multiple allergic diseases: more than two Confounding factors: parental allergic disease, maternal age, maternal smoking, breastfeeding	Dose-dependent reduced risk for asthma (p-trend= 0.03), eczema, allergic rhinitis and sensitisation (p-trend< 0.001) with increased in fish consumption frequency Introducing fish at age 3-8 months reduced risk for asthma (OR 0.73; 95% CI 0.55-0.97), eczema (OR 0.77; 95% CI 0.64-0.92), allergic rhinitis (OR 0.77; 95% CI 0.60-0.97) and sensitisation (OR 0.78; 95% CI 0.64-0.95) compared to introducing fish at or after age 9 months. • Among children without eczema and /or recurrent wheeze during the 1st year of life: fish consumption ≥2 times/month compared to ≤1 time/month during the first year of life was associated with reduced risk of any allergic disease (OR 0.76; 95% CI 0.61-0.94), eczema (OR 0.78; 95% CI 0.60-1.00), rhinitis (OR 0.60; 95% CI 0.43-0.83), sensitisation (OR 0.76; 95% CI 0.57-1.00), persistent eczema (OR 0.48; 95% CI 0.32-0.68), persistent rhinitis (OR 0.43; 95% CI 0.23-0.79) and multiple allergic disease (OR 0.56; 95% CI 0.35-0.89) at 4 yr • Fish consumption ≥2 times/month compared to ≤1 time/month during the first year of life was associated with reduced risk of sensitisation (OR 0.52; 95% CI 0.35-0.76, p< 0.01) only in children without parental allergy

Study	Study population and design	Exposure measures and exposure assessment	Outcome measures and confounding factors	Results
Chatzi *et al* 2007 (161)	460 children aged 6.5 years Cross-sectional Menorca, Spain	Children's diet including fish total fish oily fish non-oily fish fried/ coated fish seafood Parental completion of semi-quantitative FFQ (modified Harvard questionnaire) gr/day	Current wheeze, atopic wheeze (parental completion of questionnaire) SPT (atopy) Confounding factors: gender, maternal and parental asthma, maternal and paternal atopy, maternal smoking, BMI at 6.5 years, maternal and paternal education and social class, breastfeeding, fish intake during pregnancy, number of siblings	There was inverse association between children's total fish intake ≥ 60 gr/day and atopy (OR 0.43; 95% CI 0.21-0.90, p< 0.05) Subgroups of fish were not significantly associated with atopy
Alm *et al.* 2009 (159)	4921 infants aged 1year Prospective Cohort 'Infants of Western Sweden' Western Sweden	Food frequency data collected at 6 and 12 months of age Parental completion Never A few times per year 1-3 times per month 1-3 times per week 3+times per week Lean fish Salmon Flatfish Mackerel or herring	Paternal reported prevalence of eczema and food allergy diagnosed by a physisian at 6 and 12 months of age. Significant risk factors (p<0.01): maternal eczema, a sibling with eczema, a bird in the home, the introduction of fish before 9 months of age and cows' milk allergy. Non significant risk factors (p>0.01): breastfeeding, smoking	In the multivariate analysis: familial occurrence of eczema, especially in siblings (OR 1.87; 95% confidence interval (CI) 1.50 to 2.33) or the mother (OR 1.54; 95% CI 1.30 to 1.84), remained an independent risk factor. Introducing fish before 9 months of age (OR 0.76; 95% CI 0.62 to 0.94) and having a bird in the home (OR 0.35; 95% CI 0.17 to 0.75) were beneficial. At 1 year of age, 20.9% of the infants had previous or current eczema. Median age at onset was 4 months. In the univariate analysis: eating fish never vs 3times/week increases the risk of eczema at 1 year of age (OR=2.73;95%CI 1.80 to 4.13,p<0.001) and never vs usually eating lean fish reduces the risk for eczema at 1 year (OR=0.81;CI 0.68 to 0.97)

OR, odds ratio; CI, confidence interval; PUFA, polyunsaturated fatty acids; HDM, house dust mite; FFQ, food frequency questionnaire; ISAAC; International Study of Asthma and Allergies in Childhood; SPT, skin prick testing; DHA, hocosahexaenoic acid; EPA, eicosapentaenoic acid; Ig, immunoglobulin; AHR, airway hyper-responsiveness; vs, versus.

Studies showing a protective effect of fish

Of the studies that found a beneficial association between fish intake during infancy/childhood and atopic/allergic outcomes, three were prospective cohort (159;165;166), two were case-control (162;163), and three were cross-sectional (160;161;164) studies. Although cross-sectional studies are able to identify associations, they are weak designs in assessing causal associations. In contrast, retrospective case-control studies and prospective cohort studies are stronger in design and better able to identify association.

The age range of children taking part in these studies was between 1-18 years (at the time point that outcomes were measured). Because of the wide age range of the study population differences in the extent of the beneficial effect can be expected. Some of the studies measured exposure and outcome at a much older age (160;162-164) than others (159;161;165;166). The two prospective cohort studies followed-up infants to age 4 years (165;166) whereas the 'Infants of Western Sweden' study followed up infants up to one year.

The reduction in atopy/allergy risk associated with fish intake ranged between 22-80%. However, the risk reduction in most cases was between 50-60%, providing consistent evidence for suggesting a protective effect of fish consumption during childhood/infancy on atopy/allergy.

However, studies were inconsistent as far as exposure assessment is concerned. All three prospective cohort studies (159;165;166) determined the time point of fish introduction during the first year of life using a parental questionnaire. In addition Kull *et al.* (165) collected information on fish consumption frequencies. Alm *et al.* collected food frequency data at two different time points (159). The cross-sectional study of Kim *et al.* (164) used a parental FFQ, that of Chatzi *et al.* (161) used a semi-quantitative FFQ and the other cross-sectional study used parental report of fish consumption without frequencies (160). The retrospective case-control study of Dunder *et al.* (162) used a 48-hour recall of intake and the case-control study of Hodge *et al*. (163) used a parental FFQ (although consumption frequency categories were not used in their analysis).

The ideal method of collecting information on fish consumption would be a FFQ with various food categories/items reflecting intake during the past 12 months, including time of introduction of fish into diet and different types of fish consumed (oily, non-oily). Also,

ideally the questionnaire should be administered to the children in collaboration with their parents and also repeated at different time points in the case of prospective cohort studies which have a long period of follow-up.

Oily fish consumption was recorded in the studies of Hodge *et al.* (163), Chatzi *et al.* (161) and Alm *et al.* (159). The rest of the studies recorded only total fish intake without specifying the types of fish. In the study of Hodge *et al.* (163) fresh oily fish consumption had a greater protective effect on current asthma than total fish consumption (74% reduction versus 48%, respectively). However, in the 'Infants of Western Sweden' study information was collected on type of fish usually ingested split into 2 different kinds of fish (lean/white or fat/oily) and no influence was found of any type of fish on eczema. Chatzi *et al.* (161) did not identify any significant association for any of the subgroups of fish included in their FFQ.

Regarding the outcomes, in the study of Nafstad *et al.* (165;166), introduction of fish early into diet suggested a protective effect on allergic rhinitis which was observed for the whole study population initially. When analysing the results for different subgroups, the protective effect on asthma and/or allergic rhinitis remained significant only among the following groups of children: breastfed for more than 6 months, without parental hay-fever or asthma, with early life atopic eczema, without an episode of lower respiratory tract infection during the first year of life. Similarly, in the prospective cohort study of Kull *et al.* (165), although there was a dose-dependent reduction in risk of atopic outcomes with increased fish consumption frequency, when conducting the analysis for different subgroups, the suggested protective effect remained significant only for children without parental allergy. In contrast to the study of Nafstad *et al.* (166), in the study of Kull *et al.* (165) the results remained significant only for children without eczema and/or recurrent wheeze during the first year of life. Interestingly, introducing fish early during the first year of life (age 3-8 months) was more beneficial than introducing fish later on (age $\geq$ 9 months) and this was associated with a lower risk of eczema at four years of age (165). This is compatible with the findings of Alm *et al.* (159) who found that the introduction of fish before nine months of age lowered eczema risk in infants at one year (by 24%), when the prevalence of eczema at 12 months on the population was reported to be 20.9%. All the rest of the studies which suggested a protective effect, conducted their analysis for the study population as a whole.

As discussed for Table 1.7, atopic outcome definitions and assessment methods differed between studies. Three of the studies performed SPT (160;161;163) and one study

determined specific IgE to identify sensitisation (165). In the study of Alm *et al*. no clinical test or biochemical measurement of allergy was conducted. Clinical outcomes measured in all of the studies were assessed with parental questionnaires which included questions on atopic outcomes. However parents were not always asked for doctor-diagnosis [for example self-reported atopic sensitisation or eczema (159)]. Clinical outcomes in all of the studies were generally well defined.

As far as confounding factors are concerned, the only studies which adjusted for all other dietary information collected were those of Kim *et al.* (164) and Alm *et al* (159). The study of Chatzi *et al.* (161) controlled for total energy intake and for maternal fish intake during pregnancy which was recorded using the Spanish version of the validated European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk FFQ. Maternal smoking was controlled for in the studies of Hodge *et al.* (163), Nafstad *et al.* (166), Andreasyan *et al.* (160), Kull *et al.* (165) and Alm *et al*. (159). Parental history of atopy and breastfeeding were controlled for in the studies of Nafstad *et al.* (166), Kull *et al.* (165), Alm *et al*. (159) and Chatzi *et al.* (161). Hodge *et al.* (163) and Nafstad *et al.* (166) adjusted for atopy/respiratory infection in early life. It should be emphasised that issues related to the methods of collecting the information about confounding factors and the validity of this information need to be considered when interpreting the results of these studies.

Studies showing a harmful effect of fish

Three studies were identified that found a negative association of fish consumption with atopic/allergic outcomes in children/infants (167-169). All three studies had a cross-sectional study design, measuring exposure and outcome at the same time point. As mentioned previously, this study design is not strong enough to infer causality. All sample sizes can be considered as large enough. Compared to the studies that suggested a lowering atopic risk, these studies were conducted in older children. Also, the increase in risk was not of great magnitude (12-21%).

All three studies used a FFQ. The FFQs in the studies of Takemura *et al.* (169) and Antova *et al.* (167) were completed by parents. The study of Huang *et al.* (168), apart from the FFQ, also used a 24-h recall and compared the effect between quartiles of intake rather than consumption frequencies. The study conducted by Huang *et al.* was the only one which included oily fish in the questionnaire.

As far as the atopic outcome measures are concerned, in the studies of Takemura *et al.* (169) and Antova *et al.* (167) questionnaires about atopy were answered by parents whereas in the study of Huang *et al.* (168) questionnaires were answered by children. All of the studies were focused on clinical outcomes. Also, all three studies included a component of doctor-diagnosis of atopic outcomes in their questionnaires. The study of Huang *et al.* (168) excluded subjects with atopic symptoms who were not doctor-diagnosed. This could potentially lead to misclassification of atopy since some of the subjects may not have access to the health care system or may have a mild or premature form of the disease.

Two of the studies adjusted for parental atopy and for children's fruit and vegetable intake (167;169). The studies of Huang *et al.* (168) and Takemura *et al.* (169) did not adjust for socio-economic factors. As mentioned previously, these are very important since fish intake may be associated with a specific lifestyle.

In the study of Takemura *et al.* (169), although there was a trend of increasing risk of current asthma with increasing fish intake, the increase in risk was significant only when comparing the effect of fish intake 1-2 times/month with that of fish intake 1-2 times/week, but not when comparing the effect of fish intake 1-2 times/month with never or ≥ 3-4 times/week. Also, in this study the validation of the FFQ was done using four 7-day records of dietary intake (from 102 males and 113 females).

In the study of Antova *et al.* (167), although the results were significant when comparing the effect of fish intake once a month or more vs. less than once a month, the significance of the results reduced when comparing the effect of more frequent fish consumption (> 2 times/week, 1-4 times/month, never or < 1 time/month).

Studies showing no effect of fish

Of the three studies that did not identify any significant association between fish intake and atopic or allergic outcomes in infants/children, two were prospective cohort (170;172) and one was case-control (not retrospective design) (171). It has to be mentioned that the cross-sectional data taken from the case-control study nested in the follow-up study of Dunder *et al.* (162) identified a null association between fish consumption and any atopic diseases (in 1980). However, as mentioned above, the retrospective follow-up showed beneficial associations. In the same study the cross-sectional data of 1986 was not presented.

All three studies used a parental/maternal FFQ. The study of Farchi *et al.* (170) was the only one including oily fish (mentioned as 'blue fish') and 'pasta with oily fish'. The other two studies collected information only on consumption frequencies of total fish. Also, frequency categories (often, sometimes, rarely, never) in the study of Hijazi *et al.* (171) were not clearly defined in the questionnaire. What is more, the two prospective cohort studies (170;172) did not include monthly fish consumption frequencies in their analysis. In contrast, they only compared weekly consumption frequencies which may not have allowed for significant associations to be identified.

All studies were conducted in children older than 1 year of age. Hijazi *et al.* (171) collected fish intake data at age 12 years, Farchi *et al.* (170) at age 6-7 years and Wijga *et al.* (172) at age 2 years.

As far as the outcome measures are concerned, the two prospective cohort studies (170;172) assessed atopic outcome based on the ISAAC questionnaires completed by parents. The ISAAC questions were also used in the study of Hijazi *et al.* (171), however, they were answered by the children themselves. Two of the studies (171;172) reported results of univariate analysis (i.e. not controlling for possible confounders). Lastly, although the two prospective cohort studies had a large sample size, their follow-up lasted only for 1 year which may not have been long enough to allow for allergy/atopy outcomes to appear.

Conclusion

The evidence from epidemiological studies investigating the association of fish intake during infancy and childhood with atopic outcomes in those infants or children is inconsistent. However, the majority of the studies (8/14) proposed a protective association between fish intake during infancy or childhood and atopic outcomes in those infants/children. The reduction in atopy/allergy risk ranged between 22 and 80%. Nevertheless, studies differed in study design, exposure and outcome measure assessment. Three studies did not observe any association and three studies observed increased risk of atopy (12-21%) with higher fish consumption. Therefore, based on the evidence available from epidemiological studies, it cannot be concluded whether fish consumption during infancy or childhood can be recommended in order to protect from atopic disease development.

1.5.2 Randomised controlled trials (RCTs) and mechanistic studies

In general, randomised controlled trials (RCTs) are study designs that provide the highest level of evidence of cause and effect. This section reviews the literature on RCTs of fish oil supplementation in pregnancy, lactation or infancy/childhood and allergic outcomes in infants/children of those pregnancies or those infants/children (Table 1.9, Table 1.10). The studies were identified through Ovid Medline (1950-2008) and Embase (1980-2008) databases by performing and combining searches with the following keywords: fish oil, EPA, DHA, fish oil trial, fish supplements, maternal, pregnancy, lactation, atopy, allergy, asthma, eczema, childhood, infancy.

1.5.2.1 Randomised controlled trials looking on the effects of fish oil supplementation during pregnancy or lactation on allergic outcomes in the offspring of those pregnancies

Table 1.9 summarizes RCTs of maternal fish oil supplementation during early life (pregnancy and lactation) and allergic outcomes in the offspring during infancy or childhood; five such studies have been published at the time of writing. One study investigated the effects of LC n-3 PUFA supplementation during pregnancy and many scientific papers have been published on this study (35;132;133;136;173-176), of which six (35;132;133;136;173;176) refer to clinical allergic outcomes in the offspring or immune markers that may modulate these outcomes (Table 1.9). A second study, also investigating the effects of fish oil supplementation during pregnancy on the immune system of the mother and the offspring, has recently published two papers (177;178) of which one (178) is included in Table 1.9. The third study was conducted on lactating women and associated the maternal fish oil intervention with allergic biomarkers, but not with clinical outcomes in the children (179). The fourth trial, conducted by Olsen *et al.* (180) investigated the effects of fish oil intake in the last trimester of pregnancy and followed up the children to assess asthma-related diagnosis at 16 years of age. The fifth study investigated the effects of n-3 fatty acid supplementation both during pregnancy and lactation, on infant allergy risk. This trial has published two relevant papers, one looking on the effects of the supplementation on the offspring during the first year of life (181) and the other looking on the effects of the supplementation during pregnancy on the immune system of the mother (182).

Study design

The study by Dunstan *et al.* (35;132;133;136;173-176) was conducted in Australia and it was a double-blinded RCT starting at week 20 of pregnancy. The study of Krauss-Etschmann *et al.* (178) was a European multicenter (Germany, Spain, Hungary) 2-factorial double-blinded RCT starting at week 22 gestation. This study was conducted by the Nutraceuticals for Healthier Life (NUHEAL) study group. The study by Lauritzen *et al.* (179) was conducted in Denmark and it was also a double-blinded parallel group RCT including women from The Danish National Birth Cohort. In this study, the women were supplemented during lactation and their children were followed up to 2.5 years of age. The study by Furuhjelm *et al.* (181) was conducted in Sweden and was a RCT as well starting at 25th week of gestation up to 3-4 months of breastfeeding. The study of Olsen *et al.* (180), conducted in Denmark, was a double blinded RCT with stratification by maternal fish intake at baseline (low/medium/high), conducted from week 30 gestation to delivery, and children were followed up at 16 years of age.

Furuhjelm *et al.* (181) compared marine n-3 fatty acid supplementation with soy oil as placebo. The studies of Dunstan *et al.* (35;132;133;136;173-176), Lauritzen *et al.* (179), and Olsen *et al.* (180) compared marine n-3 fatty acid supplementation with olive oil as placebo. However, in the study of Lauritzen *et al.* (179) there was a third group of women which was a high-fish-intake reference group and did not receive any supplement. In the study of Olsen *et al.* (180) there was also a third group which was a control group and which received no oil capsules. Krauss-Etschmann *et al.* (178) included four groups: DHA-rich fish oil, 5-methyl-tetra-hydrofolic acid (5-MTHF, 400 μg/day), both, or placebo. All were provided in a milk-based drink. The placebo was a plain milk-based supplement of minerals and vitamins recommended for pregnancy. For the intervention groups, fish oil and/or 5-MTHF were added into the placebo supplement. All groups received supplement with similar energy value, protein, fat and carbohydrate content, and identical content in rest of vitamins and minerals. Detailed analysis of individual fatty acids of the placebo and fish oil supplement was provided by the authors, and there were not significant differences in linoleic and α-linolenic acid between them.

The main inclusion criterion for the women who participated in the study by Dunstan *et al.* (132) was the presence of atopy. All women had a history of physician diagnosed allergic rhinitis and/or asthma and one or more positive skin prick tests (SPT) to six common allergens. What is more, the subjects' habitual dietary intake did not exceed two fish meals per week. This was assessed using a semi-quantitative FFQ prior to the study. On the other

hand, in the study of Krauss-Etschmann *et al.* (178) subjects were healthy pregnant women (including both atopic and non-atopic subjects). Only women who did not use fish oil, folate or vitamin B12 supplementation after week 16 gestation were included in the study. Mothers that took part in the Danish study (179) were healthy and non-atopic. The estimation of their habitual LC n-3 PUFA (g/day) intake was conducted using a semi-quantitative 300 item FFQ. As mentioned above, randomization was based on LC n-3 PUFA intake, and women with an intake below the population median (< 0.4 g/day) were randomized to one of the intervention groups. Women with an intake in the upper quartile (> 0.8 g/day) were used as a reference group. In the study of Olsen *et al.* (180) the women were healthy at study entry (atopic and non-atopic), and those with fish allergy were excluded. Food intake was assessed at baseline by a simple FFQ that categorised women in low, medium and high habitual intake of fish. In the study of Furuhjelm *et al.* (181) both atopic (55%) and non-atopic women (30%) were included (family history of allergy assessed by interview and doctor diagnosis and IgE positive test) and those with fish or soy allergy were excluded. For the purposes of the study, only women that planned to breastfeed their offspring were included. At baseline (25 weeks of gestation) 3-day dietary diaries were conducted and PUFA intakes were calculated. In both groups EPA and DHA intakes were 0.2g/day and 0.1 g/day respectively, while in the placebo group the LA intake was about 7 g/day (181).

Although the window of early life that the intervention took place differed between the five studies (pregnancy and/or lactation), the duration of intervention period prenatally was similar for the three studies: week 20 of gestation to delivery (132), week 22 of gestation to delivery (132), and week 30 of gestation to delivery (180). For one study supplementation occurred only postnatally, during the first 4 months of lactation (179) and for the study conducted by Furuhjelm *et al.* supplementation occurred perinatally, started in pregnancy (25^{th} week) and finishing in lactation (average 3-4 months of breastfeeding) (181).

It has to be noted that the fish oil supplements used contained high amounts of long chain n-3 PUFAs and supplementation dosages differed between the three studies. Women in the fish oil group in the study of Dunstan *et al.* (132) received 3.7 g/day of LC n-3 PUFAs with 56% as DHA and 28% as EPA. The control group received 4 g/day of olive oil, containing 67% n-9 MUFAs and less than 1% n-3 PUFAs. In the study of Krauss-Etschmann *et al.* (178) women received a lower dose of fish oils: 0.5 g/day DHA and 0.15 g/day EPA. Mothers in the study of Olsen *et al.* (180) received 2.7 g/day EPA and DHA (4 x 1g capsules; each capsule containing 32% EPA, 23% DHA with 2 mg tocopherol), while the placebo group (one of the 2 control groups) received 4 g/day olive oil (72% oleic acid,

12% linoleic acid). In the trial of Furuhjelm *et al.* (181) the pregnant women assigned in the intervention group consumed 4.5 g/day of marine n-3 capsules containing 1.6 g/d EPA and 1.1 g/d DHA (35% EPA, 25% DHA with 23 mg α-tocopherol) while the placebo group consumed 4.5 g/day soy oil containing mainly linoleic acid (58%; 2.5 g/day), α-linolenic (6%; 0.28 g/day) mixed with 36 mg α-tocopherol. A-tocopherol was used for its antioxidant properties, to assure the durability of the fish oil products. Mothers in the study of Lauritzen *et al.* (179), were supplemented with 4.5 g/day of fish oil which provided 1.5 g/day of LC n-3 PUFAs or with 4.5 g/day of olive oil. It has to be noted that the supplementation in the studies of Dunstan *et al.* (132), Furuhjelm *et al.* (181), and Olsen *et al.* (180) was in the form of capsules, in the study of Krauss-Etschmann *et al.* (178) in the form of powder stirred into a milk-based drink, whereas in the study of Lauritzen *et al.* (179) the fish oil was incorporated into muesli bars, home-made cookies and capsules.

Follow up and compliance

In the study of Dunstan *et al.*, 85% of the women that took part in the intervention completed the study. Compliance was monitored by measuring the incorporation of DHA and EPA into the cell membranes of erythrocytes (174).

In the European multicenter study (178), there is no reference to subject compliance rates. However, this trial has published results on DHA and EPA incorporation in the mother and offspring (177). In this paper it is mentioned that left over sachets of the supplement were asked to be returned. Also, compliance was assessed in standardised questionnaires at 30 weeks gestation, and at delivery by asking each subject how many days of dosing they had missed. The drop-out rate was 13.18% (270 of the 311 recruited pregnant women completed the study) (177).

In the study conducted by Furuhjelm *et al.* (181) the overall dropout from gestation week 25 till delivery was 17%, and the dropout was higher in the fish oil group (23%) than in the control group (12%). After birth, 52 infants were followed up in the fish oil group and 65 infants in the control group. This means that the dropout in the fish oil group was 25% and in the control group 13%, while overall, the attrition rate was 19%. Authors did not comment anything special on method of compliance, apart from the fact that the research nurses contacted the mothers twice during the last part of pregnancy to remind them of the supplementation (181).

In the study of Olsen *et al.* (180) children were followed up and assessed in terms of asthma and other related allergic symptoms at 16 years of life. The follow up rate was extremely high, as 522 were included in analyses 16 years after the intervention. According to the authors compliance was optimised by returning and weighing the empty boxes of capsules at 3 times, so that the researchers estimated amounts of capsules consumed (180).

Lauritzen *et al.* (179) stated that the overall self-reported compliance with exclusive breastfeeding in both groups was on average 91% (range 67-100%, n=64). The follow-up rates at 2.5 years of age in the randomized groups and in the high-fish-intake reference group were 72 and 58%, respectively but in total the follow up rate at 2 1/2 years of infants' age in comparison to the baseline subjects' recruitment was 48% (101 infants out of 211 pregnant women). However, the follow-up women had significantly better compliance with exclusive breastfeeding in the intervention groups compared to the follow-up women in the reference group (89 versus 85%, p=0.020) (179).

Confounding factors

In the Dunstan study so as to minimise potential confounding factors at randomization the groups were stratified by parity (no previous term birth child vs. one or more), pre-pregnancy BMI, age and maternal allergy (allergic rhinitis or asthma). Results were adjusted for gender, parity and method of delivery (35). Dunstan *et al.* (132) reported that background maternal dietary intake of fatty acids assessed by FFQ was not different between the two groups at study entry or at 30 weeks gestation.

In the study of Krauss-Etschmann *et al.* (178), after randomization the women in different groups did not differ significantly in parity, height, weight at study entry, smoking habits, and social demographic characteristics. Also, data on dietary habits were obtained at study entry (177) but no dietary data was presented nor controlled for. The neonates did not differ significantly in sex, birth weight, length, Apgar score, and parental history of allergy. For the purposes of analysis only 158 mother-child pairs were available. Their characteristics did not differ from those of the main trial (n=311). The analysis of this study was adjusted for study centre (177) and maternal percentage weight/weight DHA at week 20 gestation (baseline). Confounding factors controlled for were gravity, parity, delivery mode, and maternal smoking at 20 and 30 weeks gestation.

Olsen *et al.* (180) did not control for any factors but randomisation was stratified by maternal habitual fish intake (low, medium and high).

Furuhjelm *et al.* (181) made adjustments in their analysis for allergic symptoms in children for the following factors: ARA and LA levels in maternal phospholipids at inclusion, breastfeeding fully until 6 months, number of siblings, exposure to tobacco smoke, maternal allergic symptoms and eczema in family (181).

Lauritzen *et al.* (179), although not controlling for confounding factors in their analysis, assessed subjects' compliance with exclusive breastfeeding, and they found no differences in characteristics of children (2.5 years) between the groups (sex, parity, birth weight, duration and degree of breastfeeding, age, height, weight, family history of atopy, eczema, wheezing, food allergy, plasma IgE).

Outcome measures and results in the Australian 'pregnancy supplementation study' (35;132;133;136;173;175;176)

Plasma (cord blood) and urinary F_2-isoprostanes were measured as markers of lipid peroxidation. Barden *et al.* (136) associated urinary markers of cord blood and cord erythrocyte fatty acids with neonatal lipid peroxidation suggesting that maternal fish oil supplementation during pregnancy might be protective against oxidative stress in the infant of atopic mothers soon after birth. It was concluded that supplementation with fish oil during pregnancy significantly alters early post-partum breast milk fatty acid composition, and it was suggested that there is a relationship between fatty acid status and mucosal immune function, potentially influencing infant immune development. In accordance with this, a more recent analysis (175), showed that breast milk from women who received fish oil had proportionally higher EPA and DHA levels at 3 days and 6 weeks after delivery, but not at 6 months. Also, infant DHA status at 1 year of age was directly related to DHA levels at 3 days, 6 weeks and 6 months postpartum, but not to antenatal supplementation.

Dunstan *et al.* (133) examined the relationships between PUFA levels and immunological parameters in breastmilk, such as sCD14, IgA and a range of cytokines, suggesting a further pathway of maternal diet effect on the post-natal infant immune development.

Dunstan *et al.* (35) observed that maternal dietary supplementation with LC n-3 PUFA was associated with significantly lower IL-13 levels in cord plasma, supporting a relation between dietary n-3 PUFA and neonatal immune function.

Dunstan *et al.* (132) provided evidence that fish oil supplementation in pregnancy achieved a significant increase in LC n-3 PUFAs in neonatal red blood cell membranes compared with the control group which was associated with a general trend for decreased neonatal

immune responses to allergens and reduced expression of allergic disease at 1 year of age. This is the only intervention study to publish evidence that fish oil might offer potential clinical benefits in reducing the risk of allergic disease. However it should be noted that the children were too young to study a diagnosis of asthma reliably. In this regard, further analysis at 2.5 and 5 y of age would provide a more reliable assessment of such clinical outcomes. In addition, the small sample size makes this study an "exploratory" study. Larger scale studies are needed for neonatal clinical assessment and to investigate the effects of n-3 PUFA supplementation during pregnancy and/or early life in relation to allergy prevention in children of those pregnancies.

Denburg *et al* (173) showed that dietary LC n-3 PUFA supplementation during pregnancy in atopic mothers altered infant cord blood hemopoietic progenitor phenotype, that favours the development of allergic disease. It was shown that fish oil supplementation resulted in increased numbers of cord blood $CD34^+$ progenitors and their increased responsiveness to IL-5 in cord blood eosinophil/basophil colony forming units (Eo/B-CFU). The numbers of $CD34^+$ cells in cord blood increased significantly the risk of atopic eczema/dermatitis syndrome (AEDS) in infants at 1 year of age, and cord blood progenitor IL-5 responsiveness increased the risk of AEDS and recurrent wheeze significantly. These results support that fish oil supplementation during pregnancy may have an impact on development of atopic disease in the offspring.

Finally, Prescott *et al* (176) showed that maternal fish oil supplementation in pregnancy modifies neonatal neutrophil function, which has implications for early immune programming and provides another mechanism through which LC n-3 PUFAs may influence early immune development. Specifically, it was shown that the production of LTB_4 from neutrophils was significantly lower in neonates born to mothers who were supplemented with fish oil during pregnancy as can be seen on Table 1.9. There was a trend for lower production of 5-HETE by neutrophils in the fish oil group. There was also a trend for higher levels of less inflammatory products (LTB_5) in the fish oil group. Also, LTB_5 levels were positively correlated with n-3 PUFAs levels in maternal and neonatal erythrocytes (particularly EPA), and negatively correlated with n-6 PUFAs.

Outcome measures and results from the European multicenter pregnancy supplementation study (177;178)

The European multicenter study measured the effects of fish oil supplementation during pregnancy on maternal and cord blood plasma concentrations of EPA and DHA. It was

shown that fish oil supplementation with or without 5-MTHF resulted in increased proportion of DHA in cord blood plasma, and DHA and EPA in maternal plasma at 30 weeks of gestation and at delivery compared with placebo or 5-MTHF (177).

Outcomes on immune markers in the study are presented on Table 1.9. It was shown that fish oil supplementation during pregnancy was associated with decreased mRNA expression of IL-4, IL-13 and CCR4 in cord blood. Also, fish oil supplementation was related to decreased frequencies of cord blood NK cells and $CCR3^{+}$ $CD8^{+}$ T cells. It has to be mentioned that the data on cord blood lymphocyte subsets were analysed only for the German arm of the study (n= 30), where facilities were available. Moreover, mRNA levels of IL-1 and IFN-γ in maternal blood at delivery were decreased after fish oil supplementation. On the contrary, mRNA levels of the regulatory cytokine TGF-β were increased in both maternal blood at delivery and in cord blood following maternal supplementation. Thus, it was shown that fish oil supplementation during pregnancy downregulates Th1 responses in the mother and Th2 responses in the fetus. These results are in line with the observations made by Dunstan *et al.* (35) who also showed a reduction in cord blood IL-13 levels, a Th2 marker. In addition, Krauss-Etschmann *et al.* (178) showed that the decrease in cord blood IL-13 mRNA levels was more pronounced in non-allergic mothers. Their study showed that Th2 responses are decreased in neonates of both allergic and non-allergic mothers, as opposed to the Australian study which focused on allergic mothers. The authors suggested as a clinical implication of their study that fish oil supplementation of pregnant women might be an option for primary allergy prevention.

Outcome measures and results from the Danish follow up study (180)

Olsen *et al.* (180) conducted the supplementation trial in 1992 and related late pregnancy fish oil supplementation to prolonged gestation. Fish oil supplementation had no detrimental effects on the course of the labour or on the growth of the fetus. The dose of the supplementation was 2.7 g/day EPA and DHA, equal to 10 times the average intake (180). After 16 years, they followed up the offspring of those mothers who participated in the trial and assessed the prevalence of asthma-related diagnosis, as well as atopic dermatitis or allergic rhinitis in those children after obtaining information on occurrences of those allergic diagnoses from the Danish patient registry. The hazard rate ratio of asthma (all types) and allergic asthma decreased significantly (by 63% and 87% respectively) in the fish oil group in comparison to the olive oil group (control). Interestingly, the risk of asthma (all types) was decreased in the no oil group as well,

compared with the olive oil group, but not significantly. There was a decrease in the prevalence of asthma (all types), atopic dermatitis or allergic rhinitis (by 57%) and in the prevalence of allergic asthma, atopic dermatitis or allergic rhinitis (by 69%) in the fish oil group compared to control. Surprisingly a similar decrease in risk was observed for both of the above diagnoses in the no oil group compared with the olive oil group. The authors speculated that this would be attributed to contamination bias (taking part in that study, study fish oil available in the market, public awareness about potential health benefits of consuming marine foods or fish oils such as heart disease). Finally, stratification by maternal fish intake at baseline (low/medium/high) resulted in small number of cases and did not have any significant effect on the hazard rate ratio of asthma (all types) and allergic asthma between the 3 groups (fish oil/ olive oil/ no oil), using the olive oil group as reference (180).

Outcome measures and results from the Swedish pregnancy and lactation study (181;182)

The paper of Warstedt *et al.* (182) associated fish oil supplementation during pregnancy (25 weeks till delivery) with plasma fatty acid composition and with maternal immune function at 1 week post-partum. This is the second study investigating immune function in pregnant women after LC n-3 PUFA supplementation after Krauss-Etschmann *et al.* (177). In terms of phospholipid fatty acid changes, it was shown that the maternal serum/plasma ratio of ARA/EPA was decreased in the fish oil group. Also, in the control group plasma phospholipid %ARA increased, whereas %EPA and %DHA increased in the intervention group. These results were similar in both atopic and non-atopic mothers. Moreover LPS-induced PGE_2 secretion from whole blood cultures was decreased in the majority of the fish oil group. However, the mean decrease in the fish oil group was not significant, in contrast to the PGE_2 production in the control group that increased significantly. The non-significant decrease of PGE_2 secretion in the intervention group was probably due to the observation of higher LPS-induced PGE_2 secretion at baseline from the mothers that were randomized to the fish oil group compared to those allocated in the placebo group. The change in PGE_2 production differed significantly between the 2 groups and the decrease in PGE_2 secretion in the LC n-3 PUFA group was more pronounced among non-atopic women (not significant). The researchers tried to explain this tendency of reduction of PGE_2 in the non-atopic women, but it was not possible as no differences in fatty acid profile were observed between atopic and non-atopic women. Secretion of LTB_4, chemokines and cytokines were not affected by fish oil. Krauss-Etschmann *et al.* reported

no data on eicosanoid secretion (177). Finally, Warstedt *et al.* showed that the changes in LPS-induced PGE_2 production in the intervention group correlated positively with changes in plasma phospholipid ARA% and negatively with changes in EPA%, which was even stronger among the non-atopic (182).

The paper published by Furuhjelm *et al.* (181) associated the fish oil supplementation during pregnancy and lactation (25 weeks gestation to 3-4 months lactation) with atopic outcomes in the offspring at 6 months and 1 year of age. Regarding allergic sensitization, the prevalence of any positive SPT during the first year of life was significantly lower in the fish oil group compared to the placebo. More specifically, the prevalence of positive SPT to egg was lower in the fish oil group at 12 months. Moreover, in terms of clinical symptoms IgE associated eczema (eczema in the presence of detectable IgE antibodies or positive SPT towards egg, milk or wheat) and food allergy (reaction to egg or milk) during the first 12 months of life were significantly higher in the placebo group. IgE associated eczema was higher in the placebo group at 6 months of age as well but not significantly. In the regression analysis, after controlling for the confounding factors described above, it was found that the risk of developing any positive SPT, a positive SPT to egg or IgE associated eczema was 3-4 times less in the fish oil group compared to the placebo. The risk of developing food allergy was reduced 10 times in the LC n-3 PUFA group in comparison to the control. The researchers also found that these significant relations for any positive SPT, food allergy and IgE mediated eczema were reserved after separate analysis for the non-allergic mothers but not for the allergic mothers. One disadvantage of the study was the incomplete blinding because of belching with fishy taste that some mothers in the fish oil group reported. Nevertheless, the examiners were blinded. The study had enough power to detect significant differences in the incidence of egg sensitization and IgE-mediated symptoms but not in sensitization to milk as the incidence was very low (181).

Outcome measures and results in the 'Danish National Birth Cohort'(179)

Erythrocyte LC n-3 PUFAs at 4 months were higher in infants from the mothers in the fish oil group compared with the olive oil control group. These differences were no longer evident at 2.5 years of age. The study was not powered to look at clinical outcomes i.e. atopic sensitization and no differences in atopy and plasma IgE were observed between the groups. No association was found between in vitro cytokine production and plasma IgE levels and there was no significant association as well between plasma IgE and eczema, wheezing or food allergy, although both associations tended to be positive. Cytokine

responses and erythrocyte fatty acid composition in children of mothers with a high fish intake (reference group) were intermediate in comparison to those in the randomized groups. However, the subjects were healthy children in the sense that were not selected to have a high risk of atopy. Furthermore, plasma IgE is a very crude measure of atopy and does not give an indication of allergen sensitization. Lauritzen *et al.* (179) stated that because of the immunomodulatory and anti-inflammatory properties that olive oil has the differences between the fish oil and the control group may have been larger if they had chosen a more 'neutral' oil. On the other hand, in the Australian pregnancy supplementation trial, the amount given (4 g of olive oil /day) did not significantly alter the average daily intake of oleic acid (26 g/day) in Australian diets.

Conclusion

The studies of Dunstan *et al.* and Krauss-Etschmann *et al.* (132;178) both investigated the effects of maternal fish oil supplementation during pregnancy on blood immune markers in the offspring. It is clearly evident from the Australian study of Dunstan *et al.* (35) that maternal fish oil supplementation resulted in higher LC n-3 PUFA status (higher EPA and DHA in cord blood erythrocytes) and lower n-6 PUFA status in the neonates. What is more, in relation to immune markers, fish oil supplementation resulted in lower cytokine levels in cord blood plasma (IL-13), lower cytokine production from cord blood mononuclear cells (IL-10), and lower leukotriene production from cord blood neutrophils (LTB_4). In addition, fish oil significantly altered cord blood hemopoietic progenitor phenotype which may impact on the development of atopic disease. Lastly, although the study was not designed to assess clinical outcomes, at 1 year of age there were signs of beneficial effects on atopic outcomes as a result of maternal fish oil supplementation during pregnancy (less severe atopic dermatitis, lower risk of positive SPT to egg).

The study of Krauss-Etschmann *et al.* (178), also demonstrated that fish oil supplementation during pregnancy results in higher levels of DHA in maternal and cord blood. The intervention resulted in differential affects on maternal and fetal immune parameters. There was a significant decrease in maternal Th1-related cytokines (IL-1, INF-γ) at birth, and in fetal Th2-related cytokines (IL-13, IL-4, CCR4). The latter results are in accordance with those from the Australian study. However results in the study of Krauss-Etschmann *et al.* (178) were significant irrespective of the atopy status of the mothers. The effects observed might be mediated through TGF-β, which mRNA levels were increased in both maternal blood at birth and in cord blood.

The Danish study of Olsen *et al.* (180) conducted in late pregnancy (week 30-delivery), supplemented pregnant women with either fish oil (2.7g/day), olive oil or no oil and assessed asthma and other allergic manifestations on the children of those pregnancies 16 years later. They observed a large reduction of asthma and allergic asthma by 60-80% in the fish oil group (comparison to olive oil group). The trial had a large sample size (522 children identified at 16 years of age), with a follow up rate of 97%.

The study conducted in Sweden (181;182) is the first study to present with fish oil supplementation in both pregnancy and lactation. The results presented referred to outcomes in the mothers (plasma fatty acid composition and immune outcomes relevant to allergies at 1week post partum) (182) and in the infants (clinical outcomes on infant allergy during the first year of life) (181). LC n-3 PUFA supplementation decreased the ARA/EPA ratio and this was associated with reduced LPS-induced PGE_2 secretion, which is related with the Th2 polarization in the mother and subsequently less allergic inflammation in the mother and probably less allergic inflammation in the immunologically naïve offspring. Clinical outcomes suggest that the infants from mothers in the fish oil group had reduced risk of developing allergic sensitization to egg, IgE associated eczema and food allergy during the first year of life. However, repeatability of such observations is essential in order to draw more definite conclusions about the benefit of LC n-3 PUFA towards allergic manifestations.

The study of maternal fish oil supplementation during lactation (179) is also the only one to date investigating outcomes on immune markers in the offspring. As in the previous study, infants of lactating mothers who received fish oil supplementation had a higher n-3 PUFA status at 4 months of age. What is more, IFN-γ production at 2.5 years of age increased significantly in the fish oil group, an observation which may reflect faster maturation of the immune system. This study was also not powered to assess clinical outcomes.

In conclusion, the evidence available supports that fish oil supplementation during pregnancy and lactation results in higher provision of n-3 PUFAs to the offspring perinatally, and thus, it can increase LC n-3 PUFA status in the offspring. The increase in LC n-3 PUFA status of the offspring may potentially affect immune markers in early life indicating a possible protective effect of maternal fish oil supplementation during pregnancy or lactation on atopic disease in the offspring. The study of Krauss-Etschmann *et al.* (178) points out the clinical implications of their study: ‘fish oil supplementation of pregnant women might be an option for primary allergy prevention’. The follow-up of the

European multicenter study and its effects on incidence of allergic diseases in the offspring of fish oil supplemented mothers are anticipated. The follow up study by Olsen *et al.* (180) suggests the importance of fish oil supplementation in early life, particularly during pregnancy, as the results were observed in adolescence. However, interpretation of such results should be done with caution especially when the follow up of the study is of long duration and not controlled for confounding factors such as lifestyle (physical activity), gender, weight, and most importantly diet (fish, fruit, vegetable intake) of the children. The unique Swedish supplementation study in pregnancy and lactation implies protective effect and clinical signs of reducing the allergic sensitisation in the infants at 6 months and 1 year of age (181;182).

Well designed maternal fish oil supplementation trials would possibly be more effective during pregnancy. The observations of these studies need to be confirmed by future trials powered adequately to examine clinical outcomes in the offspring later on in life in order to be able to draw more definite conclusions and form recommendations.

Table 1.9: Maternal fish oil supplementation during pregnancy or lactation and allergic outcomes in infants/ children of those pregnancies

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Barden *et al.* 2004 (136) Double-blinded RCT Perth, Australia	83 Atopic pregnant women n= 40 FO n= 43 Control	FO: 4 (1 g) capsules/day providing 3.7 gr/day n-3 PUFA (56% DHA, 27,7% EPA) Control: 4 (1g) olive oil capsules/day (66.6% n-9 oleic acid, <1% n-3 PUFA)	From week 20 of pregnancy till delivery	Lipid peroxidation: CB plasma and infant urinary F_2-isoprostanes CB erythrocyte membranes FA levels Markers of APC function (HLA-DR expression and cytokine responses)	Maternal fish oil supplementation lowered CB plasma ($p< 0.0001$) and urinary F_2-isoprostanes ($p= 0.06$) HLA-DR expression on APC was not different between the groups 28.8% of the variance in CB plasma F_2-isoprostanes was explained by positive relationships with erythrocyte ARA and monocyte HLA-DR expression and a negative relationship with erythrocyte EPA	This study shows that maternal supplementation with fish oil can attenuate neonatal lipid peroxidation.
Dunstan *et al.* 2003 (35) Double-blinded RCT Perth, Australia	Same as above	Same as above	Same as above	CB plasma cytokine levels (IL4,IL5,IL6,IL10,IL12,IL13, TNF-α, TNF-γ) CB plasma total IgE CB erythrocyte membranes FA levels	Maternal fish oil supplementation resulted in : increased total n-3 PUFA, EPA and DHA levels, and decreased n-6 ARA levels in CB erythrocyte membranes ($p< 0.001$) Significantly lower CB plasma IL-13 levels ($p< 0.05$) in neonates from intervention group compared to control Significant inverse relationship between n-3 PUFA in CB erythrocyte membranes and CB plasma IL-13	Increasing neonatal n-3 PUFA levels with maternal fish oil supplementation can achieve subtle modification of neonatal cytokine levels.
Dunstan *et al.* 2003 (132) Double-blinded RCT Perth, Australia	Same as above	Same as above	Same as above	CB erythrocyte membranes FA levels CBMNC cytokine responses to allergen and mitogen (IL-5, IL-10, IL-13, INF-γ) SPT of infants at 1 year of age Symptoms of allergic disease (asthma, wheeze, food allergy, atopic dermatitis)	Fish oil supplementation resulted in higher proportions of total n-3 PUFAs in CB erythrocyte membranes (17.75% ± 1.85%) compared with control group (13.69% ± 1.22%, $p< 0.001$), and lower proportions of total n-6 PUFA ($p< 0.001$) CBMNC cytokine (IL-5, IL-13, IL-10, and INF-γ) responses tended to be lower in the fish oil group (statistically significant only for IL-10 in response to cat allergen, $p= 0.046$) Amongst subjects with atopic dermatitis, those in the FO group were 10 times less likely to have severe disease (SCORAD>25) (OR 0.09, 95% CI 0.01-0.94, $p= 0.045$) Infants in the fish oil were 3 times less likely to have a positive SPT to egg at 1 year of age (OR 0.34, 95% CI 0.11-1.02, $p= 0.055$)	These data suggest a potential reduction in subsequent infant allergy after maternal PUFA supplementation.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Dunstan *et al.* 2004 (133) Double-blinded RCT Perth, Australia	Same as above	Same as above	Same as above	Immunomodulatory factors in breast milk collected 3 days post-partum: sCD14, IgA, cytokines (IL-5, IL-6, IL-10, TNF-α and INF-γ) Breast milk FA levels	DHA and EPA were in higher proportions in breast milk from women supplemented with fish oil (DHA 1.15% ± 0.47%, EPA 0.16% ± 0.07%) than in samples from the control group (DHA 0.50% ± 0.17%, EPA 0.05% ± 0.02%, $p< 0.001$) Breast milk ARA levels were significantly lower ($p= 0.045$) in the fish oil group (mean 0.55%, SD 0.12%) compared with the control group (mean 0.61%, SD 0.14%) Breast milk IgA was positively correlated with DHA ($p= 0.046$) and 22:5n-3 ($p= 0.003$), but inversely correlated with linoleic acid ($p= 0.034$) No differences were observed in breast-milk IgA, sCD14, and cytokines between the groups	Supplementation with fish oil during pregnancy significantly alters early post-partum breast milk fatty acid composition. n-3 PUFA levels were positively associated with IgA and sCD14 levels, suggesting a relationship between fatty acid status and mucosal immune function.
Denburg *et al* 2005 (173) Double-blinded RCT Perth, Australia	Same as above	Same as above	Same as above	CB $CD34^+$ hemopoietic progenitors CB $CD34^+$ cell expression of cytokine (IL-5Rα, IL-3Rα) or chemokine receptors (CXCR4, CCR3). CB cells were also cultured for Eo/BCFU Clinical assessment of infants for atopic disease/ SPT at 1 year of age	Percentages of CB $CD34^+$ cell numbers were higher after n-3 PUFA than placebo ($p< 0.002$) CB $CD34^+$ cell expression of cytokine and chemokine receptors was not different between groups There were significantly more IL-5 responsive CB Eo/BCFU in the fish oil, compared to the control group ($p< 0.003$) Overall, there was a positive association between the percentage of $CD34^+$ cells, and the number of IL-5 responsive Eo/BCFU in CB, and 1 year clinical outcomes, including atopic dermatitis and wheeze ($p< 0.05$)	Dietary n-3 PUFA supplementation during pregnancy in atopic mothers alters infant cord blood hemopoietic progenitor phenotype, that favors the development of allergic disease. This may have an impact on development of atopic disease.
Prescott *et al* 2007 (176) Double-blinded RCT Perth, Australia	Same as above	Same as above	Same as above	Leuktriene production by CB neutrophils after stimulation CB and maternal (37 weeks gestation) erythrocyte FA CBMNC cytokine production (IL-5, IL-10, IL-13, INF-γ)	CB neutrophil LTB_4 (isomer 2) production reduced in FO group compared to controlled ($p= 0.031$) LTB_4 (isomer 2) production levels were inversely related to the n-3 PUFA content of maternal erythrocyte membranes ($p< 0.05$) Production of IL-6 responses and IL-10 responses by CBMNC was lower in neonates with lower neutrophil LTB_4 production	Fish oil supplementation can lead to modification of neutrophil function, which may have implications for early immune programming

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Krauss-Etschmann *et al*. 2008 (178) Double-blinded 2-factorial RCT Multicenter: Spain Germany Hungary	311 Pregnant women This analysis on subcohort n= 45 FO n= 49 5-MTHF n= 49 FO + 5-MHTF n= 50 Control	4 groups: 1. FO: 0.5 g/day DHA, 0.15 g/day EPA 2. 5-MTHF 3. FO + 5-MTHF 4. Control: plain milk based supplement FO and 5-MTHF added to control supplement	From week 22 of pregnancy till delivery	Th1/Th2 related molecules in maternal blood at delivery and in CB: mRNA expression of CCR4, IL-13, IL-4, CRTH2, CXCR3, INF-γ, IL-1, TGF-β CB lymphocyte subsets	Maternal FO supplementation compared to control was associated with: Increased mRNA expression of TGF-β in maternal blood at birth (χ 0.85, 95% CI 0.80-0.89; placebo χ 0.68, 95% CI 0.64-0.72), and in CB (χ 0.85, 95% CI 0.81-0.90; placebo χ 0.75, 95% CI 0.71-0.79) (all p< 0.001) Decreased mRNA expression of INF-γ (χ 0.54, 95% CI 0.51-0.57; placebo χ 0.65, 95% CI 0.61-0.69) and IL-1 (χ 0.69, 95% CI 0.66-0.73; placebo χ 0.83, 95% CI 0.79-0.88) in maternal blood at birth (all p< 0.001) Decreased CB mRNA levels of IL-4 (χ 0.54, 95% CI 0.52-0.57; placebo χ 0.64, 95% CI 0.61-0.68), IL-13 (χ 0.61, 95% CI 0.58-0.65; placebo χ 0.85, 95% CI 0.80-0.89), and CCR-4 (χ 0.70, 95% CI 0.67-0.73; placebo χ 0.88, 95% CI 0.84-0.92) (p< 0.001) Decreased CB NK (χ 9.0%, 95% CI 7.5-11.0%; placebo χ 14.6%, 95% CI 12.3-17.2%, p< 0.001) and $CCR3^{+}$ $CD8^{+}$ T-cells (χ 0.2%, 95% 0.1-0.6%; placebo χ 1.5%, 95% 0.5-4.5, p< 0.04)	FO supplementation during pregnancy was associated with decreased mRNA levels of Th2-related molecules in CB and decreased maternal Th1 cytokines at birth. These effects may be mediated by TGF-β.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Furuhjelm *et al.* 2009 (181) Double blinded RCT Linkoping, Sweden	145 Pregnant women with allergic family history: n=70 n-3 PUFA n=75 control 117 offsprings followed up: n=52 n-3PUFA group n=65 control	2 groups: 1. FO: 1.6 g/day EPA, 1.1 g/day DHA 2. Control: soy oil capsules (2.5g/dayLA, 0.28g/day LNA)	From week 25 of pregnancy till end of lactation (3-4 months of breastfeeeding)	Clinical examinations of infants: -Skin prick testing to cows' milk, egg, and wheat at 6 and 12 months of age -IgE associated eczema and food allergy at 3, 6, and 12 months of age Detection of plasma specific IgE to egg/milk/wheat at 3 and 12 months age	**Sensitisation within the 1st year of life:** -prevalence of food allergy was lower in the n-3 group (2%) compared to the placebo group (15%, p<0.05) -prevalence of IgE-associated eczema was lower in the n-3 group (8%) compared to placebo (24%, p<0.05). -prevalence of any positive SPT was 15% in the n-3 and 32% in the control group(p= 0.04). -the prevalence of positive egg SPT in the n-3 group at 12 months was 12% compared to 29% in the placebo group (p= 0.02). **Logistic regression analysis**: -Reduced risk of developing any positive SPT (OR=0.36, 95% CI 0.14-0.95, p<0.05), a positive SPT to egg (OR=0.31, 95% CI 0.11-0.89, p<0.05), and IgE-associated eczema (OR=0.22, 95% CI 0.06-0.81, p<0.05) during the first year of life -The risk of developing food allergy was reduced 10 times in the n-3 group compared to the placebo group (OR=0.09, 95% CI 0.01-0.74, p<0.05)	Fish oil supplementation during pregnancy and lactation may reduce the risk of developing allergic sensitization to egg, IgE associated eczema and food allergy during the first year of life.
Warstedt *et al.* 2009 (182) Double blind RCT Linkoping, Sweden	The same	The same	From week 25 of pregnancy till 1 week after birth	**Maternal outcomes only** Whole blood cultures: -analysis of PGE2 and LTB4 -analysis of cytokines and chemokines (IFNγ, IL-5, IL-6, TNF, IL8, IL10, CCL2, CCL3) Analysis of plasma or serum phospholipids Analysis of serum IgE antibodies	Phospholipid fatty acid proportions altered after supplementation (1 week after birth): -Increase in EPA (by 6.3%) and DHA (by 2.4%) in the n-3 group (p<0.001) -Decrease in ARA (by 1.1%) in the n-3 group (p<0.001), whereas it increased in the control grp (by 1.6%; p<0.001) From 25wk gestation till 1wk after delivery: -LPS-induced PGE2 secretion decreased in 64% of the n-3-supplemented mothers and increased in 77% of the control group (*p*= 0.002). -The decreased PGE2 production was more pronounced among non atopic (80%) than atopic mothers (69%) (not significant) The LPS induced cytokine and chemokine secretion was not affected.	Fish oil supplementation during the last trimester decreases the ARA/EPA ratio and is associated with reduced secretion of pro inflammatory and Th2-promoting PGE2, which in turn may contribute to an allergy-preventing effect.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Olsen *et al.* 2008 (180) Double blinded RCT Copenhagen, Denmark	533 Pregnant women n=266 FO n=136 control n=131 no oil capsules Stratification by maternal baseline fish intake (2:1:1): low/ medium/ high 522 children followed up at 16 years after birth	3 groups: 1.FO: 2.7 g/day n-3PUFA (32% EPA, 23% DHA) 2. Control: olive oil 4 g/day (72% oleic acid, 12% LA) 3. No oil capsules	From week 30 of pregnancy till delivery	Offspring asthma-related diagnosis at 16 years old: allergic asthma, asthma of mixed type, atopic dermatitis or allergic rhinitis Data taken from the National patient registry in Denmark	In the fish oil compared to the olive oil group: -the hazard rate of asthma was reduced by 63% (95% CI 8%-85%, p= 0.03) -the hazard rate of allergic asthma was reduced by 87% (95% CI 40%-97%, p= 0.01) -the hazard rate of asthma all types, atopic dermatitis or allergic rhinitis was reduced by 57% (95% CI 4%-81%, p= 0.04) -the hazard rate of allergic asthma, atopic dermatitis or allergic rhinitis was reduced by 69% (95% CI 16%-89%, p= 0.02) Stratification by maternal baseline fish intake (low, medium, high): -no differences between stratification groups -almost no differences between fish oil and control group within stratification categories, although in the low fish intake group the risk of asthma (all types), atopic dermatitis or allergic rhinitis was decreased by 90% (95% CI 13-99%, p=0.04) in the n-3 group	Increasing n-3 PUFA intake in late pregnancy may be an important prophylactic potential in relation to offspring asthma in adolescence.
Lauritzen *et al* 2005 (179) Double-blinded parallel group RCT Denmark	Lactating mothers with fish intake below the population median: n= 37 FO n= 28 Control Lactating women with fish intake in the highest quartile: n= 26 Reference	Intervention: 1.5 g/day of n-3 LC PUFA (equivalent to 4.5 g/day of fish oil) Placebo: olive oil. Supplement was incorporated in muesli bars, home-made cookies, or capsules	First 4 months of lactation	Infant erythrocyte membranes FA (4 months and 2.5 years of age) Infant plasma IgE levels and cytokine production in endotoxin-stimulated whole-blood cultures (2.5 years old) Parental report of allergy diagnosis or tendency to allergy	Erythrocyte n-3 PUFA at 4 months were higher in infants from the fish oil group compared with the olive oil group (p< 0.001) but were no longer different at 2.5 years old. The median production of INF-γ in the fish oil group was 4fold higher than that in the control group (p= 0.034) IL-10 production was similar between groups. The INF-γ/ IL-10 ratio was 2 fold higher in the fish oil group (p= 0.019) and was positively correlated with 20:5n-3/ 20:4n-6 in erythrocytes at 4 months (p= 0.050)	Fish oil supplementation during lactation resulted in increased in vitro INF-γ production in children 2 years after the supplementation was given, which may reflect a faster maturation of the immune system.

RCT, randomized control trial; OR, odds ratio; CI, confidence interval; SD, standard deviation; FA, fatty acids; PUFA, polyunsaturated fatty acids; LC, long chain; FO, fish oil; 5-MTHF, 5-methyl-tetra-hydrofolic acid; SPT, skin prick testing; DHA, hocosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; CB, cord blood; CBMNC, cord blood mononuclear cells; NK, natural killer cells; APC, antigen presenting cells; HLA-DR, human leukocyte antigen-DR; sCD14, soluble CD14; Eo/BCFU, eosinophil/basophil colony forming units; Ig, immunoglobulin; INF, interferon; IL, interleukin; TNF, tumour necrosis factor; TGF-β, transcription growth factor β; χ, geometric mean; vs, versus.

1.5.2.2 Randomized controlled trials investigating the effects of fish oil supplementation during infancy/childhood on allergic outcomes in those infants/ children

Table 1.10 summarizes the randomized controlled trials investigating the effects of fish oil supplementation during infancy/childhood on allergic outcomes in those infants/children (atopy, asthma, eczema, dermatitis, cough). Five studies were identified. Six scientific papers have been published from the Childhood Asthma Prevention Study (CAPS), of which one describes extensively the study protocol (183). Table 1.10 includes the results at 18 months (184;185), at 3 years (186), and at 5 years of age (187;188). Damsgaard *et al.* (189) also studied the impact of LC n-3 PUFA supplementation on allergy risk in infants. Studies done by Hodge *et al.* (190), Nagakura *et al.* (191), and Vaisman *et al.* (192) investigated the effect of LC n-3 PUFA supplementation in children covering a wide age range.

Study design

The study carried out by Hodge *et al.* (190) and the CAPS study took place in Australia (183). In the latter, infants were recruited from 6 different hospitals in Sydney. The study done by Nagakura *et al.* (191) took place in Japan in a hospital/clinical setting; the study done by Vaisman *et al.* (192) took place in Israel; and the study conducted by Daamsgaard *et al*. (193) was conducted in Denmark in a community setting.

Infants who participated in the CAPS study (183) were at high risk of developing asthma, whereas infants that took part in the Danish study were not (189). Children who participated in Vaisman *et al*. (192) study were healthy but children in the Hodge *et al*. and Nagakura *et al.* studies were asthmatic (190;191).

All 5 trials were of parallel design, meaning that they compared LC n-3 PUFAs with placebo. Three of the studies were double-blinded controlled trials. CAPS was a single blinded study and the Danish study (189) was an unmasked randomized trial. Of the 5 trials, 2 compared LC n-3 PUFA supplements with placebo (191;192) and the other three compared LCn-3 PUFAs with n-6 PUFAs (183;189;190). The CAPS study intervention consisted not only of a diet intervention but of a house dust mite (HDM) avoidance intervention as well. It has to be noted for the CAPS study that there was no blinding for the HDM modification. Some of the volunteers reported ‘fishy’ smell of the fish oil supplements, so it might have been that that the

subjects were un-blinded. Also, the CAPS study tried to achieve an intake of n-3 to n-6 fatty acids of ratio 1:5 in the intervention group. Parents were asked to add the fish oil supplement to milk formulas and after six months of age also to favourite foods and beverages. Parents were finally asked to avoid using specific formulas that had added LC n-6 PUFAs and to cook with oils and margarines provided (183).

The constituents of the placebos used in the RCTs studies varied and are described in Table 1.10. Hodge *et al*. (190) used capsules containing n-6 PUFAs; these were a mix of palm oil, olive oil, safflower oil (plus usual dietary fat replaced by sunflower oil); Nagakura *et al*. (191) used capsules containing olive oil; the CAPS study used capsules containing sunola oil and dietary modification; Vaisman *et al*. (192) used capsules containing canola oil as a placebo; Daamsgaard *et al*. (189) used milk or formula without added oils. Thus the different studies provided different sources and amounts of n-6 PUFAs to the control group.

The LC n-3 PUFA content of the fish oil capsules used in the different studies and the relative proportions of EPA and DHA differed between studies (Table 1.10). It is also worth noting that in the study of Nagakura *et al.* (191) the daily dose of LC n-3 PUFAs varied between subjects due to the fact that it was adjusted to the body weight of the children, which was of a wide range. Also, the supplementation period varied between the studies, ranging from 3 months (192;193), 6 months (190), 10 months (191), to 5 years (183). The differences in supplementation dosages and period of supplementation between the studies may have contributed to the heterogeneity of the effect of fish oils on allergic and atopic disease during infancy/childhood seen in the studies.

Finally, as has been said above, infants and children taking part in these five studies were supplemented during different periods of life. The CAPS study conducted the intervention from onset of formula feeding (or 6 months of age) to 5 years of age (183). The study of Damsgaard *et al.* (189) supplemented infants between months 9 and 12 of life. The other three studies of Hodge *et al.* (190), Nagakura *et al.* (191), and Vaisman *et al.* (192) conducted the intervention on children of a wide age range (8-12, 4-17, and 8-12 years of age respectively). The age differences of the subjects between the studies may also partially explain the different effects observed between the studies.

Sample size, follow up, compliance

Referring to sample size, in the CAPS study, it is possible that failure to show a clinical benefit resulted from lack of power (188). In the study conducted by Hodge *et al.* (190), authors suggested that significant changes in TNF-α production may have been detected if the sample size was larger. Finally, in the study of Damsgaard *et al.* (189), it was recognized that sample size was small and that the rate of completion with blood samples was low.

Regarding the follow up of the trials, in the CAPS study, 68% of the children remaining in the study (excluding those lost to follow-up) were available for assessment and had their blood taken at 18 months of age. At 5 years of age, 84% of the children that participated in the randomized cohort were available for assessment. In the study of Damsgaard *et al.* (189), the attrition rate was 32% (30 subjects out of 94 dropped out), and the completion rate was 88% (and it did not differ significantly between the groups). In the study of Hodge *et al.* (190) 6 children out of 45 dropped out at baseline (13% withdrawal). Vaisman *et al.* (192) did not have any dropouts given the fact that the sample size was small. In the study of Nagakura *et al.* (191) only one child out of thirty dropped out.

In the CAPS study, the moderate compliance rates might be related to the lack of effect of the intervention on allergic outcomes at 3 and 5 years of age. In the CAPS study, compliance was assessed by counting the number of capsules used. The proportion of parents who reported to have remembered to use the study spreads and oils all or most of the time of the study was 88%, and this proportion was not different between groups (188). However, when the weight change of the used capsule containers was measured, the median adherence to oil capsules during the period after age 2.5 years was only 56% and was higher in the control versus the fish oil group (62% vs. 51%, p= 0.004).

As far as compliance is a major issue in supplementation studies, Damsgaard *et al.* (189) asked volunteers to return remaining bottles and report any waste. They reported that mean fish oil consumption was 3.4 ml/day (range 0.8-5 ml/day), which was according to the advice given to the subjects (1-2 teaspoonfuls/day of the oil supplement into milk or formula). Hodge *et al.* (190) assessed compliance by counting the number of unused capsules by the participants and by food diary records repeated at 3 time points after dietary modification and supplementation. Mean number of capsules taken per day was 3 instead of 4 (for both groups), and there were no children with an average of less that 2 capsules per day. Moreover, subject

compliance was confirmed by the observed changes in plasma EPA levels over the whole period of the trial. Vaisman *et al.* (192) ensured compliance of the participants by recording the empty containers of chocolate spread in both groups on a weekly basis. However, the compliance rate was not reported. In the study of Nagakura *et al.* (191) compliance was controlled within the hospital setting.

Confounding factors

Potential confounders in the CAPS study include those related to the family (age of parents, socioeconomic status, ethnicity, parental smoking, number and age of siblings), the home environment, lifestyle and diet (intake of potential allergens, foods high in n-3 fatty acids), duration of breastfeeding, and maternal diet during late pregnancy and breastfeeding (including fish eating habits, oils/margarines use, organ meat eating habits as well as use of vitamin/mineral supplements) (183). The clinical setting (hospital) in the study carried out by Nagakura *et al.* (191), and the fact that subjects stayed in the hospital ward for almost 85% of the intervention, was ideal as the environment was highly controlled. However, in this study the two groups differed significantly in the amount of medication used for acute asthma attacks during the study. In the Danish study, sex, parity and breastfeeding duration were tested as covariates because they are known to modify immune function and development of allergies. In the formula groups, parents were asked to use formulas with a ratio of LA to ALA of about 8:1 (189).

Hodge *et al.* (190) assessed the dietary intake of children using dietary records of 1 week (at baseline, 3 months, and 6 months after intervention). It was found that the fish oil group ate significantly more fresh fish than the control group (370 ± 148 vs. 109 ± 70 g/month, p= 0.0045). Oil and margarine use did not differ between the two groups throughout the study. However, like with the studies of Nagakura *et al.* (191) and Vaisman *et al.* (192), the study of Hodge *et al.* (190) did not control for fish intake or for any other confounding factors in their analyses.

Outcome measures

The CAPS study assessed clinical outcomes of allergic diseases in children at three different time points (18 months, 3 years and 5 years of age) (188). Primary outcomes were asthma and cough at 3 years of age, and probable current asthma at 5 years of age. Wheeze was a secondary outcome measure at 5 years of age (Table 1.10). Clinical outcomes were also measured in the study conducted by Hodge *et al.* (190) in which lung function, atopy and asthma severity were assessed. Asthma was also assessed in the study of Nagakura *et al.* (191). No clinical allergy outcomes were evaluated in the studies conducted by Vaisman *et al*. and Damsgaard *et al.* Blood immune markers were determined in the studies conducted by Vaisman *et al.* (192), Damsgaard *et al.* (189), the CAPS study and in the study of Hodge *et al.* (190).

Effect of fish oil on outcome measures

In the CAPS study, primary results at 18 months of age suggested that increasing LC n-3 PUFAs in the diet had a beneficial effect on wheezing in high-risk infants (184). The results from the follow-up at 3 years of age suggested fish oil supplementation from infancy to childhood could modulate the development of allergic sensitization and airway disease at an early age, as fish oil supplementation reduced cough, but not wheeze (186). However, no effect was reported on the other end points measured such as eczema, serum IgE or doctor diagnosis of asthma. At 5 years of age there were no significant beneficial effects on clinical outcomes (lung function, allergy) (188). Almqvist *et al.* (187) reported that increased intake of LC n-3 PUFAs from early life did not affect atopy and asthma at age 5 years, which agrees with the results published by Marks *et al.* (188) about the CAPS study. Almqvist *et al* (187) reported that dietary and plasma LC n-3 PUFAs were significantly higher in the intervention group in comparison to the control group at all ages. Also, plasma n-6 PUFA levels were lower in the fish oil group.

It has to be noted that the CAPS study (188) was the first study to examine the effects of dietary modification of n-3/n-6 balance during infancy and childhood on the incidence of asthma or allergic disease in children. The infants participated in that study had a family history of asthma, which put them in higher risk of developing asthma. Possible reasons for the lack of beneficial effects of LC n-3 PUFAs on the incidence of any allergic outcome at 5 years of age may be related to suboptimal adherence to the intervention (50% and 56%

compliance in the intervention and control group, respectively), as well as to the dose of fish oil used, loss to follow-up and lack of power. The investigators estimated that the diet supplied 30 mg/day EPA and 128 mg/day DHA, an amount 3 times higher than the reference intakes for children in Australia and New Zealand (National health and medical research council, 2006). Higher doses might have resulted in more evident effects and clinically significant differences.

Damsgaard *et al.* (189) concluded that a relatively high dose of LC n-3 PUFAs from 9 to 12 months of age did not affect markers of innate immunity or inflammation in healthy infants. The tendency for an increased IFN-γ production in the fish oil group ($p = 0.08$) could be related to acceleration of immune system maturation. The accompanying tendency of a reduced IL-10 production could indicate a decrease in Th2 responses, with possible implications for the development of allergies (189), although clinical outcomes were not assessed.

In the study of Hodge *et al.* (190) fish oil supplementation along with the addition of canola oil and canola oil margarine to the diet over 6 months increased plasma n-3 fatty acid levels, but had no effect on clinical severity of asthma in the children. On the contrary, in the study of Nagakura *et al.* (191), a positive effect of the dietary supplementation with fish oil on asthma severity score and lung function in children with bronchial asthma was observed. Vaisman *et al.* (192) found that long term supplementation of moderate amounts of LC n-3 PUFAs increased the production of both pro- and anti-inflammatory cytokines which may result in a better clinical response, although clinical outcomes were not assessed. However, in this study, no baseline data on population characteristics and diet were given. Both of the groups were provided with canola oil (which is high in the n-3 PUFA α-linolenic acid). Also, the amounts of n-3 and n-6 fatty acids (mg/day) coming from the oil added to the chocolate spread were not reported for the control group.

Conclusion

According to the findings of the CAPS study which was conducted on high risk infants (184), fish oil supplementation during infancy and childhood resulted in increased n-3 PUFA status and decreased n-6 PUFA status at 18 months, 3 years and 5 years of age. However, this was not associated with the expected beneficial effects on atopic outcomes and asthma. At 18 months of age, fish oil resulted in decreased prevalence of wheeze, and higher plasma n-3

PUFA levels were associated with lower bronchodilator use, irrespective of the supplementation group. At 3 years of age, there was a lower prevalence of cough only in atopic children. Finally, at 5 years of age, no significant effects were detected on asthma, wheezing, eczema, atopy (SPT positivity) and IgE levels. The CAPS study concluded that fatty acid supplementation in infants cannot be recommended as an effective measure for primary prevention of asthma. The authors suggested that influential dietary, socioeconomic and lifestyle factors should further be explored in order to explain the apparent protective effect of oily fish consumption on asthma among children (187).

The Danish study of fish oil supplementation in low risk infants (189) also showed increased LC n-3 PUFA status, and in addition a tendency to increased IFN-γ production in the fish oil group that may be linked to faster immune system maturation. However, no other immune parameters were affected.

The other three studies were conducted in older children. Two of the studies included asthmatic children and showed that fish oil supplementation increased n-3 PUFA status. Also, Hodge *et al.* (190) showed a decrease in TNF-α production in the fish oil group, but clinical outcomes of asthma were not affected. On the other hand, Nagakura *et al.* (191) showed a decrease in asthma symptom scores, although the study took place in a controlled environment. Finally, the study of Vaisman *et al.* (192) did not provide a clear effect as fish oil supplementation resulted in increased pro- and anti-inflammatory cytokine production. It has to be noted that these children were healthy/free of atopic disease.

In conclusion, larger long-term randomized infant trials are needed to further explore the immuno-modulating effects of long-chain n-3 PUFAs and the associated impact on disease indicators and manifestations.

Table 1.10: Fish oil supplementation in infants/children and allergic outcomes in those infants/children (data from RCTs)

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Mihrshahi *et al* 2003 (184) CAPS study Single-blinded 2×2 RT Sydney, Australia (6 Hospitals)	616 Infants with family history of asthma (at least one parent or sibling with asthma) 554 Children of 616 completed the study at 18 months	1. HDM reduction, placebo supplement 2. No HDM reduction, placebo supplement 3. HDM reduction, fish oil supplement 4. No HDM reduction, fish oil supplement Fish oil group: 500 mg/day tuna oil capsules (37% n-3 PUFAs, 6% n-6, 24% MUFAs, 28% SFAs, 5% minor FAs), plus canola margarines and oil (16% n-6, 40% n-9, 6% n-3 PUFAs)	From 6 months old or onset of bottle-feeding till 5 years old Placebo group: 500 mg/day sunola oil capsules (0.3% n-3, 7% n-6 PUFAs, 82% MUFAs, 9% SFAs, 1.7% minor FAs), plus margarines and oil (40% n-6, 20% n-9, 1.2% n-3) Results at 18 months of age	At 18 months: Plasma FA SPT Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema Total serum IgE Lymphocyte cytokine responses to allergen stimulation	Plasma n-3 PUFAs were higher in the FO compared to the control group (6.7%, 95% CI 6.5-7.0, vs 5.0%, 95% CI 4.8-5.2, p< 0.0001) Plasma n-6 PUFAs were lower in the FO compared to the control group (35% vs 32.5%, p< 0.0001) Significant difference in the ratio of n-3 to n-6 FAs between the FO and control group (1:5 vs 1:7.14, p< 0.0001) The diet intervention resulted in a 9.8% absolute reduction (95% CI 1.5-18.1, p = 0.02) in the prevalence of any wheeze, and a 7.8% absolute reduction (95% CI 0.5-15.1, p= 0.04) in prevalence of wheeze for more than 1 week No effect of FO on serum IgE, SPT, doctors' diagnosis of asthma, cytokine responses The HDM intervention did not affect outcomes	Increasing dietary n-3 fatty acids might have a beneficial effect on the prevalence of wheeze during the first 18 months of life.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Mihrshahi *et al* 2004 (185) CAPS study Single-blinded 2×2 RT Sydney, Australia (6 Hospitals)	Same as above 376 Children of 616 completed the study at 18 months and had blood taken	Same as above	Same as above Results at 18 months of age	At 18 months: Plasma FAs (expressed in quintiles of n-3 exposure, regardless randomization) Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema SPT Total serum IgE	Wheeze ever (p-trend 0.031), doctor visits for wheeze (p-trend 0.047), bronchodilator use (p-trend <0.001) and nocturnal coughing (p-trend 0.032) were significantly reduced in children in the higher n-3 exposure quintiles After adjusting for breastfeeding and smoking during pregnancy, the highest exposure quintiles were only protective for bronchodilator use (OR 0.46, 95% CI 0.30-0.71, p< 0.0001) Serum IgE was reduced in the highest quintile of n-3 exposure but not significantly There was no difference in diagnosed asthma or atopy (SPT) between the exposure quintiles	Although wheeze at this age may not be a good indicator of asthma in later childhood, it is encouraging that some symptoms have been reduced in children with high n-3 fatty acid concentrations in plasma.
Peat *et al* 2004 (186) CAPS study Single-blinded 2×2 RT Sydney, Australia (6 Hospitals)	Same as above 526 Children of 616 completed the study at 3 years of age	Same as above	Same as above Results at 3 years of age	At 3 years: Plasma FAs Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema SPT Total serum IgE	Plasma n-3 PUFAs were higher in the FO compared to the control group (6.3% vs 4.9%, p< 0.0001) Plasma n-6 PUFAs were lower in the FO compared to the control group (33.3% vs 35.7%, p< 0.0001) Significant difference in the ratio of n-3 to n-6 FAs between the FO and control group (1:5.9 vs 1:7.7, p< 0.0001) In the active diet group there was a 10.0% (95% CI, 3.7-16.4) reduction in the prevalence of cough only in atopic children (p= 0.003; number needed to treat, 10) In the active allergen avoidance group there was a 7.2% (95% CI 10.1-14.3) reduction in sensitization to HDM (p= 0.05; number needed to treat, 14) No significant differences in wheeze, eczema, asthma, and serum IgE were found with either intervention	These results suggest that such interventions may have a role in preventing the development of allergic sensitization and airways disease in early childhood. This offers the prospect of reducing allergic disease in later life.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Marks *et al* 2006 (188) CAPS study Single-blinded 2×2 RT Sydney, Australia (6 Hospitals)	Same as above 516 Children of 616 completed the study at 5 years of age	Same as above	Same as above Results at 5 years of age	At 5 years: Plasma FAs Parental questionnaire: wheeze, cough, asthma history, rhinitis, eczema Clinical assessment for eczema Spirometric lung function, respiratory system resistance measurements SPT Total serum IgE	Dietary intervention resulted in: Significant difference in the ratio of n-3 to n-6 fatty acids in plasma between FO and control group (1:5.8 vs 1:7.4, p< 0.0001) The prevalence of asthma, wheezing, eczema, atopy (SPT), and IgE levels did not differ between the diet groups (p> 0.1) HDM avoidance intervention had no effect on the prevalence of asthma, wheeze or atopy at 5 years The prevalence of eczema was higher in the active HDM avoidance group (26% vs 19%, RR 1.39, CI 1.00-1.93, p= 0.06)	HDM avoidance measures and dietary fatty acid modification, in infancy and early childhood to prevent the onset of asthma and allergic disease in children at increased risk of asthma cannot be recommended. Indeed, the former may be contraindicated because of an increased risk of eczema.

Study and study design	Subjects	Intervention	Exposure period	Outcome measures	Results	Conclusions
Almqvist *et al* 2007 (187) CAPS study Single-blinded 2×2 RT Sydney, Australia (6 Hospitals)	Same as above 516 Children of 616 completed the study at 5 years of age	Same as above	Same as above Results at 5 years of age	At 5 years: Plasma FAs (regardless of randomization) Parental questionnaire: wheeze, cough, asthma history, eczema Clinical assessment for eczema Spirometric lung function SPT **Dietary intake of FA:** At 18 months with 3-day weighed food record At 3 years with semi-quantitative FFQ	Plasma n-3 FA were higher and plasma n-6 FAs were lower in the FO group than in the control group ($p< 0.0001$) Dietary intake of n-3 was higher and that of n-6 was lower in the FO compared to control group n-3 and n-6 FAs intake from the diet at 18 months and 3 years, and from FAs supplementation throughout the 5 years were not associated to respiratory and allergic outcomes at 5 years ($p= 0.39$-0.85) Plasma n-3 or n-6 FA levels were not associated with wheeze, eczema or atopy at 18months, 3 years, and 5 years ($p= 0.11$-0.96) The overall effect of plasma FA, dietary intake and supplements compliance was not associated with any respiratory or allergic outcomes at 5 years ($p= 0.35$-0.59)	Dietary n-3 supplementation and n-6 restriction during early childhood in children with high risk of developing asthma did not prevent atopy and asthma, despite the significant increase in n-3 plasma levels.
Damsgaard *et al* 2007 (189) Randomized, unmasked 2 x 2 factorial design Denmark	64 Healthy Danish infants **Cows' milk** n= 13 FO n= 20 Control **Infant Formula** n= 20 FO n= 11 Control	4 Intervention groups: Cows' milk and FO Cows' milk only Infant formula and FO Infant formula only Mean fish oil consumption 3.4 mL/day (571 mg EPA and 381 mg DHA)	From 9^{th} to 12^{th} month of age	Before and after intervention: FA composition of erythrocyte membranes, plasma IgE, CRP, sIL-2R TNF-α, INF-γ, and IL-10 production in whole-blood cultures Fecal IgA at 10 months of age	**FO supplementation**: Raised erythrocyte n-3 PUFA ($p< 0.001$) Increased induced INF-γ production ($p= 0.05$) Tended to reduce stimulated IL-10 production ($p= 0.08$) The FO intervention did not affect any of the other analyzed immune variables. Erythrocyte EPA was negatively associated with stimulated IL-10 production ($p= 0.02$)	FO supplementation suggests a faster immune maturation with no apparent reduction in immune activation, since the capacity to produce INF-γ has been proposed as a maturation marker for the immune system in early life.

Hodge *et al* 1998 (190) Double-blinded RCT Sydney, Australia	39 Asthmatic children aged 8-12 years n= 20 FO n= 19 Control	Intervention: 4 fish oil capsules (0.18g EPA, 0.12g DHA/capsule) providing 1.2 g/day n-3 PUFAs **Diet**: canola oil and canola-oil based margarines and salad dressing Control: 4 capsules (0.45g safflower, 0.45g palm, 0.1 g olive oil/capsule) **Diet**: sunflower oil and sunflower-oil based margarines and salad dressing	6 months	At baseline, 3, and 6 months after dietary modification: Plasma FAs, PBMNC TNF-α production, circulating eosinophil numbers, lung function and AHR (FVC, FEV1) SPT Asthma severity (record of expiratory flow rate, symptoms, medication)	Plasma phospholipid n-3 FAs were significantly higher in the n-3 group at 3 and 6 months compared to the n-6 group ($p< 0.001$) In the n-3 group TNF-α production decreased significantly compared to baseline ($p= 0.026$) but the magnitude of change between groups was not significant ($p= 0.075$) No significant changes in clinical outcome measures	Dietary enrichment of n-3 fatty acids over 6 months increased plasma levels of these fatty acids, reduced stimulated TNF-α production, but had no effect on the clinical severity of asthma in these children.
Nagakura *et al* 2000 (191) Double-blinded RCT Japan (Hospital setting)	29 Asthmatic children aged 4-17 years n = 15 FO n = 14 Control	Intervention: 300 mg fish oil in capsules (84 mg EPA, 36 mg DHA) Control: 300 mg olive oil capsules The number of capsules were adjusted to body weight: daily dosages of EPA and DHA were 17.0-26.8 and 7.3-11.5 mg/Kg body weight, respectively.	10 months	Asthma scoring (observation by paediatricians or nurses) Acetylocholine inhalation tests (FEV1) Plasma EPA at 1, 5, 7, 10 months after baseline	In the fish oil group but not in control : Asthma symptom scores decreased at 6, 7, 8, 9, 10 months after administration compared to baseline ($p< 0.05$) Responsiveness to acetylcholine decreased in 6, 8, 10 month after administration ($p< 0.03$) Plasma EPA levels increased only in fish oil group ($p< 0.008$)	Dietary supplementation with fish oil rich in EPA and DHA was beneficial for children with bronchial asthma in a strictly controlled environment in terms of inhalant allergens and diet.
Vaisman *et al* 2005 (192) Double-blinded RCT Israel	21 Healthy children aged 8-12 years n = 7 FO n = 14 Control	Intervention: 300 mg/day n-3 PUFA (180 mg EPA, 120 mg DHA) + 700 mg canola oil Control: 1 g of canola oil Oils were blended in chocolate spread	3 months	PBMNC cytokine production, with and without endotoxin stimulation: Pro-inflammatory: IL-1β, TNF-α, IL-6 Anti-inflammatory: IL-10, IL-1Rα	The levels of all cytokines were higher in non-stimulated and stimulated cultures, in n-3 PUFA-treated subjects compared to controls: Without stimulation: IL-lβ, TNF-α,IL-6 ($p= 0.003$, $p= 0.052$, $p= 0.004$, respectively), IL-10 and IL-1Rα ($p= 0.004$, $p= 0.02$, respectively) With stimulation: IL-β, TNF-α and IL-6 ($p= 0.002$, $p= 0.011$ and $p= 0.006$, respectively) IL-10 and IL-1Rα ($p= 0.073$ and $p= 0.008$, respectively) There was no difference in the IL-1b/ IL-1Rα ratio between the two groups, with and without stimulation ($p= 0.76$ vs $p= 0.20$, respectively)	Children on n-3 PUFA supplementation had increased production of both pro-inflammatory and anti-inflammatory cytokines, with and without endotoxin stimulation.

RCT, randomized control trial; CAPS; Childhood Asthma Prevention Study; OR, odds ratio; CI, confidence interval; FA, fatty acids; PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; SFA; saturated fatty acids; FO, fish oil; SPT, skin prick testing; HDM; house dust mite; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; sCD14, soluble CD14; CRP, C-reactive protein; Ig, immunoglobulin; IFN, interferon; IL, interleukin; TNF, tumour necrosis factor; vs, AHR, airway hyper-responsiveness; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; versus.

1.6 Summary, conclusions, aims and hypotheses

LC n-3 PUFAs from oily fish and fish oils affect inflammation and immune function. They act, in part, by opposing the actions of LC n-6 PUFAs. Dietary intake of n-6 PUFAs has increased in the previous 40 years or so. Prevalence of atopy and its clinical manifestations has also increased over this period. It is suggested that the increase in n-6 PUFA intake is causally linked to the increase in atopy, with ARA-derived eicosanoids playing a role. Studies of fatty acid composition in atopy provide some support for a predisposing effect of n-6 PUFAs and propose a protective effect of LC n-3 PUFAs, but these data are rather inconsistent. Epidemiological studies provide consistent evidence that fish intake in pregnancy is associated with reduced risk of atopy in infancy and childhood. This is important because atopic sensitisation occurs early in life, perhaps *in utero*, and so it may be that very early exposures to n-6 or n-3 PUFAs might be critical. Indeed, supplementation studies with fish oil in pregnant women demonstrate some immunological effects in cord blood although longer term effects on disease are less clear. One reason for discrepancy between epidemiological studies of fish consumption and supplementation studies with fish oil is that fish provide not only LC n-3 PUFAs, but other nutrients as well, and these other nutrients could play a role. It could be speculated that it is the nutrients of the oily fish as a whole or the combination of these nutrients with LC n-3 PUFAs that is important. To test this idea early intervention with oily fish is required, as the epidemiology only suggests associations and is not proof of a causal effect.

There have been no intervention trials conducted with oily fish in pregnant women. This thesis describes such a study: The Salmon in Pregnancy Study (SIPS). SIPS is a randomised controlled trial of oily fish (farmed salmon) in pregnant women looking at fatty acid status, immune functions and atopic outcomes in their offspring. The hypotheses being investigated in SIPS are that: 'increased consumption of oily fish during pregnancy by women at risk of having offspring who will develop atopy will increase their LC n-3 PUFA status and antioxidant status and that of their developing baby and will ameliorate the development of atopic markers and manifestations in the infants'. The work described in this thesis is part of SIPS. The aims of my study are:

1. to increase oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/week) during pregnancy, in women whose offspring are at a high risk of developing atopic disease;

2. to investigate the effect of increased maternal oily fish consumption on cord plasma and cord blood mononuclear cell (CBMC) LC n-3 PUFA status;
3. to investigate the effect of increased maternal oily fish consumption on markers of infant immunity at birth (i.e. in cord blood);
4. to investigate the effect of increased maternal oily fish consumption on atopic sensitization and atopic dermatitis in infants at 6 months of age.

The research hypotheses of my study are that increased consumption of farmed salmon during pregnancy by women with high risk of having an atopic offspring will:

1. increase maternal LC n-3 PUFA intake;
2. increase cord plasma LC n-3 PUFA status;
3. increase CBMC LC n-3 PUFA status;
4. alter immune markers in cord blood in a way that would indicate decreased atopy risk;
5. reduce sensitisation to common allergens and reduce atopic dermatitis score in infants at 6 months of age.

2 Salmon in pregnancy study (SIPS): Aims & hypotheses, study design and characteristics of the subjects

2.1 Aims and research hypotheses of my study

In section 1.3 the early life origins of atopic disease were described, including some of the evidence that sensitisation occurs *in utero*. In section 1.4 the effects of polyunsaturated fatty acids (PUFAs) on the immune system were summarised; through these effects n-6 and n-3 PUFAs might differentially influence sensitisation and the immunological processes associated with atopic diseases. Based on these effects long-chain (LC) n-3 PUFAs might reduce the risks of developing atopic diseases. Oily fish and fish oils are good sources of these fatty acids. Epidemiological studies, reviewed in section 1.5.1, suggest a protective effect of fish consumption by pregnant women or by children towards atopic diseases, while intervention studies with fish oil supplements in pregnant women, reviewed in section 1.5.2, demonstrate some immunological effects in cord blood but longer term effects on disease outcomes are less clear. One reason for discrepancy between epidemiological studies of fish consumption and supplementation studies with fish oil is that fish provide not only LC n-3 PUFAs, but other nutrients as well, and these other nutrients could play a role. It could be speculated that it is the nutrients of the oily fish as a whole or the combination of these nutrients with LC n-3 PUFAs that is important. To test this idea early intervention with oily fish is required, as the epidemiology only suggests associations and is not proof of a causal effect.

Currently in the UK it is recommended that pregnant women and women of reproductive age should consume oily fish but not more than two portions weekly, because of the presence of contaminants in some fish (23). However, this concern can be reduced by consuming farmed fish in which contaminant levels can be controlled and made to be very low since the feeds of the fish can be controlled as well as the waters in which they breed and grow.

There have been no intervention trials conducted with oily fish in pregnant women. The Salmon in Pregnancy Study (SIPS) described here is the first randomised controlled trial of oily fish (farmed salmon) in pregnant women; the study was single blind (investigator blind) and the babies of the women recruited were at high risk of developing atopic disease. SIPS is registered at www.clinicaltrials.gov website with the official title: 'The effects of oily fish in pregnancy on markers and manifestations of allergic diseases in infants at risk of atopy' (clinical trials identifier number: NCT00801502) (http://www.clinicaltrials.gov/ct2/show/NCT00801502?term=sips&rank=1).

This study sets out to identify the effects of increasing salmon intake in pregnant women. The hypotheses being investigated, as stated at www.clinicaltrials.com are that: 'increased consumption of oily fish during pregnancy by women at risk of having offspring who will develop atopy will increase their LC n-3 PUFA status and antioxidant status and that of their developing baby and will ameliorate the development of atopic markers and manifestations in the infants'.

The primary outcome measures of SIPS (as stated at www.clinicaltrials.gov) are LC n-3 PUFA fatty acid status in maternal and umbilical cord plasma [Time Frame: weeks 20, 34 and 38 of pregnancy and at birth (in cord)].

The secondary outcome measures of SIPS (as stated at www.clinicaltrials.gov) are:

1. Antioxidant status in maternal and umbilical cord blood [Time frame: weeks 20, 34 and 38 of pregnancy and at birth (in cord)];
2. Allergic sensitisation of infants [Time frame: 6 months of age];

The aims of my study are:

1. to increase oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake during pregnancy (two portions/week) in women whose offspring are at a high risk of developing atopic disease;
2. to investigate the effect of increased maternal oily fish consumption on cord plasma and cord blood mononuclear cell (CBMC) LC n-3 PUFA status;
3. to investigate the effect of increased maternal oily fish consumption on markers of infant immunity at birth (i.e. in cord blood);
4. to investigate the effect of increased maternal oily fish consumption on atopic sensitization and atopic dermatitis in infants at 6 months of age.

The research hypotheses of my study are that increased consumption of farmed salmon during pregnancy by women with high risk of having an atopic offspring will:

1. increase maternal LC n-3 PUFA intake;
2. increase cord plasma LC n-3 PUFA status;
3. increase CBMC LC n-3 PUFA status
4. alter immune markers in cord blood in a way that would indicate decreased atopy risk;
5. reduce sensitisation to common allergens and reduce atopic dermatitis score in infants at 6 months of age.

The research hypotheses are presented (description, methodology and results) and discussed in chapters 3-6.

The core SIPS investigators were:

Professor Philip C. Calder	Program coordinator and principal investigator
Dr Elizabeth A. Miles	Senior investigator
Dr Paul S. Noakes	Postdoctoral investigator
Mrs Norma D. Diaper, MSc	Senior research nurse
Miss Lefkothea-Stella Kremmyda, MSc	PhD student and nutritionist
Miss Maria Vlachava (MV), MSc	PhD student and nutritionist

It is important to make a clear statement of my involvement. Table 2.1 lists the activities in which I (MV) participated and the activities from which results were generated for inclusion in this thesis.

Table 2.1: Statement of the activities of MV in the SIPS

Activities performed by MV	Activities generating results in MV thesis
Recruitment and screening appointment:	
Allergy and lifestyle questionnaires	Yes
Body composition (BC) measurements	No
20, 34, 38 week maternal appointment:	
Ultrasound scan	No
Health and lifestyle questionnaires	Yes
FFQ	Yes
BC measurements	No
Urine sample collection	No
Blood samples: processing and analysis	Yes
Fish diary during pregnancy: given at 20 week appointment (started at 21st week) and collected at 38 week appointment	Yes
Stool sample collection at 38 weeks	No
Birth:	
Anthropometry measures	Yes
Cord blood samples: processing and analysis,	Yes
Cord & placental tissue: processing	No
Within 2 weeks after birth:	
DXA scan: maternal and infant	Yes (infant only)
Maternal BC	No
Maternal BC measured at home for 14 days	No
3 month visit:	
Maternal FFQ:	No
Partial collection of diaries: infant feeding and health, post partum maternal fish diary	No
Collection of infant stool samples from days: 1, 7, 14, 28 after birth	No
Collection of breast milk samples from days: 1, 5, 7, 14 after delivery	No
6 month visit: clinical assessment of allergy:	
Infant, maternal, paternal SPT, infant SCORAD	Yes
Paternal blood sample	No
Infant anthropometry	Yes
Infant blood sample: processing and analysis	Yes
Infant FFQ	Yes
Collection of diaries: post partum maternal (fish diary) and infant (feeding and health diary) (the rest)	No

2.2 Study design, power calculation, population studied and data collection

2.2.1 Study design

SIPS was a randomised, controlled intervention trial with oily fish in pregnant women. A group of 123 pregnant women with high risk of giving birth to an atopic infant and with a self-reported low habitual intake of oily fish were randomly assigned to consume two portions of oily fish (salmon) per week or to continue their habitual diet low in oily fish, from week 20 of gestation until the end of their pregnancy.

Eligibility criteria for inclusion in the study were:

1. In the early stages of pregnancy (around 13 weeks).
2. Habitual intake of ≤ 2 portions of oily fish per month (excluding tinned tuna).
3. Family history of atopy within the last 5 years (including asthma, atopic dermatitis (itchy rash/eczema), seasonal rhinitis (hay-fever), perennial rhinitis (other allergies)) in any first degree relative (mother, father or sibling of the fetus).
4. Not consuming fish oil supplements.
5. Aged between 18-40 years.

Exclusion criteria were:

1 Habitual consumption of oily fish (> 2 portion of oily fish per month excluding tinned tuna).
2 Use of fish oil supplements within the previous 3 months.
3 Use of non-steroidal anti-inflammatory drugs (NSAIDs) (e.g. aspirin, diclofenac, ibuprofen).
4 Multiple pregnancy.
5 Not willing for essential identifiable information being stored for tracking purposes.
6 Participation in another research study.
7 Known diabetic.
8 Known to suffer from auto-immune disease, e.g., multiple sclerosis, thyroid disease, systemic lupus erythematosis.

9 Having a learning disability.

10 Having a terminal illness.

11 Having a mental health problem.

Ethical approval for the study was obtained from the Southampton and South West Hampshire Research Ethics Committee (07/Q1704/43).

2.2.2 Power calculation

The SIPS study was powered according to two outcomes: fatty acid composition of maternal plasma phospholipids and infant sensitisation to at least one common allergen.

Fatty acid composition of plasma phospholipids

Based upon previous work, the content of eicosapentaenoic acid (EPA) in plasma phospholipids in these women was expected to be approx. 1% of fatty acids (196). Two salmon meals per week were anticipated to provide a total of approximately 4 g EPA plus docosahexaenoic acid (DHA)/week; of this about 2.8 g would be EPA, equivalent to an increased intake of 0.4 g EPA/day (background intake was expected to be approx. 0.1 g/day). This was expected to increase the EPA content of plasma phospholipids by 50% to about 1.5% of fatty acids. Using known standard deviations for the EPA content of plasma phospholipids in adults in the Southampton area, a sample size of 50 per group was anticipated to have a 99% power to detect this difference with $P < 0.05$ (90% power to detect with $P < 0.001$). This power calculation was performed before the study salmon had been produced and analysed. It turned out that the EPA plus DHA content of the salmon was lower than anticipated (equivalent to 3.46 g/week), but this difference was not considered to affect the power of the study to identify a significant effect of the salmon twice per week on the EPA content of maternal plasma phospholipids.

Sensitisation to at least one common allergen

Infants born to women recruited into the study were considered to be at high risk of becoming sensitized to allergens. Based upon personal communication with Professor John Warner it was anticipated that 45% of infants would be sensitized at 6 months of age. It was estimated that infants of women in the oily fish group would be 30% less likely to have been sensitized (based upon data from Dunstan *et al.* (132) using fish oil in at risk pregnant women). A sample size of 50 per group would have a 70% power to detect this

difference with $P < 0.05$. It turned out that a much lower percentage of infants than this were sensitised (see Chapter 6).

2.2.3 Subject appointments - data collection and follow-up

Women taking part in the study were seen at 13, 19-20 (referred to as 20 weeks hereafter), 32-34 (referred to as 34 weeks hereafter), and 38 weeks of pregnancy. At 13 weeks gestation women were screened for eligibility during their routine ultrasound scan appointment at the Southampton Women's Survey Ultrasound Unit (SWS UU) of Princess Anne Hospital in Southampton, UK. Prior to their appointment, they had received an initial information sheet about the SIPS (Appendix 1), along with their appointment notes sent by the SWS UU. Women were initially approached by a researcher of the SIPS team (myself, Stella Kremmyda or Norma Diaper) and were asked if they would like to hear more about the study and be screened for eligibility. Women who agreed to be screened were asked to sign a consent form for initial screening (Appendix 2). Initial screening included a health and lifestyle questionnaire administered by the researcher including questions on eligibility criteria, weight and height measurement (Appendix 3), and body composition measurement performed by bioelectrical impedance.

Women who were eligible for the study were provided with a second information sheet (Appendix 4), the study was further explained to them and their intention to take part in the study irrespective of randomization group was confirmed. A randomization plan was created through the website http://randomization.com, which randomized each subject (in the order that they were found to be eligible) to one of the two groups by using the method of randomly permuted blocks (2 blocks: control and salmon). Women who were interested in the study but had a preference for either of the two groups were not included. At 15 weeks of pregnancy women were phoned to remind them about the study, discuss any concerns and confirm their next appointment at 19-20 weeks of pregnancy at SWS UU.

Women attended the 19-20 week appointment in the fasted state. They were asked to sign the consent form for participating in SIPS (Appendix 5) and they were randomly assigned to one of the two groups, intervention or control. The appointment included an ultrasound scan, weight measurement and body composition analysis by bioelectrical impedance, a researcher-administered health and lifestyle questionnaire (Appendix 6), blood collection, urine collection, and a food frequency questionnaire (FFQ) covering the past three months administered by an appropriately trained researcher (myself or Stella Kremmyda; Appendix 7). Women were given breakfast after sample collection and before the FFQ was

administered. Women in the intervention group were also provided with a cook book with salmon recipes and a fish diary to record their consumption of the study salmon (and any other fish they may consume), how it was cooked, and whether there were any leftovers from the standard portion (Appendix 8). Women in the control group were provided with a general cook book including information on healthy eating during pregnancy, and a fish diary (Appendix 9) to record their fish consumption (type, way of cooking, portion size). Fish diaries were used to measure compliance of the subjects in both groups and to estimate nutrient intake from fish consumption.

The fasting appointments at 32-34 and 38 weeks of pregnancy were identical to the one at 19-20 weeks of pregnancy, except that at week 38 a stool sample was also collected and there was no FFQ administration or body composition measurement (Appendices 10 and 11).

The health and lifestyle questionnaire was administered by an appropriately trained member of the SIPS team (myself, Lefkothea-Stella Kremmyda, Norma Diaper) at 13, 20, 34, and 38 weeks of pregnancy. It included questions on medication, use of nutritional supplements/vitamins/minerals in the previous 4 weeks, smoking, alcohol consumption, atopic symptoms of the mother, father and sibling(s) (only at 13 weeks), pet ownership, visiting households with pets, oily and non-oily fish consumption frequencies (only at 13 weeks), level of education and occupation (only at 20 weeks), and problems with eating the salmon if in the intervention group (at 32-34 and 38 weeks) (Appendices 3, 6, 10, and 11). Any problems related to the salmon were reported to the research nurse to ensure that both nutritionists remained blind to the treatment group of the subjects. Characteristics of the women at study entry were identified from information gathered from the week 13 and 20 questionnaires.

Physical activity questions were incorporated in the Health and Lifestyle questionnaire and were administered by an appropriately trained member of the SIPS team at 20, 34 and 38 weeks of pregnancy. They included questions on frequency of performing physical activities of different intensity (vigorous, moderate, gentle exercise), as well as hours spent sleeping/lying down, sitting, standing/walking over a typical 24 h day, and walking speed at present. The questions reflected average physical activity in the three months prior to the time point of the interview. The questions were adopted from the Southampton Women's Survey (SWS) questionnaire for estimating physical activity, which has been used before on women in Southampton (194-196).

Height was measured at 13 weeks gestation using a stadiometer. Subjects were asked to remove their shoes and stand straight. The head was placed in the Frankfort Plane, such that an imaginary line joining the upper margin of the external auditory meatus and the lower border of the orbit of the eye was horizontal. The measurement was taken once and to the nearest 0.1 cm.

Weight and body composition were measured using a MC-180MA Tanita, at 13, 20, 34, and 38 weeks gestation. This is a bioelectrical impedance device using multi-frequency 8-electrodes to measure body composition. Electrolytes within the body are naturally conductive. More electrolytes are contained in muscle and blood, but few in fat. Electricity flows more easily through body tissue with more muscle and less fat while, conversely, electricity experiences more resistance in body tissue with less muscle and more fat. Using this fact, the component ratios of each can be estimated by measuring body impedance. Using the 8-electrode method, an electric current is supplied from electrodes at the ends of the fingers on both hands and the toes on both feet, and the voltage is measured at the ball of the thumbs on both hands and at the heels of both feet. This method enables measurement of impedance per segment – in other words for the whole body, right leg, left leg, right arm or left arm – by switching the points at which the current is applied and the voltage is measured. A multi-frequency measurement is used to separate between intercellular and extracellular water, and estimate total body water. Subjects were measured in the fasting state and having emptied their bladder. They were asked to remove their shoes and socks, and wipe their hands and the soles of their feet before making the measurements. They were also asked to remove rings, watches and metallic accessories when possible, as well as heavy clothing. They were then asked to stand on the electrode panel, placing their feet apart so as to avoid inner thighs contact. Similarly, subjects were asked to avoid contact between arms and torso. The measurement was performed once following the instructions given by the manufacturer. The results of the body composition measurements are not included in the present thesis.

Midwives at the Princess Anne Hospital, Southampton and the rest of birth centres (if a subject was planning to deliver in a birth centre different from the Princess Anne Hospital or at home) were provided with information about the study and were given laminated instructions to remind them to collect cord blood (at room temperature and at 4˚C). They were asked to contact the SIPS team once any samples were collected. At birth or very soon after occipito-frontal circumference (head circumference) (cm), crown-heel length (cm) and weight (kg) of the baby were measured by the SIPS research nurse (Norma Diaper) (Appendix 19).

The neonate's crown-heel length was measured with a neonatometer (CMS Ltd., London, United Kingdom), and length was measured with a portable stadiometer (Leicester height measurer; CMS Ltd.). Within 14 days of birth infant body composition was assessed by dual-energy X-ray absorptiometry (DXA) with a Lunar DPX-L instrument (GE Corp, Madison, WI, USA). Fat mass and fat-free mass were derived with a whole-body scan by using pediatric software. The total X-ray dose for the whole-body scans was 10.5 microsieverts (pediatric scan mode), equivalent to 1–2 d background radiation (197). The infant body composition information is included in this chapter (characteristics of the infants at birth).

At 3 months after delivery, a home visit was arranged with all subjects. This appointment included administration of the maternal FFQ, partial collection of the fish and infant feeding diaries (Appendices 8/9 and 12) which women were asked to complete until 6 months after birth, collection of breast milk and infant stool samples and provision of the information sheet corresponding to the final visit of the study at 6 months of baby age (Appendix 13). The breast milk and stool samples were stored in the freezers of the volunteers and were transferred to the IDS Building, University of Southampton in a portable cooler on the day of the 3-month visit. The mothers were also asked to measure their body composition for the first 14 consecutive days after birth (body weight and percentage of body fat) using Tanita portable scales (UM-014S) provided to them (Appendix 15). None of this information is included in the present thesis.

At 6 months of baby age, a clinical visit was arranged. The mother, father and study infant were invited to attend the Wellcome Trust Clinical Research Facility (WTCRF) at Southampton General Hospital. The main purpose of the visit was the clinical assessment of atopic disease of all the family members including the infant. In addition to members of the SIPS research team, a paediatric nurse was involved. The duration of this visit was 1.5 hours. Initially the research nurse gave an overview of the visit to the parents and obtained written informed consent from them (Appendix 14). The appointment included skin prick testing (SPT) (Appendices 16 & 17) of both parents and infant, measuring infant weight and length, assessment of atopic dermatitis using SCORAD and the UK Working Party Diagnostic Criteria for Atopic Dermatitis adapted for use in infant (Appendix 18), administering a clinical assessment questionnaire (adapted from the questionnaire used by SWS at age 6 months) and assessment of infant diet (using an infant FFQ adapted from the FFQ used by SWS at age 6 months) all included in the 6 month questionnaire (Appendix 20) as well as collection of the rest of the fish diaries covering maternal fish consumption from month 3 to month 6 post-natally and a diary recording infant feeding and health. On

completion of clinical examination, the research nurse discussed any significant clinical findings with the parents and explained options available for further clarification and management issues. The infant clinical results and other (diet, anthropometry) information are included in Chapter 6.

Maternal and paternal SPT were performed at 6 months post partum at the 6 month appointment. All the different appointments and assessments during the study described above are summarised in Figure 2.1.

SPT was performed by the research nurse of the study (N.D. Diaper) on the flexor aspect of the forearm using a standardized technique and allergen extracts, as well as histamine as a positive control and glycerine diluent (Soluprick SQ, ALK Abello) as a negative control. A drop of each allergen and the controls was introduced into the epidermis using a lancet, without drawing blood. A wheal diameter of ≥ 2 mm greater than the negative control was considered positive after the application of the allergen extracts. Table 2.2 presents the allergens and controls used. The potency is expressed in 'Histamine Equivalent in skin Prick testing' (HEP) which is related to the allergenic activity of the allergen. This data is included in this chapter (baseline characteristics).

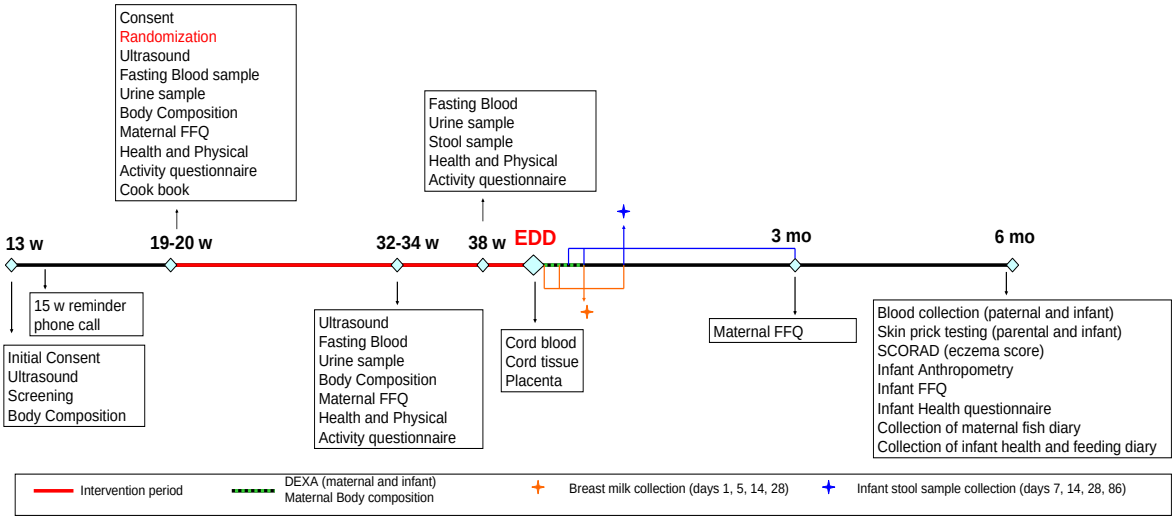


Figure 2.1: Flow chart of the study design and the different assessments conducted at the different timepoints.

Table 2.2: Allergens used in maternal and paternal SPT

Type	Strength
Negative control (glycerine)	Glycerine diluent
Dermatophagoides pteronyssinus (house dust mite (HDM))	10 HEP
Felix domesticus (cat fur; referred to as cat)	10 HEP
Canis familiaris (dog fur; referred to as dog)	10 HEP
6 Grass pollen mix (Avena/Dactylis/Poa/Festuca/Lolium/Phleum)	10 HEP
3 Tree pollen mix (Alnus/Betula/Corylus)	10 HEP
Aspergillus fumigatus (mould)	1:20 w/v
Alternaria alternata (mould)	1:20 w/v
Cladosporium herbarum (mould)	1:20 w/v
Penicillum notatum (mould)	1:20 w/v
Positive control (histamine)	10 mg/ml

2.2.4 The intervention – farmed salmon

Salmon for use in the SIPS were reared at Skretting Aquaculture Research Centre, Stavanger, Norway using high quality feed ingredients in order to be low in contaminants (dioxins and dioxin-like polychlorinated biphenyls (PCBs), dichloro diphenyl trichloroethanes (DDTs)) including heavy metals (arsenic, cadmium, mercury, lead). The diet contained (g/kg): fish meal (298.6), soya concentrate (141.3), maize gluten (99.5), wheat gluten (29.9), wheat (120.1), Southern Hemisphere fish oil (132.1), rapeseed oil (132.1), flaxseed oil (33.5), DL-methionine (0.32), L-lysine (0.9), monocalcium phosphate (5.64), and vitamin and mineral mix. The dietary ingredients used were selected to be low in contaminants. Salmon were farmed until they averaged 4 kg in weight and were then killed; a total of 2280 kg (gutted) of salmon were produced. They were then filleted into 150 g portions and the portions frozen in vacuum sealed bags in Norway, from where they were shipped to Southampton and stored at -30˚C (Figure 2.2). The salmon were delivered to the intervention group, on a monthly basis, and enough to cover intake of two portions (150 g/portion) per week by the pregnant women and their partners was provided. After delivery salmon were stored in home freezers.

Figure 2.2: Vacuum sealed salmon as delivered to the pregnant women.

Frozen portions of salmon were analysed by the National Institute for Nutrition and Seafood Research, Bergen, Norway. Thirty (30) portions were randomly sampled for analysis. The individual portions were pooled into 3 samples of 10 portions each for analysis of nutrient and contaminant content. Table 2.3 presents the fatty acid content of the tailored salmon with data expressed as g/100 g of total fatty acids, as g/kg of salmon fillet, and as g/per standard 150 g portion of salmon. The sum of EPA plus DHA was 1.73 g/portion and these two fatty acids provided 10.6% of the total fatty acids in the salmon.

Table 2.4 presents selected vitamin, mineral, total protein and fat content in the tailored salmon expressed as per kg of salmon, as well as per standard 150 g portion of salmon. Table 2.5 presents selected contaminant and heavy metal content of the salmon, expressed also per standard portion of salmon. As a result of the controlled farming environment and the high quality feeds used, the contaminant content of the salmon fillets was low, with two portions providing much less than the provisional tolerable weekly intake of all contaminants.

Table 2.3: Fatty acid composition of tailored salmon (mean ± standard deviation; n = 3)

FATTY ACID	% OF TOTAL FATTY ACIDS	G/KG FILLET	G/150 G FILLET
C14:0	2.3 ± 0.1	2.4 ± 0.3	0.36
C16:0	12.3 ± 0.3	13.2 ± 0.7	1.98
C18:0	3.3 ± 0.1	3.6 ± 0.2	0.54
Sum of SFAs	**18.6 ± 0.3**	**20.1 ± 1.2**	**3.02**
C16:1n-7	2.5 ± 0.1	2.7 ± 0.3	0.41
C18:1n-9	33.1 ± 0.4	35.7 ± 2.5	5.31
C18:1n-7	2.7 ± 0.1	2.9 ± 0.2	0.44
C20:1n-9	2.8 ± 0.1	2.9 ± 0.4	0.44
Sum of MUFAs	**43.3 ± 0.4**	**46.8 ± 3.8**	**7.02**
C18:2n-6	11.6 ± 0.2	12.5 ± 1.1	1.88
C20:2n-6	1.2 ± 0.1	1.3 ± 0.1	0.20
C20:4n-6	1.3 ± 0.1	1.4 ± 0.1	0.21
Sum of n-6 PUFAs	**14.3 ± 0.1**	**15.4 ± 1.2**	**2.31**
C18:3n-3	7.4 ± 0.3	7.9 ± 0.3	1.19
C18:4n-3	0.6 ± 0.1	0.7 ± 0.1	0.11
C20:4n-3	1.1 ± 0.1	1.2 ± 0.1	0.18
C22:5n-3	2.1 ± 0.1	2.3 ± 0.2	0.35
C20:5n-3 (EPA)	3.5 ± 0.2	3.8 ± 0.4	0.57
C22:6n-3 (DHA)	7.1 ±0.1	7.7 ± 0.5	1.16
Sum of EPA + DHA	**10.6**	**11.5**	**1.73**
Sum of n-3 PUFAs	**22.0 ± 0.2**	**23.7 ±1.5**	**3.56**
Sum of total PUFAs	**36.5 ± 0.2**	**39.4 ± 2.7**	**5.91**
Total Fatty Acids	**100 ± 0.00**	**107.9 ± 7.8**	**16.19**
n-3 PUFA/n-6 PUFA	1.53 ± 0.02		

Table 2.4: Vitamin, mineral, total protein and total fat content of tailored salmon (mean ± standard deviation; n = 3)

VITAMINS	**MG/KG FILLET**	**MG/150 G FILLET**
α-tocopherol	27.3 ± 0.1	4.1
γ-tocopherol	10.9 ± 1.2	1.6
Vitamin A (sum of retinols)	0.039 ± 0.006	0.006
Vitamin D_3	0.093 ± 0.001	0.014
Vitamin K_1	< 1	
Vitamin K_2	< 1	
Minerals	**mg/kg fillet**	**mg/150 g fillet**
Iodine	0.044 ± 0.008	0.006
Iron	2.49 ± 0.21	0.373
Selenium	0.288 ± 0.008	0.043
Zinc	2.85 ± 0.06	0.428
	g/kg fillet	**g/150 g fillet**
Total protein	203 ± 2	30.5
Total fat	109 ± 6	16.4

Table 2.5: Contaminant and heavy metal content of tailored salmon (mean ± standard deviation; n=3)

COMPOUND	**PER KG FILLET**	**PER 150 G FILLET**
Sum of dioxin and dl-PCBs	0.35 ± 0.06 pg WHO TEQ/g	52.5 pg
Sum of DDTs	3.28 ± 0.4 µg	0.492 µg
Arsenic	0.34 ± 0.05 mg	0.051 mg
Cadmium	0.001 ± 0.01 mg	0.15 µg
Mercury	0.023 ± 0.00 mg	3.45 µg
Lead	0.001 ± 0.001 mg	0.15 µg

PCBs, polychlorinated biphenyls; dl, dioxin like; DDTs, dichloro diphenyl trichloroethanes; WHO TEQ, World Health Organisation toxic equivalent

Table 2.6 presents the calculated total intake of selected compounds from consumption of two 150 g portions of the tailored salmon per week. As can be seen from the table, consumption of two portions of the salmon per week will result in an intake of 493 mg of EPA plus DHA per day, which meets the proposed intake set by the SACN (23), which is 450 mg/day. The calculated weekly contaminant intake expressed as per kg body weight is lower than the provisional tolerable weekly intake (PTWI) established by the Joint Food and Agricultural Organisation/World Health Organisation Expert Committee on Food Additives (198;199).

Table 2.6: Weekly and daily intake of DHA plus EPA, and weekly intake (crude and per kg of body weight) for selected contaminants from consumption of two portions (each 150 g) of tailored salmon per week

COMPOUND	PER WEEK[1]	PER DAY[1]	PER KG BODY WEIGHT/WEEK[2]	PTWI
EPA+DHA	3.45 g	493 mg		
Sum of dioxin and dl-PCBs	105 pg		1.3-1.75 pg WHO TEQ	14 pg WHO TEQ
Sum of DDTs	0.984 µg		0.012-0.016 µg	
Arsenic	0.102 mg		1.3-1.7 pg	15 µg
Cadmium	0.3 µg		0.004-0.005 pg	7 µg
Mercury	6.9 µg		0.09-0.115 pg	5 µg
Lead	0.3 µg		0.004-0.005 pg	25 µg

PTWI, provisional tolerable weekly intake; PCBs, polychlorinated biphenyls; dl, dioxin like; DDTs, dichloro diphenyl trichloroethanes; WHO TEQ, World Health Organisation toxic equivalent

[1] Based on consumption of 2 × 150 g tailored salmon fillets per week

[2] Calculations based on a range of 60-80 kg body weight

2.3 Progress of the study and subject follow-up

Figure 2.3 shows the recruitment process of the subjects who participated in SIPS in both treatment groups. 692 women were sent the initial information sheet about SIPS together with the appointment letter for their 13 week gestation ultrasound scan. Out of those, 377 agreed to and were screened for eligibility for SIPS. The major reason for not agreeing to be screened was lack of interest in the study. The recruitment period lasted for 12 months (from July 2007 until July 2008). From the 377 women who agreed to be screened, 166 were eligible to participate to SIPS and the other 211 were ineligible mainly because of absence of atopy. Of the 166 eligible, 123 women were finally randomized at 15 weeks (by telephone contact) to participate. The other 43 women were either randomized at 15 weeks but not enrolled at the first SIPS appointment (20 weeks of gestation) (n=35) or were not anymore interested at 15 weeks to participate in the study (n=8). The number of women recruited (n=123) was to assure that approximately 100 mother-neonate pairs would remain in the study at birth on the assumption that about 20% would drop-out. In fact there were 107 mother-neonate pairs still in the study at birth (see below).

Figure 2.4 presents the progress of women and their neonates through the study from the first appointment (20 weeks gestation; i.e. baseline) until the end of the study at 6 months post-partum (clinical assessment of the infants). After signing the SIPS consent form, 62 and 61 pregnant women were randomly assigned to the salmon and control groups, respectively. Before delivery 9 subjects withdrew from the salmon group and 7 from the control group. 14 subjects delivered before their 38 week appointment. Between delivery and 6 months 6 more subjects withdrew from the salmon group and 15 more from the control group and finally 86 subjects completed the 6 month appointment (n=47 in salmon group and n=39 in control group). In total the attrition rate was 30%; in the salmon group this was 24% and in the control group 36%. At the 6 month appointment the parents were also offered SPT and this was completed by 86 mothers and 76 fathers.

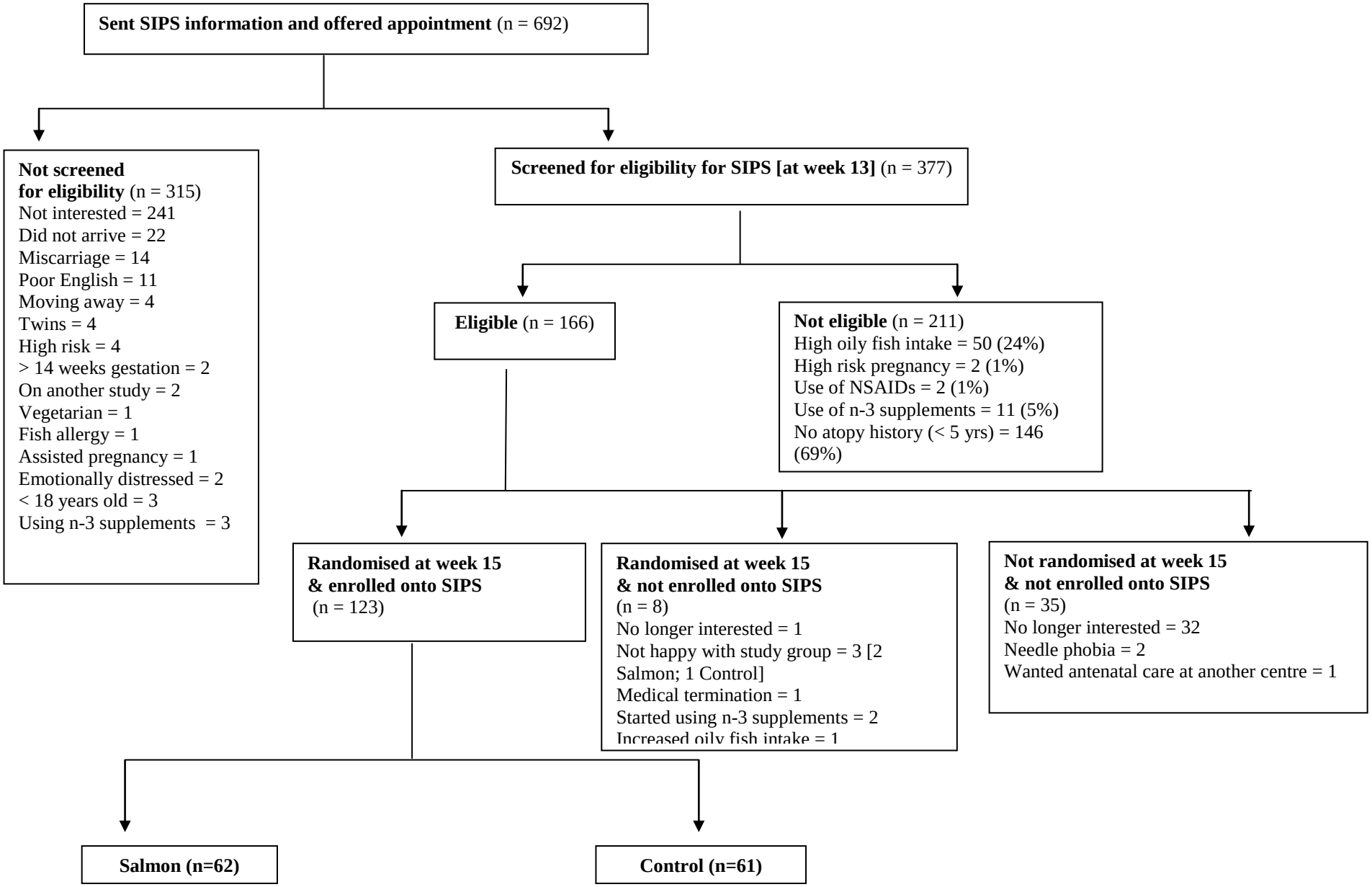


Figure 2.3: Recruitment of women into SIPS

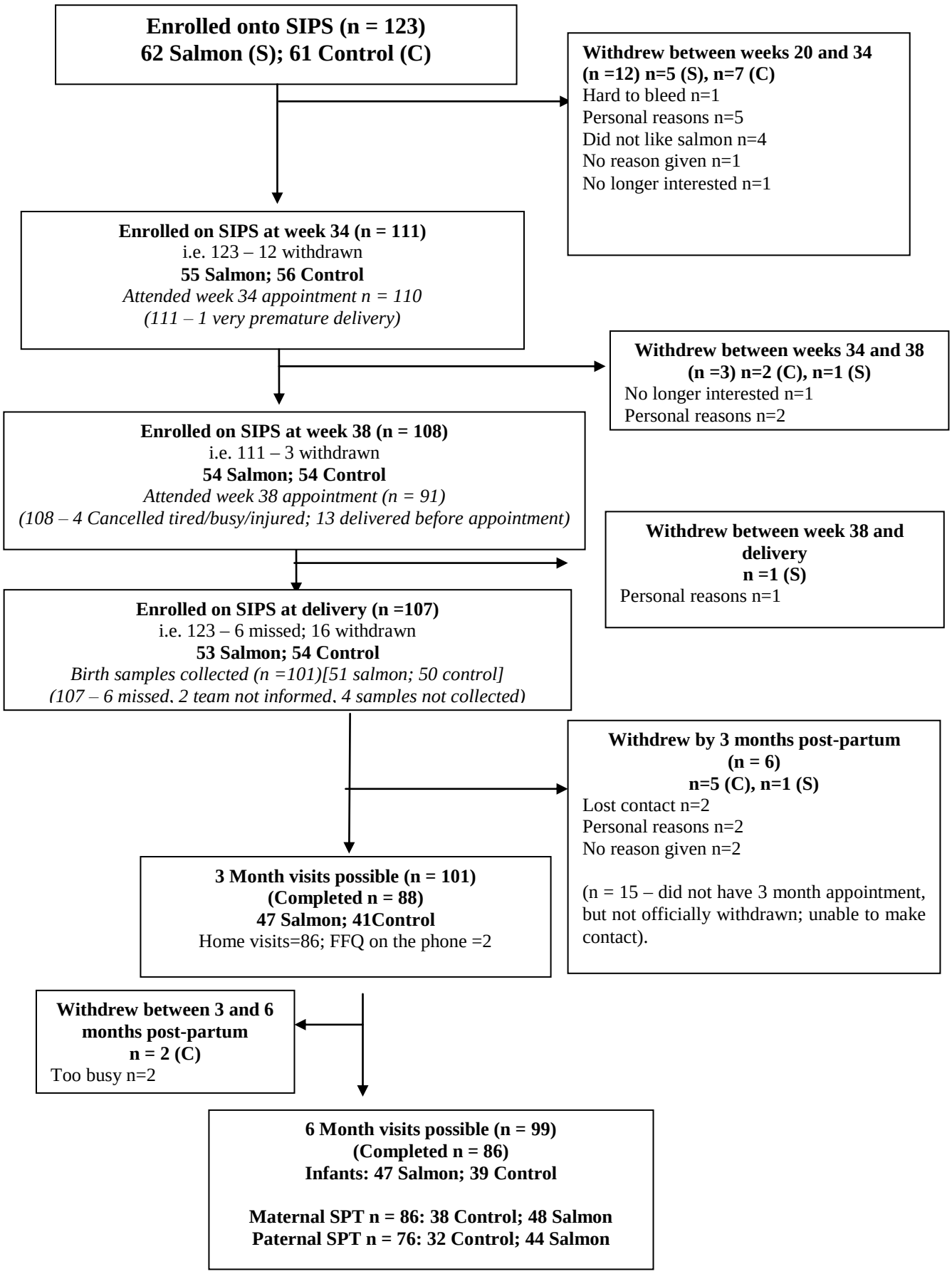


Figure 2.4: Progress of enrolled subjects through the study

2.4 Subject characteristics

2.4.1 Maternal characteristics at study entry

Table 2.7 describes some characteristics of the pregnant women at study entry (information acquired at 13 and 20 weeks of gestation) according to the group to which they were randomised. Maternal age, weight, and height, number of pregnancies (gravida), maternal smoking, nutritional supplement use, alcohol intake, and education, and keeping pets at home did not differ between the two groups, but significantly more women in the salmon group did not already have a child (p=0.013). Among those still in the study at 6 months after birth (n=86; control group n=39, salmon group n=47), birth order remained significant (p=0.042) with more women in the salmon group having their first child (n=31 out of 47) than those in the control group (n=17 out of 39). The difference was smaller when comparing the numbers of women who now had a second child (n=22 out of 39 for the control group and n=16 out of 47 for the salmon group). Paternal education was almost significantly different between the 2 groups at baseline (p=0.055), but this marginal difference disappeared when those remaining in the study at 6 months postpartum were considered.

Table 2.8 describes the characteristics of the pregnant women who dropped out of the study at any time from the start of the the intervention (week 20 of pregnancy) until the end of the follow-up (6 months postpartum). There are no significant differences between the study groups.

Figure 2.5 shows the distribution of the groups in conducting gentle, moderate and strenuous physical activity. There was no difference between the two groups in any type of activity.

Table 2.9 shows that the total duration of gestation and blood loss during delivery did not differ significantly between the two study groups, although women in the salmon group had an average of a 4 day longer pregnancy compared with those in the control group (p=0.094). Table 2.10 presents the different modes of birth, with most frequent being the normal vaginal delivery. There were no differences in mode of delivery between the two groups.

Table 2.11 shows self reported maternal, paternal and sibling (any type) atopy in the two groups. Paternal self reported atopy was different between the two groups with more fathers in

the salmon group reporting atopy (p=0.015). Among those remaining in the study 6 months after birth, paternal atopy remained different between the two groups; 25 fathers in the control group and 40 in the salmon group self-reported atopy (p=0.042). Maternal and sibling self reported atopy were not different between groups when those remaining in the study at 6 months post-partum were considered.

Table 2.7: Baseline characteristics of SIPS parents according to study group

	Control group (n=61)		Salmon group (n=62)		p
	Mean	SD	Mean	SD	
Maternal age (years) (at 13 weeks) [1]	28.4	4.7	29.5	4.3	0.174
Maternal height (cm) (at 13 weeks) [1]	165.6	6.8	165.4	6.2	0.885
Maternal weight (kg) (at 13 weeks) [1]	71.4	15.4	67.4	11.9	0.117
Number of children (at 13 weeks) [1]					
none	26	42.6	38	61.3	**0.013**
1	23	37.7	9	14.5	
≥2	12	19.7	15	24.2	
Pregnancies including the current (at 13 weeks) [2]					
1	23	37.7	27	43.5	0.509
≥2	38	62.3	35	56.5	
Maternal smoking (at 13 weeks) [2]					
never	30	49.2	33	53.2	0.460
past	23	37.7	25	40.3	
current	8	13.1	4	6.5	
Maternal alcohol intake (at 13 weeks) [2]					
no	46	78	46	76.7	0.866
yes (1-7 units/week)	13	22	14	23.3	
Maternal supplement use (at 20 weeks) [2]					
no	15	24.6	14	22.6	0.966
containing folic acid	45	73.8	47	75.8	
not containing folic acid	1	1.6	1	1.6	
Maternal education (at 20 weeks) [2]					
school	9	15.5	9	14.5	0.513
further	24	41.4	20	32.3	
higher	25	43.1	33	53.2	
Paternal education (at 20 weeks) [2]					
school	17	29.8	16	25.8	**0.055**
further	25	43.9	17	27.4	
higher	15	26.3	29	46.8	
Pets at home (at 13 weeks) [2]					
Cat					
no	43	70.5	44	71	0.954
yes	18	29.5	18	29	
Dog					
no	49	80.3	50	80.6	0.965
yes	12	19.7	12	19.4	
Other					
no	42	68.9	49	79	0.198
yes	19	31.1	13	21	

[1]Values are mean (standard deviation (SD)), independent t-test p-values
[2]Values are number of subjects (within group percentage (%)), Chi-square test p-values

Table 2.8: Baseline characteristics of the SIPS parents who dropped out of the study according to study group

	Control group (n=22)		Salmon group (n=15)		p
	Mean	SD	Mean	SD	
Maternal age (years) (at 13 weeks) [1]	25.6	5.04	28.4	4.28	0.076
Maternal height (cm) (at 13 weeks) [1]	164.9	7.05	162.8	4.8	0.292
Maternal weight (kg) (at 13 weeks) [1]	70.8	11.9	65.4	9.9	0.152
Number of children (at 13 weeks) [1]					
none	13	59.1	5	33.3	0.107
1	1	4.5	4	26.7	
≥2	8	36.4	6	40	
Pregnancies including the current (at 13 weeks) [2]					
1	9	40.9	3	20	0.286
≥2	13	59.1	12	80	
Maternal smoking (at 13 weeks) [2]					
never	8	36.4	3	20	0.545
past	10	45.5	8	53.3	
current	4	18.2	4	26.7	
Maternal alcohol intake (at 13 weeks) [2]					
no	19	90.5	13	86.7	0.560
yes (1-7 units/week)	2	9.5	2	13.3	
Maternal supplement use (at 20 weeks) [2]					
no	10	55.6	4	44.4	0.858
containing folic acid	5	27.8	3	33.3	
not containing folic acid	3	16.7	2	22.2	
Maternal education (at 20 weeks) [2]					
school	5	22.7	7	46.7	0.311
further	13	59.1	6	40.0	
higher	4	18.2	2	13.3	
Paternal education (at 20 weeks) [2]					
school	8	36.4	7	46.7	0.064
further	13	59.1	4	26.7	
higher	1	4.5	4	26.7	
Pets at home (at 13 weeks) [2]					
Cat					
no	18	81.8	12	80	0.890
yes	4	18.2	3	20	
Dog					
no	18	81.8	12	80	0.890
yes	4	18.2	3	20	
Other					
no	17	77.3	12	80	0.843
yes	5	22.7	3	20	

[1]Values are mean (standard deviation (SD)), independent t-test p-values
[2]Values are number of subjects (within group percentage (%)), Chi-square test p-values

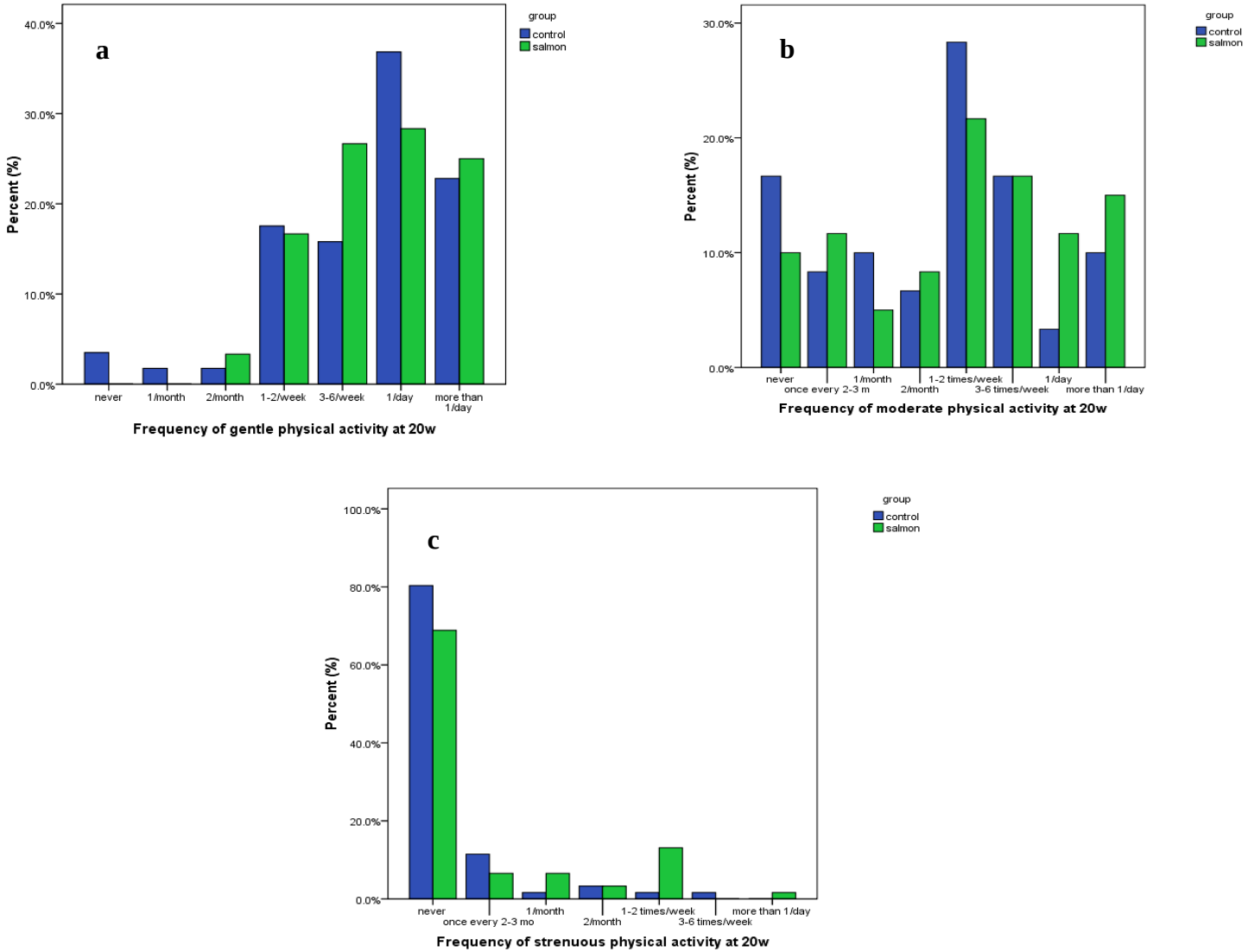


Figure 2.5: Distribution of frequency of conducting: a) gentle (p=0.448), b) moderate (p=0.499), c) strenuous physical activity (p=0.102) according to study group
Values are percentages of subjects, Chi-square test p-values

Table 2.9: Characteristics of SIPS mothers at birth according to study group

	Control group (n=61)		Salmon group (n=62)		p
	Mean	SD	Mean	SD	
Duration of gestation (days)	277	9	282	8	0.094
Blood loss at delivery (mL)	332	193	446	429	0.086

Values are mean ± standard deviation (SD), independent t-test p-values

Table 2.10: Mode of delivery according to study group and overall

Delivery mode	Control group (n=54)	Salmon group (n=53)	p	Total (n=107)
Normal Vaginal	35	35	0.914	70
Elective Lower Segment Caesarian Section (LSCS)	2	3		5
Emergency LSCS	7	5		12
Instrumental (ventouse/forceps)	10	10		20

Values are number of subject, Chi-square test p-values

Information on self-reported allergy history (different manifestations of atopy) in the two groups is presented in Figure 2.6. Maternal and paternal atopy characteristics did not differ between the two groups, but in the salmon group there was less self-reported asthma amongst siblings of fetus (p=0.011) compared to the control group.

Table 2.11: Self-reported atopy at 13 weeks screening according to study group

Self-reported atopy	Control group (n=61)		Salmon group (n=62)		p
	counts	% within group	counts	% within group	
Maternal (any)					
yes	38	62.3	43	69.4	0.409
no	23	37.7	19	30.6	
Paternal (any)					
yes	37	60.7	50	80.6	**0.015**
no	24	39.3	12	19.4	
Sibling (any)	**(n=36)**		**(n=25)**		
yes	17	27.9	10	16.1	0.610
no	19	31.1	15	24.2	

Values are number of subjects (within group percentage), Chi-square test p-values

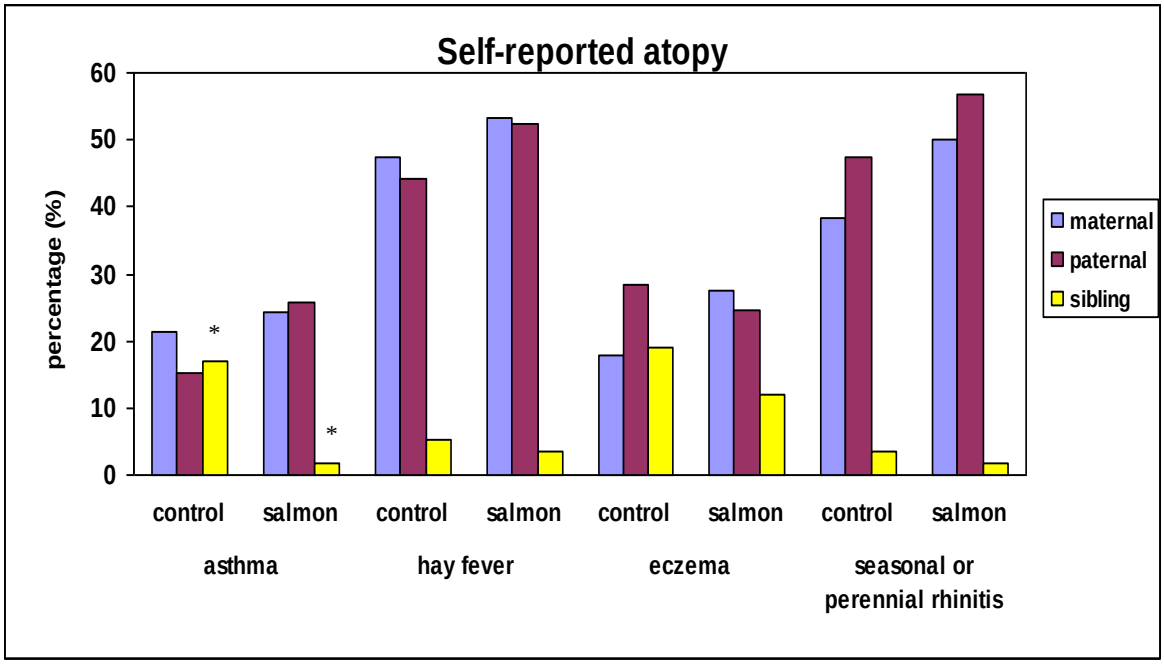


Figure 2.6: Distribution of self-reported atopy categories according to study group at 13 weeks screening
* Chi-square test p=0.011

Table 2.12 shows that the two groups did not differ according to maternal and paternal positive SPT. The breakdown of the positive maternal/paternal SPT results is shown on Table 2.13.

Table 2.14 shows how maternal and paternal self-reported atopy (at 13 weeks) is matched with SPT (determined at 6 months post-partum). 44.7 % and 52.1 % of the mothers in the control and salmon groups who were tested, were confirmed by positive SPT to be atopic while for fathers the figures were 59.4% and 68.2%, respectively.

Table 2.12: Any positive maternal or paternal skin prick tests (SPT) according to study group

Positive SPT	Control group (n=38)		Salmon group (n=48)		p
Maternal (any)	counts	% within group	counts	% within group	
yes	21	55.3	31	70.5	0.380
no	17	44.7	17	35.4	
Paternal (any)	**(n=32)**		**(n=44)**		
yes	20	62.5	31	70.5	0.466
no	12	37.5	13	29.5	

Values are number of subjects (within group percentage), Chi-square test p-values

Table 2.13: Percentage (%) of subjects sensitized to each allergen assessed by skin prick testing (SPT)

Skin Prick Test Allergen	Maternal – Control group (n = 38)	Maternal – Salmon group (n = 48)	Paternal – Control group (n = 32)	Paternal – Salmon group (n = 44)
Dermatophagoides pteronyssinus	36.8	47.9	40.6	56.8
Felis domesticus (cat)	28.9	35.4	18.8	38.6
Canis familiaris (dog)	31.6	39.6	34.4	45.4
6 Grass mix	47.3	54.2	50.0	59.0
3 Tree mix	15.8	29.2	15.6	36.4
Alternaria alternata	5.3	12.5	6.25	18.2
Aspergillus fumigatus	10.5	4.2	0	11.4
Cladosporium herbarum	0	2.1	3.2	9
Penicillum notatum	2.6	0	0	0

Table 2.14: Maternal and paternal self reported atopy confirmed by skin prick testing (SPT)

	Control group (n=38)		Salmon group (n=48)		p
Maternal	counts	% within group	counts	% within group	
Reported atopy confirmed by +SPT	17	44.7	25	52.1	0.433
No reported atopy confirmed by -SPT	12	31.6	8	16.7	
Reported atopy but no +SPT	5	13.2	9	18.8	
No reported atopy but +SPT	4	10.5	6	12.5	
Paternal	**(n=32)**		**(n=44)**		
Reported atopy confirmed by +SPT	19	59.4	30	68.2	0.057
No reported atopy confirmed by -SPT	9	28.1	3	6.8	
Reported atopy but no +SPT	3	9.4	10	22.7	
No reported atopy but +SPT	1	3.1	1	2.3	

Values are number of subjects (within group percentage of those skin prick tested), Chi-square test p-values

2.4.2 Infant characteristics at birth

Table 2.15 shows the infant gender distribution in the two study groups at birth. There were more male infants in the control group and more female infants in the salmon group. However, this difference was not statistically significant. Table 2.16 presents the birth characteristics of the infants. Weight at birth, apgar score and head circumference did not differ between the two groups. Infants in the salmon group were almost significantly longer (measured from crown to heel) (1.5 cm; p=0.057). After adjustment for gestational age and gender by univariate analysis (General Linear Model), birth weight did not differ between the 2 groups (p_{group}=0.527, p_{gender}< 0.001, $p_{gestational\ duration}$< 0.001). Apgar score ($p_{group}$=0.557, p_{gender}=0.231, $p_{gestational\ duration}$=0.091) and head circumference (p_{group}=0.078, p_{gender}=0.313, $p_{gestational\ duration}$=0.034) were not different between groups after adjustment for gender and length of gestation. Crown heel length was significantly different between the two groups after adjustment (p_{group}=0.037, p_{gender}=0.042, $p_{gestational\ duration}$=0.091), with neonates in the salmon group being longer. Table 2.17 shows some body composition measurements (bone mineral content, bone mineral density, whole body mass, fat mass, fat percentage, and lean mass) of the infants assessed by DXA within days of being born. There was no difference in any of these measurements between the two groups even after controlling for gender, gestational length and age of the neonates at DXA.

Table 2.15: Distribution of newborn gender according to study group and overall

Gender by group	Group		Total	p
	Control	Salmon		
Male	33 (61.1%)	23 (43.4%)	56 (52.3%)	0.067
Female	21 (38.9%)	30 (56.6%)	51 (47.7%)	

Values are number (% within group), Chi-square test p-value

Table 2.16: Characteristics of infants at birth according to study group

	Control group			Salmon group			p
	n	Mean	SD	n	Mean	SD	
Weight at birth (g)	54	3425	605	53	3449	525	0.83
Apgar score 5 min after birth	53	9.1	0.6	51	9.1	0.7	0.9
Occipito-frontal circumference (head circumference) (cm)	32	36.0	4.3	44	34.9	1.5	0.188
Crown-heel length (cm)	32	49.0	3.9	44	50.5	2.1	0.057

Values are mean and SD, independent t-test p-values

Table 2.17: Infant body composition measured by DXA within 14 days of birth

	Control group (n=33)		Salmon group (n=35)		p
	Mean	SD	Mean	SD	
Whole body global bone mineral content (g)	62.2	11.5	63.8	15.6	0.63
Whole body global bone mineral density (score)	0.20	0.02	0.21	0.03	0.72
Whole body global fat (g)	479.5	285.3	578.9	374.8	0.22
Whole body global fat (%)	13.1	6.4	15.7	7.5	0.14
Whole body global lean (g)	3007.7	338.5	2964.9	391.5	0.63
Whole body global mass (g)	3487.2	511.8	3543.9	558.1	0.66

Values are mean and SD, independent t-test p-values

3 Salmon in pregnancy study (SIPS): Compliance to the salmon intervention, maternal fish and nutrient intake, dietary patterns

3.1 Introduction

The richest dietary source of the long chain n-3 polyunsaturated fatty acids (LC n-3 PUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is seafood. Among different types of seafood, oily fish have a higher content of EPA and DHA compared to non-oily fish, and these LC n-3 PUFAs are found mainly in the flesh of the fish and a smaller amount is accumulated in the liver. Examples of oily fish are salmon, sardines, pilchards, kippers, mackerel, herring, trout and fresh tuna. One portion/serving of oily fish (140 g) will provide between 1.5 g and 3.5 g EPA and DHA (24;25). In comparison one portion/serving of white fish will provide about 0.3 g of EPA and DHA (1;2). LC n-3 PUFAs are also found in fish oil supplements.

Data from the last National Diet and Nutrition Survey (NDNS) of adults aged 19-64 years showed that most people in the UK consume very little fish. Mean consumption of total fish was 217 g per week, white fish was 103 g per week and oily fish was 50 g per week (23). The latest NDNS (2008/2009), from which the first results were published in 2010, showed that women aged 19-64 years consume on average 49 g/week coated and fried white fish, 105 g/week other white fish, shellfish and tinned tuna, and 77 g/week oily fish. This latest data also showed that over 70% of women aged 19-64 years old do not consume any oily fish, although 30% of consumers' intake is in accordance with the minimum recommendation (200). EPA and DHA intakes were not reported in the first report of the latest NDNS (2008/2009). The previous NDNS data showed that mean intake of EPA plus DHA for adults in the UK was 0.244 g/day from fish and animal sources. So, typical daily intakes of EPA plus DHA in adult individuals in UK who do not regularly consume either oily fish or fish oil capsules are probably less than 0.25 g per day (23).

Data (27) from the Australian National Nutrition Survey in 1995 showed that the average daily intakes of EPA and DHA acids were 0.056 and 0.106 g, respectively, and LC n-3 PUFA intake (addition of EPA, DPA, and DHA) was 0.189 g, similar to the above data for the UK. However, median intakes were considerably lower (0.008 g EPA, 0.015 g DHA, and 0.029 g LC n-3 PUFAs) (27). Thus 50% of the adult Australian population was consuming < 0.03 g LC n-3 PUFAs/day. Similar low intakes seem likely in the UK. As expected, seafood was the main source of LC n-3 PUFAs in Australia (5), contributing 71%, while meat and eggs contributed 20% and 6%, respectively. These results indicate that the majority of Australians are failing to meet intake recommendations for LC n-3

PUFAs. This emphasises the need for strategies to increase the availability and consumption of LC n-3 PUFA containing foods.

Recommended intakes for n-3 PUFAs are not uniform. In 2000, Simopoulos recommended daily intakes for EPA plus DHA at 0.65 g, with at least 0.222 g for each of EPA and DHA, and 2.22 g/d for α-linolenic acid (ALA) (28). The American Heart Association recommends adults to eat fish (in particular fatty fish) at least two times per week (29). The guidelines proposed by the US Food and Nutrition Board (Food and nutrition board 2005) established adequate intakes for ALA at 1.6 g/d and 1.1 g/d for adult men and women, respectively, and that EPA plus DHA could account for up to 10% of the total n-3 fatty acid intake as a contribution toward the adequate intake for ALA (30). The latest report from World Health Organisation and Food Agriculture Organisation (WHO/FAO) in 2008 on total fat and fatty acids concluded that for adult males and non-pregnant/non-lactating adult females 0.25 g/day of EPA plus DHA is recommended, and that, with insufficient evidence to set a specific minimum intake of either EPA or DHA alone, both should be consumed (9). For adult pregnant and lactating females, the minimum intake for optimal adult health and fetal and infant development is 0.3 g/d EPA+DHA, of which at least 0.2 g/d should be DHA (199).

Current UK recommendations propose that people should eat at least 2 portions of fish, of which one should be oily, per week; this recommendation also applies to pregnant women (23). This was equated to a daily intake of at least 0.45 g of LC n-3 PUFAs (23). There are several published recommendations concerning the intake of n-3 fatty acids during pregnancy and lactation:

- Mele & Miggiano (31) recommended a DHA intake of 100 mg/day (with an extra 50-60 mg/day in the third trimester) in pregnant women;
- Makrides & Gibson (32) suggest an intake of LC n-3 PUFAs of 200-400 mg/day in pregnant and lactating women;
- the European Food Safety Authority suggests an intake of 250 mg/day of DHA (33) during pregnancy and lactation;
- the (UK) Scientific Advisory Committee on Nutrition (SACN) recommends 450 mg/day intake of LC n-3 PUFAs in pregnant and lactating women (23);
- the latest EFSA report on reference values for fats and fatty acids proposed to set an Adequate Intake of 250 mg/day for EPA plus DHA for adults based on cardiovascular considerations. To this 100 to 200 mg of preformed DHA should be

added during pregnancy and lactation to compensate for oxidative losses of maternal dietary DHA and accumulation of DHA in body fat of the fetus/infant (201).

Despite these recommendations, based upon an assumed need for LC n-3 PUFAs, especially DHA, in pregnancy and lactation, concern about contaminants such as methylmercury, dioxins and dioxin like PCBs in some species of oily fish, has lead to the Food Standards Agency (FSA) to advise that pregnant women, women intending to become pregnant, and girls under 16 years old should avoid eating shark, marlin and swordfish. The Committee on Toxicology advices that "women of reproductive age and girls should aim to consume within the range of one to two portions of oily fish per week, based on maintaining consumption of dioxins and dioxin like PCBs below the tolerable daily intake (TDI) of 2 pg WHO-TEQ/kg bodyweight per day. Women past reproductive age, boys and men should aim to consume within the range of one to four portions of oily fish a week, based on maintaining consumption of dioxins and dioxin-like PCBs below the guideline value of 8 pg WHO-TEQ/kg bodyweight per day" (23).

The provision of EPA and DHA to the fetus is important for fetal growth and development. Preformed LC n-3 PUFAs, particularly DHA, but also EPA, may need to be provided in the diets of infants to meet the high requirements of rapidly growing tissues and organs (23). The mother is the primary source of these fatty acids for the fetus and breast-fed infant. These fatty acids are important for brain and visual development, and may also be important for optimal development and functioning of the vascular, cardiac and immune systems. Regarding the immune system, there is evidence (see literature review; Section 1.5) that early fish and LC n-3 PUFA exposure reduces the risk of immune dysfunctions, such as allergic disease and asthma in infancy and childhood (202;203).

According to data reviewed by SACN in 2004 (23), there is some evidence that many women have a marginal status of LC n-3 PUFAs during pregnancy and lactation. However, there is a lack of agreed markers which are suitable for defining LC n-3 PUFA status. The markers usually reported relate to concentrations of fatty acids within the circulation. They may be expressed as a percentage of total fatty acids or as their absolute concentrations in different circulating pools. Concentrations of fatty acids in maternal or umbilical blood have been used as measures of LC n-3 PUFAs during pregnancy. These measures have been related to maternal dietary intake (35), as well as to pregnancy outcomes such as gestation duration and birthweight (36-39). During pregnancy a woman must meet the additional demands related to the accretion of maternal, placental, and fetal tissues.

Although, the formation of DHA and EPA appears to be tightly regulated, a marginal state for many women during pregnancy and lactation cannot be excluded. The additional demands for EPA and DHA or other fatty acids during a normal pregnancy have not been adequately defined. It was concluded that the extent of dietary dependence on increased levels of consumption of LC n-3 PUFAs, or specifically DHA, to improve pregnancy outcome needs to be demonstrated (23).

Oily fish are also good sources of vitamin D and antioxidants such as vitamin E and selenium. Early fish and vitamin D exposure improves bone health in infancy and childhood, and there is evidence that vitamin D supplementation in pregnant women may reduce the risk of osteoporotic fracture in the offspring (204). Also, more recently, it has been suggested that increased dietary exposure to antioxidants in the early stages of fetal and infant development influence the risk of childhood asthma and atopic sensitization possibly by affecting the first interactions between the neonatal immune system and allergens (205). Evidence from birth cohort studies has suggested that there are potentially beneficial associations between maternal intake of vitamin D and some antioxidants (vitamin E, Se, Zn) during pregnancy and early childhood wheezing/asthma (206-209). On the contrary, there is limited data suggesting associations between maternal antioxidant intake and childhood atopic dermatitis and allergic rhinitis. The exact nature of these associations remain unclear and more interventions in the window or early life are needed to be proven (205). A recent publication by Nurmatov *et al.* has reviewed and analysed systematically whether intake of vitamins A, C, D, E, selenium and zinc can modify the risk of children to develop allergy (210).

Data from birth cohort studies showed beneficial associations between maternal vitamin E intake and childhood asthma/wheeze outcomes (206;209;211). Meta-analysis of the data from three cohort studies showed higher maternal vitamin E intake during pregnancy to be associated with a significant decrease in the odds of wheezing in children at age two years: OR=0.68 (95%CI 0.52-0.88), p=0.004 (210). However, most cross-sectional studies reported no associations between intake or serum levels of vitamin E in children and sensitization, atopic eczema, current wheeze, allergic rhinoconjunctivitis, lung function and asthma outcomes (212-214).

A number of studies investigate associations with vitamin D (208;210;215-218). Cohort studies reported that higher maternal vitamin D intake during pregnancy may decrease the risk of wheezing in early childhood (208;216). Two cohort studies reported on the

association between maternal vitamin D intake and childhood asthma at age five years, with one study reporting no association (208) and one reporting a beneficial inverse association (217): higher maternal vitamin D intake was associated with reduced odds of wheezing (i.e. either recurrent wheezing/wheeze in the previous year: OR=0.56, $p<0.001$). Two other cohort studies reported adverse associations between maternal blood vitamin D levels during pregnancy or high dose vitamin D supplementation during infancy and childhood asthma, atopic dermatitis and/or allergic rhinitis (218).

Many studies have assessed whether there is an association between selenium exposure and atopic risk (171;207;214;219;220). Three reports, including two birth cohort studies related blood selenium in pregnant women, umbilical cord and early childhood to childhood outcomes (207;219). One cohort study concluded that higher plasma selenium during pregnancy (maternal and cord) were associated with a reduced risk of childhood wheezing at age 2 years but not at 5 years (207). Another cohort study also found higher cord selenium to be associated with a reduced risk of persistent childhood wheeze: (OR=0.67 (95%CI 0.45-0.99)) (219). Case-control studies reported no associations between serum/urine selenium levels or selenium intake and atopic dermatitis or asthma-related outcomes (220;221). Overall, the body of evidence in relation to selenium was unsupportive of an association between childhood asthma and allergy and selenium status during pregnancy or childhood.

The body of evidence from studies examining vitamin A in relation to childhood allergy risk was weak, but possibly suggestive of an association between reduced vitamin A and childhood asthma, but not between maternal vitamin A intake during pregnancy and childhood wheeze (210).

The evidence from studies examining vitamin C in association with allergy risk in children was weak and unsupportive to show possible effectiveness of vitamin C to prevent asthma or atopic outcomes (210). The evidence from studies examining zinc in association with atopy risk in childhood was also weak but suggestive of the possible effectiveness of zinc in relation to the prevention of asthma (210).

Overall, it seems that a higher consumption of oily fish during pregnancy will result in better provision of several nutrients (LC n-3 PUFAs, vitamin D, antioxidants such as Se and vitamin E) to the fetus and that this likely to be associated with improved health in infancy and childhood. There is evidence of the protective effect of these nutrients based on

supplementation and cohort studies (132;178;207-209). However, there are no published intervention trials with fish during pregnancy. Since eating fish is advised, but fish are also a source of contaminants, aquaculture of fish to have low contaminant levels could be an ideal solution to enable oily fish consumption by pregnant women and possibly the intake of the recommended amounts of LC n-3 PUFAs. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish in pregnant women. SIPS focuses on pregnant women whose offspring have increased risk of developing atopic disease, aiming to identify whether there is an effect on atopy outcomes in the offspring. Also, consumption of tailor-made salmon by pregnant women could prevent the depletion of the mother's EPA and DHA and could improve the fetal/newborn status of EPA and DHA. A total of 123 women were randomly assigned to consume either 2 portions of farmed salmon per week (from week 20 till delivery) or to continue their habitual diet low in oily fish (less or equal to two portions per month) (see Chapter 2).

The aim of this chapter is to describe the diets of the subjects of both groups at baseline (frequencies of intake of specific foods and nutrient intakes) and to find out whether and how the diet and nutrient intakes changed over the course of pregnancy in the salmon and the control groups. Moreover, the compliance of the salmon and the control groups to the intervention and their habitual dietary habits, respectively, is assessed.

The primary hypotheses of the work described in this chapter are that the oily fish intervention will:

1. increase oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/week) during pregnancy in women whose offspring is at a high risk of developing atopic disease;
2. increase maternal LC n-3 PUFA intake during pregnancy, and that this intake will be higher in the salmon than in the control group.

The secondary hypotheses of the work described in this chapter are that the oily fish intervention will:

1. change the dietary patterns in the salmon group;

2. increase the maternal intake of vitamin D and selenium, and that their intakes will be higher in the salmon than in the control group.

3.2 Materials and methods

3.2.1 Study design and subjects

Chapter 2 described the study design, the subjects' characteristics, and the intervention. In summary, the population studied were pregnant women with high risk of having an atopic offspring. The study groups were two, of which the control group (n=61) was asked to keep their habitual diet unchanged during the remainder of pregnancy (from 20 weeks of pregnancy until delivery) and the salmon group (n=62) consumed two portions of farmed salmon per week (from 20 weeks of pregnancy until delivery). Each portion of salmon provided 1.73 g of EPA plus DHA, 14 μg of vitamin D, and 43 μg of selenium. The nutrient analysis of salmon is described in Chapter 2; Table 2.3. Women of both groups completed a Food Frequency Questionnaire (FFQ) at weeks 20 and 34 of pregnancy. They also completed the FFQ 3 months after delivery (data not included in this thesis). Moreover, the women were asked to complete fish diaries to record any fish intake during intervention (from week 19/20 until delivery). These diaries were also completed by most of the women from delivery till 6 months post-partum (data not included in this thesis).

3.2.2 Dietary assessment (fish, whole diet and nutrient intakes)

3.2.2.1 General Comments: Selection of methodology - connection with SWS

In SIPS, diet was assessed retrospectively by FFQ. As for all dietary assessment methods, FFQs are subject to error since they depend on individuality and memory. The FFQ has some advantages and disadvantages. Compared to the gold standard prospective method of weighed records, which are considered to be more accurate for determining nutrient intake, FFQs can be applied to a larger number of people and can assess diet for longer periods. Weighed records can cover a small period of time (3-7 days) and can be implemented on small groups of subjects, as they are labour intensive, and more expensive. Whilst cheaper to administer, FFQs also have the advantage that by summarising data over a longer period of time they may better describe habitual diet than assessments over a shorter period. In this way, FFQs have become the principal method for dietary assessment in large population studies. However, there are concerns that the nutrient intake estimates derived from FFQs are not very accurate, to the point that disease-nutrient associations may be concealed (222).

SIPS was based in the Southampton area and had a small sample size (n=123). The maternal FFQ used by SIPS was initially developed for a large cohort study, the Southampton Women's Survey (SWS) that was also conducted in the Southampton area. The use of a tool that had already been implemented in the same population seemed appropriate. The FFQ included questions on fish consumption and reflected diet over the previous three months. In addition, so as to assess compliance to the intervention, volunteers in both groups were asked to complete a daily record of fish consumption throughout pregnancy, here termed the "fish diary". The combination of the FFQ retrospectively and of the fish diary prospectively, could provide more accurate assessment of nutrient intake, information on fish consumption frequencies and dietary patterns.

The SWS FFQ has been validated to assess nutrient intakes and dietary patterns in pregnant women in Southampton (223). The SWS is an on-going population study of initially 12,579 non-pregnant women aged 20-34 years in Southampton. Women who subsequently became pregnant were followed up; 2,270 women in early and 2,649 in late pregnancy completed dietary information (224). The SWS children are being followed through infancy and childhood to examine how their pre-natal growth interacts with their postnatal growth, and how both affect the children's risk factors for a range of chronic diseases (195).

3.2.3 Maternal food frequency questionnaire (FFQ)

The semi-quantitative 100 item FFQ was administered by an appropriately trained nutritionist (myself or Stella Kremmyda) at 20 and 34 weeks gestation and reflected food intake over the past 12 weeks (3 months). The FFQ at 20 weeks reflected dietary intake prior to the intervention (i.e. at baseline (from 8th week till 20th week of pregnancy), and the FFQ at 34 weeks reflected intake during the intervention period (from 22nd week till 34th week). Both nutritionists were blinded to the treatment group of the subjects.

The FFQ (Appendix 7) reflected intake of a hundred different food items including the following categories: bread, cereals, different types of fruit, fruit juices, types of vegetables, pasta, rice, potatoes, beans/pulses, different types of meat and meat products, fish, eggs, diary products, milk, nuts, types of spreading fat and cooking oils, desserts/ sweets, savoury snacks, tea/coffee, soft drinks. Amongst those, the FFQ included questions of consumption frequency of different types of fish in four categories: 'oily fish', 'non-oily fish' (including tinned tuna), 'fish fingers and fish dishes' and 'shellfish'. Also the FFQ included questions on composite food dishes, including foods they ate out or cooked at

home. Moreover, there were questions about nutritional supplement intake (vitamins/minerals/fibre) and alcohol consumption. An additional question was added to the FFQ asking about intake of any 'functional food products' with added n-3 and/or n-6 fatty acids, probiotics, prebiotics or combinations of those. Subjects were asked to indicate the brand, quantity and weekly frequency of any functional food they had consumed on a systematic weekly basis in the 12 weeks prior to the FFQ administration.

3.2.3.1 Consumption frequencies & nutrient intakes

Subjects were asked to recall their intake during the three months prior to the time point of the interview. To help their recall, subjects were given prompt cards with examples of food items included in each food category and they were asked to think about composite food dishes, including foods they ate out or cooked themselves. Categories of consumption frequency were also given in a prompt card and were: 'more than once a day', 'once a day', '3-6 times per week', '1-2 times per week', 'once a fortnight', 'once a month', 'once every 2-3 months', 'never'. The FFQ administered at 20 and 34 weeks was used to calculate the weekly consumption frequencies for each food group for all subjects. The 8 categories of consumption frequency described above, correspond to a different multiplication factor as described in Table 3.1 below so as to calculate weekly frequencies.

Table 3.1: Factors used to calculate frequencies and number of portions eaten per week

Frequency eaten	Multiplication factor
Never	0
Once every 2-3 months	0.1
Once a month	0.25
Once a fortnight	0.5
1-2 times per week	1.5
3-6 times per week	4.5
Once a day	7
More than once a day	7 x times per day

Because of the interest in fish consumption, the frequencies of consumption of the 4 fish categories included in the FFQ (questions 68-71 of the FFQ; white fish, fish fingers and fish dishes (FFFD), oily fish and shellfish) were calculated.

Standard portion sizes were assigned for each food item on the FFQ according to a published list for the UK (225). The nutrient content of a standard portion of each food was multiplied by its reported frequency (Table 3.1) so as to calculate weekly nutrient intake. To get the daily nutrient intake, weekly nutrient intake was divided by seven. Total intake for each nutrient was obtained by summing up the specific nutrient intake from all FFQ food items. The macro- and micro-nutrients that had complete values (12;13;226), and could provide a reliable estimate of intake are included in Table 3.2. Selenium values were complete, however they were used with caution, as selenium content varies depending on soil content (227).

Table 3.2: Nutrients for which intake can be calculated (*Se estimated intake is crude)

	Fat	Vitamins	Minerals
Energy (kJ/kcal)	Total fat	A (retinol/carotene), D, E, K	Ca, Mg P, Zn
Carbohydrate	MUFAs	C	Fe, Se*
Protein	PUFAs (total)	B1, B2, B3, B6, B12	Na, K
Dietary fibre	Total n-3 PUFAs	Folate	
	Total n-6 PUFAs		
	EPA, DHA		

For fish intake (for the 4 fish FFQ categories; Questions 68-71; see Appendix 7) and for seven nutrients (EPA, DHA, n-3 PUFA, n-6 PUFA, vitamin D, vitamin E and Se) myself and Lefkothea-Stella Kremmyda calculated the intake using the weekly frequencies (using the multiplication factors) from the FFQ analysis but different fish recipes from the ones that SWS used. The reason for doing so is that the SWS recipes were not as accurate (they were rough estimates) for those nutrients. In this way, we developed different recipes for each fish category, which gave us different amounts of those nutrients per portion from the amounts calculated by SWS. The fish diaries (described below) were used to identify which fish types to include in each of the four fish recipes that were developed. We defined portion sizes as well for the 4 fish categories, which were slightly different from the ones that SWS used. Afterwards the average amounts of those nutrients for each fish category were calculated. When calculating the nutrients from the oily fish category for the salmon group, the nutrient composition information from the study salmon was used instead of the oily fish recipe. To make it clear, below is the table including the recipes used with the corresponding amounts to those seven nutrients (Table 3.3).

Table 3.3: Content of total n-3 PUFAs, total n-6 PUFAs, EPA, DHA, Se, vit D and vit E in fish, shellfish and fish dishes per 100 g (12;13)

Composition of fish and fish dishes per portion							
	Total n-3 PUFAs (g)	Total n-6 PUFAs (g)	EPA (g)	DHA (g)	Se (µg)	Vit D (µg)	Vit E (mg)
WHITE Fish-150 g portion							
Cod raw	0.39	0.03	0.12	0.24	42.00	0.00	0.66
Haddock raw	0.26	0.05	0.08	0.15	40.50	0.00	0.59
Plaice raw	0.48	0.02	0.24	0.15	55.50	0.00	
Tinned tuna in water, drained	0.26	0.06	0.03	0.21	117.00	5.40	0.83
Tinned tuna in oil, drained	1.83	4.89	0.09	0.41	135.00	4.50	2.91
Average amount per 150 g portion	**0.64**	**1.01**	**0.11**	**0.23**	**78.00**	**1.98**	**1.25**
OILY Fish-150 g portion							
STUDY Salmon	**3.56**	**2.31**	**0.57**	**1.16**	**43.00**	**14.00**	**4.10**
Average amount per 150 g portion							
CONTROL Salmon							
Pink/red canned in brine	2.78	0.41	0.83	1.29	37.50	13.80	2.28
Mackerel raw	4.17	0.77	1.07	1.65	45.00	12.30	0.65
Trout grilled	1.98	0.62	0.33	1.13	31.50	14.40	1.52
Average amount per 150g portion	**2.98**	**0.60**	**0.74**	**1.36**	**38.00**	**13.50**	**1.48**
SHELLFISH-75 g portion							
Prawns (boiled/frozen raw)	0.08	0.02	0.05	0.03	17.25	0.00	
Mussels boiled	0.51	0.06	0.31	0.12	32.25	0.00	0.79
Scampi breaded cooked	0.05	0.11	0.00	0.00	12.75	0.00	
Crab boiled	0.83	0.18	0.35	0.34	63.00	0.00	
Average amount per 75 g portion	0.37	0.09	0.18	0.12	31.31	0.00	0.79
FFFD-100 g portion							
Fish cakes frozen raw	0.33	0.60	0.05	0.09	NA	0.00	NA
Fish fingers frozen raw	0.17	1.28	0.04	0.03	21.00	0.00	NA
Average amount per 100 g portion	**0.25**	**0.94**	**0.05**	**0.06**	**21.00**	**0.00**	

FFFD: fish fingers and fish dishes, NA: data is not available

3.2.3.2 Dietary patterns

In order to determine possible changes occurring in the diets of the pregnant women in relation to the intervention and/or pregnancy, the FFQ items were grouped. The 98 foods and non alcoholic beverages listed on the FFQ were combined into 48 food groups on the basis of similarity of nutrient composition and comparable useage, as Crozier *et al*. had done before for the SWS FFQ (224). For example, the 4 food categories of white fish, fish fingers and fish dishes, oily fish, and shellfish were combined into the 'total fish and shellfish' group. Weekly consumption frequency of these 48 food groups was calculated by adding the weekly consumption frequency of each food item. Table 3.4 lists all the food groups, and how the separate food items were incorporated in those groups.

Table 3.4: FFQ food groupings

Food group	Food items included
Rice and pasta	Rice, pasta
White bread	White bread
Wholemeal bread	Wholemeal bread
Quiche and pizza	Quiche and pizza
Yorkshire pudding and savoury pancakes	Yorkshire puddings and savoury pancakes
Breakfast cereals	'Bran' breakfast cereal, other breakfast cereals
Cakes and biscuits	Cakes, buns, pastries, biscuits, other biscuits
Puddings	Fruit based puddings, milk based puddings, other puddings, ice cream
Cream	Cream
Full-fat milk	Full-fat liquid milk
Reduced-fat milk	Reduced-fat liquid milk
Yoghurt	Yogurt
Cheese and cottage cheese	Cheese, cottage cheese
Eggs and egg dishes	Eggs, omelette
Full-fat spread	Full-fat spread
Reduced-fat spread	Reduced-fat spread
Cooking fats and salad oils	Cooking fats and salad oils
Red meat	Pork, lamb, beef
Chicken and turkey	Chicken and turkey
Liver, liver pate and kidney	Liver and kidney, pate and liver sausage, faggots and black pudding
Offal Processed meat	Bacon and gammon, meat pies, sausages, ham and canned meats
Fish and shellfish	White fish, fish fingers and fish dishes, oily fish, shellfish
Salad vegetables	Salad, coleslaw, tomatoes, green peppers, watercress
Green vegetables	Spinach, brussels sprouts and broccoli, cabbage and cauliflower, peas, courgettes, marrow and leeks
Root vegetables	Carrots, parsnips, swedes and turnips
Other vegetables	Sweetcorn, onions, mushrooms
Tinned vegetables	Tinned vegetables

Vegetable dishes	Vegetable dishes, vegetarian foods
Beans and pulses	Beans and pulses
Chips and roast potatoes	Chips and roast potatoes
Boiled potatoes	Boiled, mashed and jacket potatoes
Crisps	Crisps and savoury snacks
Crackers	Crackers and cheese biscuits, wholemeal crackers
Citrus fruit and fruit juices	Oranges and orange juice, grapefruit and grapefruit juice
Other fruit	Apples and pears, bananas, peaches, plums, cherries and grapes, strawberries and raspberries, melon, pineapple, mango and kiwi
Other fruit juices	Other fruit juices (not squashes)
Dried fruit	Dried fruit
Cooked and tinned fruit	Cooked fruit, tinned fruit
Nuts	Nuts
Sugar (teaspoons)	Added sugar
Sweet spreads and jam	Sweet spreads and jam
Sweets and chocolate	Chocolate, other sweets and confectionery
Soft drinks	Blackcurrants and blackcurrant drinks (Ribena), fizzy drinks and squashes, cola drinks
Diet coke	Diet cola drinks
Tea and coffee	Tea, coffee
Decaffeinated tea and coffee	Decaffeinated tea (including herbal tea) and decaffeinated coffee
Hot chocolate drinks	Drinking hot chocolates and milkshakes
Miscellaneous	Soups, stock cubes and marmite, mayonnaise and salad cream, pickles, chutney and ketchup, added bran to foods

Some additional food groups were created for SIPS. These were also created by adding up the consumption frequencies of either individual FFQ items or food groups. Table 3.5 shows these extra groups. The categories of fats and oils were formed after combining the different types of fats and oils consumed (specific brands and names were recorded on the FFQ).

Table 3.5: Extra FFQ food groupings created for SIPS

Extra food groups for SIPS	Food items or food groups included
Total fruit	Citrus fruits and fruit juices + other fruit+ other fruit juices + dried fruit + cooked and tinned fruit
Total vegetables	Salad vegetables + green vegetables + root vegetables + other vegetables +beans and pulses + tinned vegetables + vegetable dishes and vegeterian foods
Total meat	Red meat + processed meat+ offal + chicken and turkey + liver, liver pate and kidney
Low alcohol (alcohol units)	Low alcohol beer + low alcohol wine
Normal alcohol (alcohol units)	Normal alcohol beer + wine + spirits
Total alcohol (alcohol units)	Low alcohol + normal alcohol
Margarine	Margarines + vegetable oil spreading fats
Butter	Butter
Olive spread	Olive oil based spreading fat
Other vegetable oil	Other vegetable oils consumed (vegetable blend + sunflower + corn + hazelnut + walnut + sesame + hemp seed)
Olive oil	Olive oils (all kinds including extra virgin)
Animal fat	Animal fat (lard, goose fat)
Salad dressings	Salad dressings

3.2.4 Fish diaries

3.2.4.1 Compliance

Subjects in both groups (salmon and control) were asked to keep a record of their fish consumption (including oily and non-oily fish, shellfish and fish dishes) on a daily basis during the study, from week 21 of pregnancy until delivery and postnatally for 6 months. All subjects were asked to fill in the quantity, exact name, and cooking method of any fish consumed during pregnancy. Women in the salmon group were also asked to record exactly the days of every week that they ate study salmon, quantity eaten and method of cooking. They also recorded any other seafood that they consumed in addition to the study fish.

The fish diary was used as a tool for estimating compliance and evidence of eating two portions of salmon per week in the salmon group, as well in the control group for not eating more than two portions of oily fish per month. The only difference to the diaries provided to the control and the salmon group was that the control group was given a list of fish categorised as oily and non-oily so as to be aware and to remain within the limit of the low consumption level of oily fish (twice per month) (Appendices 8 and 9).

3.2.4.2 Consumption frequencies

The fish diaries were used to identify frequencies of fish intake as well as to aid estimation of nutrient intake. Fish diaries were analysed by myself and Lefkothea-Stella Kremmyda. Portion sizes were defined for all fish (white and oily) to be 150 g, for shellfish 75 g, for fish fingers 4 fish fingers, for fish cakes 2 fish cakes, for fish pie 1/3 portion of haddock, 1/3 portion of salmon and 1/3 portion of prawns. Similarly to the FFQ, fish were identified in 4 categories: 'oily', 'white', 'fish fingers and fish dishes', 'shellfish'. Oily fish included the sum of study salmon + fresh tuna + fresh salmon + tinned salmon + sardines + kippers + pilchards + herring + mackerel + sward + trout + anchovies. White fish included the sum of tinned tuna + cod + haddock + plaice + sole + whitebait + halibut + scabbard + hake +monkfish + pollack + sea bass + marlin. 'Fish fingers and fish dishes' included the sum of fish fingers + fish cakes. Shellfish included the sum of scampi + crab + oyster + shrimp + prawn + mussels + scallop + crayfish. Finally, 'Total fish' included the sum of white fish + oily fish + fish fingers and fish dishes + shellfish.

3.2.4.3 Nutrient calculations

Fish diaries were also used to calculate intakes of the seven nutrients of interest (EPA, DHA, n-3 PUFAs, n-6 PUFAs, Se, vitamin D and vitamin E). The fish recipes used were the same as those used for calculating nutrient intake from the FFQs (Table 3.3). The information collected from the fish diaries played a major role in deciding the specific kind of fish the nutritionists (myself and Stella Kremmyda) chose to include in the 'fish recipes'. Weekly intake of the seven nutrients of interest was calculated by multiplying the weekly fish consumption frequency by the nutrients per portion (Table 3.3), and the daily intake of those nutrients was deriving by dividing the weekly intake by seven. Mean weekly consumption frequencies of fish categories and mean daily nutrient intakes were calculated for the period 21 to 38 weeks of pregnancy, as from week 39 until birth the diary records were poor.

3.2.5 Statistical analysis

The statistical package for social sciences (SPSS) version 16 was used for all the statistical analysis, and a p value equal or less to 0.05 ($p \leq 0.05$) was considered to be statistically significant. Data that were not normally distributed were not log transformed but were subjected to anlysis using non-parametric tests.

3.2.5.1 FFQ

Where data were normally distributed they are described as mean and standard deviation (SD) and differences between treatment groups were determined using independent samples t-test. Where data were not normally distributed they are described as median and interquartile range and differences between treatment groups were determined using the Mann-Whitney test. Differences within a group were determined by Wilcoxan ranked signs test.

3.2.5.2 Fish diaries

Data from the fish diaries were not normally distributed, and are described as median and interquartile range. Differences between treatment groups were determined using the Mann-Whitney test.

3.3 Results

3.3.1 Food groups consumption frequencies based on the FFQ

Consumption frequencies of the 48 food groups according to the SWS categorization at baseline (20 weeks) and during intervention (34 weeks of pregnancy) are described in Table 3.6. Although the subjects were randomly assigned in the two groups, consumption frequencies at baseline differed significantly between groups for the following food groups: whole-meal bread, total fish and shellfish, citrus fruit and fruit juices, other fruit juices, sweets and chocolate. The salmon group had higher intake for each of these food groups apart from sweets and chocolate for which it had a lower intake. Specifically the salmon group had a median intake of total fish and shellfish of 1.75 times per week compared to that of the control group that was 0.75 times per week at baseline ($p<0.001$). During intervention the salmon group had a significantly higher consumption frequency of total fish and shellfish (2.35 vs. 0.75 times per week, $p<0.001$) and a significantly lower consumption frequency of red meat than the control group (1.75 vs. 2.5 times per week, $p=0.022$). Consumption frequencies of chips and roast potatoes and of sugar differed between the two groups at 34 weeks of pregnancy (lower in the salmon group).

Table 3.6: Weekly consumption frequency of food groups in the FFQ administered at 20 and 34 weeks gestation according to study group

Food group	20 weeks gestation			34 weeks gestation		
	Control group (n=61)	Salmon group (n=62)	p	Control group (n=54)	Salmon group (n=55)	p
Rice and pasta	3 (1.9, 3)	3 (1, 3)	0.533	2 (1, 3)	2 (1, 3)	0.729
White bread	9 (2.3, 14)	3.75 (0.9, 14)	0.096	9 (3, 14)	3 (1, 14)	0.061
Whole-meal bread	3 (0.3, 9)	4.5 (0.7, 14)	**0.048**	1.25 (0, 9)	3 (0.2, 14)	0.051
Quiche and pizza	0.5 (0.25, 1.5)	0.5 (0.25, 1.5)	0.437	0.5 (0.25, 0.5)	0.5 (0.25, 0.5)	0.967
Savory pancakes (incl. Yorkshire pudding)	0.25 (0, 0.5)	0.1 (0.1, 0.25)	0.298	0.1 (0, 0.25)	0.1 (0, 0.25)	0.287
Breakfast cereals	4.5 (0.5, 7)	4.5 (0.9, 7)	0.212	4.6 (1.5, 7)	7 (1.5, 7)	0.233
Cakes and biscuits	5.5 (3, 9.8)	4.38 (2, 9.4)	0.331	5.8 (3, 10)	8 (3.5, 11)	0.252
Puddings	1 (0.5, 2.5)	1.55 (6, 2.3)	0.691	1.7 (0.8, 2.6)	1.5 (0.8, 2.3)	0.634
Cream	0.25 (0, 0.5)	0.18 (0, 0.5)	0.652	0.25 (0, 0.5)	0.25 (0, 0.5)	0.461
Full-fat milk (pt/day)	0 (0, 0)	0 (0, 0)	0.706	0 (0, 0)	0 (0, 0)	0.851
Reduced-fat milk (pt/day)	0.5 (0.09, 0.75)	0.5 (0.15, 0.56)	0.580	0.5 (0.2, 1)	0.5 (0.25, 1)	0.649
Yogurt	1.5 (0.25, 4.5)	1.5 (0.25, 4.5)	0.758	1.5 (0.5, 4.5)	1.5 (0.25, 4.5)	0.515
Cheese and cottage cheese	4.5 (1.5, 4.5)	4.5 (1.5, 4.6)	0.222	4.5 (1.5, 4.5)	4.5 (1.5, 4.5)	0.368

Eggs and egg dishes	0.5 (0.1, 1.8)	0.5 (0.25, 1.1)	1.000	0.5 (0.2, 1.5)	0.5 (0.2, 1)	0.736
Full-fat spread	4.5 (0, 7)	1.5 (0, 7.4)	0.712	4.5 (0, 7.4)	4.5 (0.25, 9)	0.701
Reduced-fat spread	0 (0, 5.3)	1.5 (0, 7)	0.108	0 (0, 7)	0 (0, 7)	0.979
Cooking fats and salad oils	3 (1.5, 4.5)	3.1 (1.5, 6)	0.234	3 (1.5, 5.1)	4.5 (1.5, 6)	0.348
Red meat	2.5 (1, 3.5)	2.1 (0.95, 3.1)	0.354	2.5 (1.8, 3.5)	1.8 (1, 3)	**0.022**
Chicken and turkey	1.5 (0.5, 1.5)	1.5 (1.5, 1.5)	0.498	1.5 (1.5, 1.5)	1.5 (0.5, 1.5)	0.152
Liver, liver pate, and kidney	0 (0, 0)	0 (0, 0)	0.545	0 (0, 0)	0 (0, 0)	0.394
Processed meat	2.8 (1.4, 5.3)	2.8 (1.8, 4.8)	0.877	2.4 (1.2, 3.6)	2.5 (1.3, 5.1)	0.501
Total fish and shellfish	0.75 (0.35, 2)	1.75 (0.75, 2.25)	**0.001**	0.75 (0.26, 1.85)	2.4 (2, 3)	**<0.001**
Salad vegetables	4.6 (3, 9.4)	6.9 (3.8, 10.1)	0.072	4.8 (2.7, 7)	5.3 (3.3, 9.6)	0.376
Green vegetables	3.7 (2.1, 5.6)	3.7 (2.1, 6.1)	0.581	3.7 (1.7, 5.3)	3.8 (2.6, 6)	0.310
Root vegetables	1.8 (1.5, 4.5)	1.75 (1.5, 4.5)	0.711	1.75 (1.4, 3)	1.75 (1.5, 3)	0.710
Other vegetables	3.5 (2, 6.5)	4.5 (2.25, 6.7)	0.966	3.3 (2.2, 6.3)	3.3 (2, 6.3)	0.956
Tinned vegetables	0.25 (0, 1.5)	0.1 (0, 0.5)	0.192	0.2 (0, 1.5)	0 (0, 0.5)	0.189
Vegetable dishes	0.25 (0, 1.25)	0.25 (0, 0.9)	0.667	0.25 (0, 0.5)	0.25 (0, 0.5)	0.865
Beans and pulses	1.5 (0.25, 1.5)	1.5 (0.4, 1.5)	0.910	1 (0.25, 1.5)	1.5 (0.5, 1.5)	0.641
Chips and roast potatoes	3 (1.5, 6)	3 (1, 4.5)	0.314	3 (1.5, 4.5)	1.5 (1, 4.5)	**0.021**
Boiled potatoes	9 (4.1, 13.5)	4.5 (4.5, 6.8)	0.090	4.5 (3.6, 13.5)	4.5 (4.5, 13.5)	0.660
Crisps	4.5 (1.5, 4.5)	1.5 (1.25, 4.5)	0.176	1.5 (1.5, 4.5)	1.5 (0.5, 4.5)	0.158
Crackers	0.25 (0, 0.5)	0.25 (0, 0.75)	0.274	0.1 (0, 0.5)	0.25 (0, 0.5)	0.692
Citrus fruit and fruit juices	1.5 (1.5, 4.6)	4.5 (1.5, 7)	**0.047**	4.5 (1.5, 5.2)	4.5 (1.5, 7.1)	0.244
Other fruit	9.3 (5.4, 16.3)	9 (3.8, 14.4)	0.487	7.6 (3.3, 14)	8.8 (5, 14)	0.346
Other fruit juices	0.5 (0, 4.5)	1.5 (0.25, 7)	**0.018**	1.5 (1, 4.5)	1.5 (0.1, 4.5)	0.735
Dried fruit	0.1 (0, 0.5)	0.25 (0, 0.75)	0.490	0.18 (0, 0.5)	0.1 (0, 0.5)	0.695
Cooked and tinned fruit	0.2 (0, 0.6)	0.15 (0, 0.5)	0.476	0.25 (0, 0.5)	0.25 (0, 0.5)	0.522
Nuts	0 (0, 0.25)	0.1 (0, 0.5)	0.060	0.05 (0, 0.5)	0 (0, 0.5)	0.559
Sugar (tsp/day)	0 (0, 3)	0 (0, 2)	0.886	1 (0, 3)	0 (0, 1)	**0.049**
Sweet spreads and jam	0.5 (0.25, 1.5)	1.5 (0.25, 4.5)	0.071	1 (0.25, 1.5)	1.5 (0.25, 1.5)	0.347
Sweets and chocolate	5 (2, 9)	3 (1.8, 6)	**0.037**	6 (2.8, 8.6)	4.8 (2, 7)	0.216
High-energy soft drinks	6 (1.5, 10)	4.5 (0.5, 9.8)	0.354	7.4 (1.6, 14.0)	3 (0.5, 10.5)	0.058
Diet cola	0.25 (0, 1.5)	0.18 (0, 1.5)	0.464	0 (0, 0.25)	0.1 (0, 0.5)	0.209
Tea and coffee	11.5 (0.6, 21)	7 (1.5, 15.5)	0.508	8.8 (4.5, 21)	7 (1.5, 15.5)	0.187
Decaffeinated tea and coffee	0 (0, 4.5)	0 (0, 5.1)	0.864	0 (0, 2.25)	0 (0, 4.5)	0.559
Hot chocolate drinks	0.5 (0.05, 1.5)	0.25 (0, 1.5)	0.081	0.25 (0, 1.5)	0.5 (0, 1.5)	0.635
Miscellaneous	5.9 (3.1, 9)	4.8 (3.23, 8.6)	0.792	5 (3.3, 8)	5 (2.5, 8.6)	0.450

Values are median (interquartile range) number of times per week (apart from milk (pints/day) and sugar (teaspoons/day)); Mann Witney test p-values

Food grouping performed according to principal component analysis (PCA) of the SWS FFQ

Table 3.7 compares within group differences in frequency of consumption of food groups over time. Chicken intake decreased in the salmon group from 20 to 34 weeks gestation (p=0.018) as did intake of non-oily fish (p=0.014).

Table 3.7: Within group comparisons of weekly consumption frequency of food groups in the FFQ administered at 20 and 34 weeks gestation

Food group	Control group			Salmon group		
	20 weeks	34 weeks	p	20 weeks	34 weeks	p
Rice and pasta	3 (1.9, 3)	2 (1, 3)	0.097	3 (1, 3)	2 (1, 3)	0.248
White bread	9 (2.3, 14)	9 (3, 14)	0.337	3.8 (0.9, 14)	3 (1, 14)	0.721
Whole-meal bread	3 (0.3, 9)	1.25 (0, 9)	0.556	4.5 (0.7, 14)	3 (0.2, 14)	0.861
Quiche and pizza	0.5 (0.25, 1.5)	0.5 (0.25, 0.5)	0.877	0.5 (0.25, 1.5)	0.5 (0.25, 0.5)	0.103
Savory pancakes (incl. Yorkshire pudding)	0.25 (0, 0.5)	0.1 (0, 0.25)	0.092	0.1 (0.1, 0.25)	0.1 (0, 0.25)	0.817
Breakfast cereals	4.5 (0.5, 7)	4.6 (1.5, 7)	0.202	4.5 (0.9, 7)	7 (1.5, 7)	0.778
Cakes and biscuits	5.5 (3, 9.8)	5.8 (3, 10)	0.401	4.4 (2, 9.4)	8 (3.50, 11)	**0.006**
Puddings	1 (0.5, 2.5)	1.7 (0.8, 2.6)	0.552	1.6 (6, 2.3)	1.5 (0.75, 2.3)	0.485
Cream	0.25 (0, 0.5)	0.25 (0, 0.5)	0.152	0.18 (0, 0.5)	0.25 (0, 0.5)	0.486
Full-fat milk (pt/day)	0 (0, 0)	0 (0, 0)	0.972	0 (0, 0)	0 (0, 0)	0.646
Reduced-fat milk (pt/day)	0.5 (0.09, 0.75)	0.5 (0.2, 1)	**0.048**	0.5 (0.15, 0.6)	0.5 (0.25, 1)	**0.044**
Yogurt	1.5 (0.25, 4.5)	1.5 (0.5, 4.5)	0.311	1.5 (0.25, 4.5)	1.5 (0.25, 4.5)	0.223
Cheese and cottage cheese	4.5 (1.5, 4.5)	4.5 (1.5, 4.5)	0.793	4.5 (1.5, 4.6)	4.5 (1.5, 4.5)	**0.003**
Eggs and egg dishes	0.5 (0.10, 1.75)	0.5 (0.2, 1.5)	0.071	0.5 (0.25, 1.1)	0.5 (0.2, 1)	0.456
Full-fat spread	4.5 (0, 7)	4.5 (0, 7.4)	0.896	1.5 (0, 7.4)	4.5 (0.25, 9)	0.165
Reduced-fat spread	0 (0, 5.25)	0 (0, 7)	0.165	1.5 (0, 7)	0 (0, 7)	0.555
Cooking fats and salad oils	3 (1.5, 4.5)	3 (1.5, 5.1)	0.708	3.1 (1.5, 6)	4.5 (1.5, 6)	0.404
Red meat	2.5 (1, 3.5)	2.5 (1.8, 3.5)	0.736	2.1 (0.95, 3.1)	1.8 (0.95, 3)	0.263
Chicken and turkey	1.5 (0.5, 1.5)	1.5 (1.5, 1.5)	0.718	1.5 (1.5, 1.5)	1.5 (0.5, 1.5)	**0.018**
Liver, liver pate, and kidney	0 (0, 0)	0 (0, 0)	0.369	0 (0, 0)	0 (0, 0)	0.719
Processed meat	2.8 (1.4, 5.3)	2.4 (1.2, 3.6)	**0.013**	2.8 (1.8, 4.8)	2.5 (1.25, 5.1)	0.214
Total fish and shellfish	0.75 (0.35, 2)	0.75 (0.25, 1.85)	0.519	1.75 (0.75, 2.25)	2.35 (2, 3)	**<0.001**
Salad vegetables	4.6 (3, 9.4)	4.8 (2.7, 7)	0.743	6.9 (3.8, 10.1)	5.3 (3.3, 9.6)	0.028
Green vegetables	3.7 (2.1, 5.6)	3.7 (1.7, 5.3)	0.510	3.7 (2.1, 6.1)	3.8 (2.6, 6)	0.763
Root vegetables	1.8 (1.5, 4.5)	1.8 (1.4, 3)	0.334	1.8 (1.5, 4.5)	1.8 (1.5, 3)	0.314
Other vegetables	3.5 (2, 6.5)	3.3 (2.2, 6.3)	0.337	4.5 (2.3, 6.7)	3.3 (2, 6.3)	0.271
Tinned vegetables	0.25 (0, 1.5)	0.18 (0, 1.5)	0.705	0.1 (0, 0.5)	0 (0, 0.5)	0.290
Vegetable dishes	0.25 (0, 1.25)	0.25 (0, 0.5)	0.293	0.25 (0, 0.94)	0.25 (0, 0.5)	0.025
Beans and pulses	1.5 (0.25, 1.5)	1 (0.25, 1.5)	0.494	1.5 (0.44, 1.5)	1.5 (0.5, 1.5)	0.322
Chips and roast potatoes	3 (1.50, 6)	3 (1.5, 4.5)	0.735	3 (1, 4.5)	1.5 (1, 4.5)	0.273
Boiled potatoes	9 (4.1, 13.5)	4.5 (3.6, 13.5)	0.111	4.5 (4.5, 6.8)	4.5 (4.5, 13.5)	0.105

Crisps	4.5 (1.5, 4.5)	1.5 (1.5, 4.5)	0.089	1.5 (1.3, 4.5)	1.5 (0.5, 4.5)	0.232
Crackers	0.25 (0, 0.5)	0.1 (0, 0.5)	0.239	0.25 (0, 0.75)	0.25 (0, 0.5)	**0.010**
Citrus fruit and fruit juices	1.5 (1.5, 4.6)	4.5 (1.5, 5.2)	0.956	4.5 (1.5, 7)	4.5 (1.5, 7.1)	0.662
Other fruit	9.3 (5.4, 16.3)	7.6 (3.3, 14)	0.079	9 (3.8, 14.4)	8.8 (5, 14)	0.883
Other fruit juices	0.5 (0, 4.5)	1.5 (1, 4.5)	0.888	1.5 (0.25, 7)	1.5 (0.1, 4.5)	**0.001**
Dried fruit	0.1 (0, 0.5)	0.2 (0, 0.5)	0.949	0.25 (0, 0.75)	0.1 (0, 0.5)	0.183
Cooked and tinned fruit	0.2 (0, 0.55)	0.25 (0, 0.5)	0.086	0.15 (0, 0.5)	0.25 (0, 0.5)	0.064
Nuts	0 (0, 0.25)	0.05 (0, 0.5)	0.075	0.1 (0, 0.5)	0 (0, 0.5)	0.183
Sugar (tsp/day)	0 (0, 3)	1 (0, 3)	0.079	0 (0, 2)	0 (0, 1)	0.240
Sweet spreads and jam	0.5 (0.25, 1.5)	1 (0.25, 1.5)	0.178	1.5 (0.25, 4.5)	1.5 (0.25, 1.5)	0.675
Sweets and chocolate	5 (2, 9)	6 (2.8, 8.6)	0.991	3 (1.8, 6)	4.8 (2, 7)	0.151
High-energy soft drinks	6 (1.5, 10)	7.4 (1.6, 14.0)	0.951	4.5 (0.5, 9.8)	3 (0.5, 10.5)	0.577
Diet cola	0.25 (0, 1.5)	0 (0, 0.25)	**0.005**	0.18 (0, 1.5)	0.1 (0, 0.5)	0.304
Tea and coffee	11.5 (0.6, 21)	8.8 (4.5, 21)	0.248	7 (1.5, 15.5)	7 (1.5, 15.5)	**0.018**
decaffeinated tea and coffee	0 (0, 4.5)	0 (0, 2.25)	0.516	0 (0, 5.13)	0 (0, 4.5)	0.686
Hot chocolate drinks	0.5 (0.05, 1.5)	0.25 (0, 1.5)	0.936	0.25 (0, 1.5)	0.5 (0, 1.5)	0.317
Miscellaneous	5.9 (3.1, 9)	5 (3.3, 8)	0.434	4.8 (3.3, 8.6)	5 (2.5, 8.6)	0.266

Values are median (interquartile range) number of times per week (apart from milk (pints/day) and sugar (teaspoons/day)); Wilcoxon test p-values

Food grouping performed according to principal component analysis (PCA) of the SWS FFQ

Table 3.8 describes the between group comparisons for consumption frequencies of additional food groups. There were no differences between the groups in consumption frequencies of total fruit, total vegetables, total meat and total alcohol at any time point. However, as shown in

Table 3.9, within the salmon group total meat consumption decreased (p=0.048) from 20 to 34 weeks gestation, and total vegetable consumption showed a strong trend of decrease (non-significant) within the salmon group. Table 3.9 also includes information on consumption frequencies of different types of spreading fat, cooking fat, and salad dressings. The only significant difference between the two groups was in olive oil consumption at 34 weeks gestation (p=0.038).

Table 3.8: Between group comparisons of weekly consumption frequency of additional food groups in the FFQ at 20 and 34 weeks

Food group	20 weeks gestation			34 weeks gestation		
	Control	Salmon	p	Control	Salmon	p
Total fruit	15.5 (10.2, 23.8)	18.9 (10.9, 28.2)	0.268	11.8 (8.4, 24.5)	18.1 (10.5, 27.1)	0.096
Total vegetables	18.6 (14.3, 28.3)	21.2 (15.3, 27.8)	0.491	16.8 (12.9, 26.5)	19.3 (13.7, 25.5)	0.476
Total meat	7.4 (5.1, 11.7)	7.3 (5.5, 10.2)	0.638	7.3 (4.4, 10.6)	6.5 (4.6, 9.5)	0.495
Total alcohol	0 (0, 0.33)	0.6 (0, 0.26)	0.711	0 (0, 0.5)	0 (0, 0.25)	0.898
Margarine/ Vegetable oil based spreading fat	0 (0, 4.5)	0 (0, 7)	0.810	0.13 (0, 4.5)	0 (0, 6)	0.773
Butter	1.5 (0, 7)	1.5 (0, 7)	0.781	4.5 (0, 7)	2 (0, 7)	0.943
Olive oil based spreading fat	0 (0, 0)	0 (0, 0.44)	0.191	0 (0, 0)	0 (0, 0)	0.582
Vegetable oils	0.5 (0, 1.5)	0.38 (0, 1.5)	0.435	0.5 (0, 1.5)	0.25 (0, 1.5)	0.159
Olive oil	1.5 (0, 3.75)	1.5 (0, 4.5)	0.067	0.13 (0, 3.4)	1.5 (0, 4.5)	**0.038**
Animal fat	0 (0, 0)	0 (0, 0)	0.152	0 (0, 0)	0 (0, 0)	0.322
Salad dressing	0 (0, 0)	0 (0, 0)	0.800	0 (0, 0)	0 (0, 0)	0.538

Values are median (interquartile range) number of times per week; Mann Witney test p-values

Table 3.9: Within group comparisons of weekly consumption frequency of additional food groups in the FFQ at 20 and 34 weeks

Food group	Control group			Salmon group		
	20 weeks	34 weeks	p	20 weeks	34 weeks	p
Total fruit	15.5 (10.2, 23.8)	11.8 (8.4, 24.5)	0.241	18.9 (10.9, 28.2)	18.1 (10.5, 27.1)	0.080
Total vegetables	18.6 (14.3, 28.3)	16.8 (12.9, 26.5)	0.201	21.2 (15.3, 27.8)	19.3 (13.7, 25.5)	**0.059**
Total meat	7.4 (5.1, 11.7)	7.3 (4.4, 10.6)	0.386	7.3 (5.5, 10.2)	6.5 (4.6, 9.5)	**0.048**
Total alcohol	0 (0, 0.33)	0 (0, 0.5)	0.202	0.6 (0, 0.26)	0 (0, 0.25)	0.683
Margarine/ Vegetable oil based spreading fat	0 (0, 4.5)	0.13 (0, 4.5)	0.461	0 (0, 7)	0 (0, 6)	0.611
Butter	1.5 (0, 7)	4.5 (0, 7)	0.064	1.5 (0, 7)	2 (0, 7)	0.309
Olive oil based spreading fat	0 (0, 0)	0 (0, 0)	1.000	0 (0, 0.44)	0 (0, 0)	0.700
Vegetable oils	0.5 (0, 1.5)	0.5 (0, 1.5)	0.422	0.38 (0, 1.5)	0.25 (0, 1.5)	0.194
Olive oil	1.5 (0, 3.75)	0.13 (0, 3.4)	0.656	1.5 (0, 4.5)	1.5 (0, 4.5)	0.908
Animal fat	0 (0, 0)	0 (0, 0)	0.180	0 (0, 0)	0 (0, 0)	0.317
Salad dressing	0 (0, 0)	0 (0, 0)	0.440	0 (0, 0)	0 (0, 0)	0.529

*Values are median (interquartile range) number of times per week; Wilcoxon test p-values

Due to the fact that differences were observed at baseline for total fish and shellfish consumption between the two groups, the different fish categories were further analysed (Table 3.10, Table 3.11). At week 20 the salmon group was consuming more frequently non-oily fish (medians 0.5 and 0.25 times per week, for salmon and control group respectively, p=0.005) as well as oily fish (medians 0.25 and 0 times per week, for salmon and control group respectively, p<0.001) in comparison to the control group. At week 34 (during intervention) there were no significant differences in non-oily fish intake between

the two groups, but there was a significant increase in the oily fish intake in the salmon group, (medians 1.5 and 0 times per week for salmon and control group respectively, p<0.001). This is very important because it shows that the women in the salmon group had increased their oily fish intake as required by the study protocol.

Table 3.10: Between group comparisons of weekly consumption frequency of meat and fish categories in the FFQ

Food group	20 weeks gestation			34 weeks gestation		
	Control group	Salmon group	p	Control group	Salmon group	p
Non-oily fish	0.25 (0.25, 0.5)	0.5 (0.25, 1.5)	**0.005**	0.25 (0.08, 1.5)	0.5 (0.25, 1.5)	0.204
Fish fingers and fish dishes	0.25 (0, 0.5)	0.25 (0, 0.5)	0.597	0.18 (0, 0.5)	0.1 (0, 0.25)	0.866
Oily fish	0 (0, 0.25)	0.25 (0, 0.5)	**<0.001**	0 (0, 0.14)	1.5 (1.5, 1.5)	**<0.001**
Shellfish	0 (0, 0.1)	0 (0, 0.1)	0.855	0 (0, 0)	0 (0, 0.1)	0.0760
Pork	0.25 (0, 0.5)	0.17 (0, 0.5)	0.526	0.25 (0.8, 0.5)	0.25 (0.1, 0.5)	0.189
Chicken	1.5 (0.5, 1.5)	1.5 (1.5, 1.5)	0.498	1.5 (1.5, 1.5)	1.5 (0.5, 1.5)	0.152
Lamb	0.1 (0, 0.5)	0.17 (0, 0.5)	0.894	0.25 (0, 0.5)	0.1 (0, 0.5)	0.92
Beef	0.5 (0.1, 1.5)	0.4 (0.25, 1.5)	0.959	0.5 (0.25, 1.5)	0.5 (0.1, 0.50)	0.351
Minced meat dishes	1.5 (0.25, 1.5)	0.5 (0.43, 1.5)	0.315	1.5 (0.5, 1.5)	0.5 (0.25, 1.5)	0.301
Sausages	0.5 (0.25, 1.5)	0.5 (0.25, 1.5)	0.463	0.5 (0.21, 0.5)	0.5 (0.25, 0.5)	0.395
Ham and luncheon meat	1.5 (0.25, 1.5)	1.5 (.43, 1.5)	0.467	0.5 (0.25, 1.5)	1.5 (0.25, 1.5)	0.156

Values are median (interquartile range) number of times per week; Mann Witney test p-values

Table 3.11: Within group comparisons of weekly consumption frequency of meat and fish categories in the FFQ at 20 and 34 weeks of gestation

Food group	Control group			Salmon group		
	20 weeks	34 weeks	p	20 weeks	34 weeks	p
Non-oily fish	0.25 (0.25, 0.5)	0.25 (0.08, 1.5)	0.736	0.5 (0.25, 1.5)	0.5 (0.25, 1.5)	**0.014**
Fish fingers and fish dishes	0.25 (0, 0.5)	0.18 (0, 0.5)	0.766	0.25 (0, 0.5)	0.1 (0, 0.25)	0.132
Oily fish	0 (0, 0.25)	0 (0, 0.14)	**0.025**	0.25 (0, 0.5)	1.5 (1.5, 1.5)	**<0.001**
Shellfish	0 (0, 0.1)	0 (0, 0)	0.066	0 (0, 0.1)	0 (0, 0.1)	0.841
Pork	0.25 (0, 0.5)	0.25 (0.8, 0.5)	0.787	0.17 (0, 0.5)	0.25 (0.1, 0.5)	0.167
Chicken	1.5 (0.5, 1.5)	1.5 (1.5, 1.5)	0.718	1.5 (1.5, 1.5)	1.5 (0.5, 1.5)	**0.018**
Lamb	0.1 (0, 0.5)	0.25 (0, 0.5)	0.909	0.17 (0, 0.5)	0.1 (0, 0.5)	0.735
Beef	0.5 (0.1, 1.5)	0.5 (0.25, 1.5)	0.603	0.4 (0.25, 1.5)	0.5 (0.1, 0.50)	0.756
Minced meat dishes	1.5 (0.25, 1.5)	1.5 (0.5, 1.5)	0.962	0.5 (0.43, 1.5)	0.5 (0.25, 1.5)	0.670
Sausages	0.5 (0.25, 1.5)	0.5 (0.21, 0.5)	**0.040**	0.5 (0.25, 1.5)	0.5 (0.25, 0.5)	0.558
Ham and luncheon meat	1.5 (0.25, 1.5)	0.5 (0.25, 1.5)	0.064	1.5 (.43, 1.5)	1.5 (0.25, 1.5)	0.723

Values are median (interquartile range) number of times per week; Wilcoxon test p-values

Figure 3.1 and Figure 3.2 show the distribution of intake of non-oily and oily fish, respectively, according to the FFQ frequency categories, for both study groups at baseline (20 weeks gestation) and during intervention (34 weeks gestation). In Figure 3.2 all women in the salmon group reported consuming oily fish 1-2 times per week, which was interpreted as 1.5 times per week.

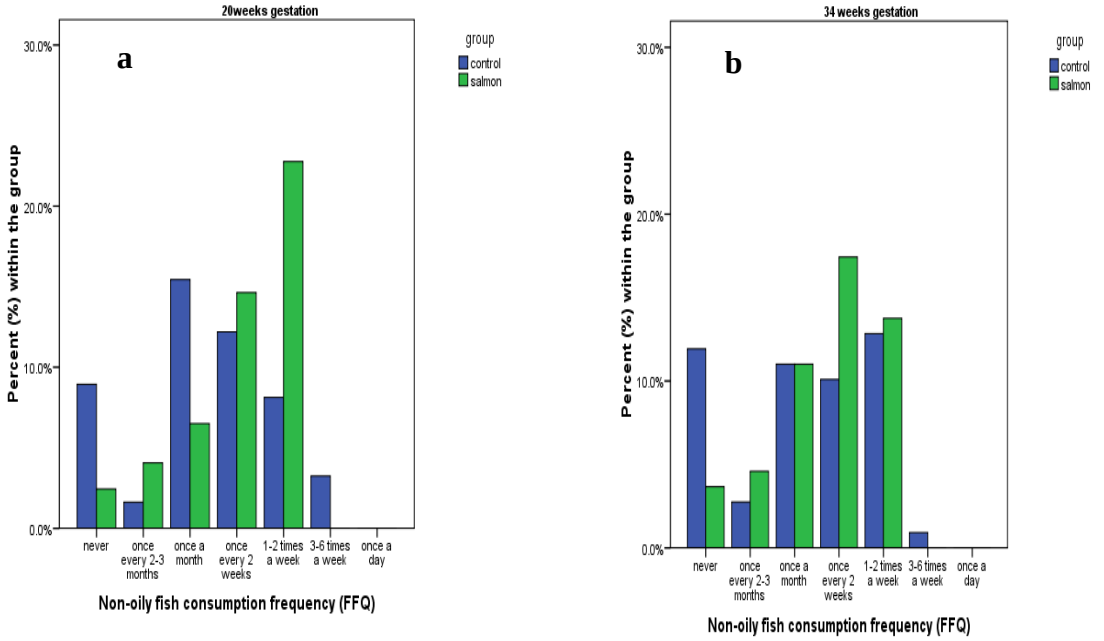


Figure 3.1: Distribution of non-oily fish consumption into FFQ categories at a) 20 weeks gestation, b) 34 weeks gestation

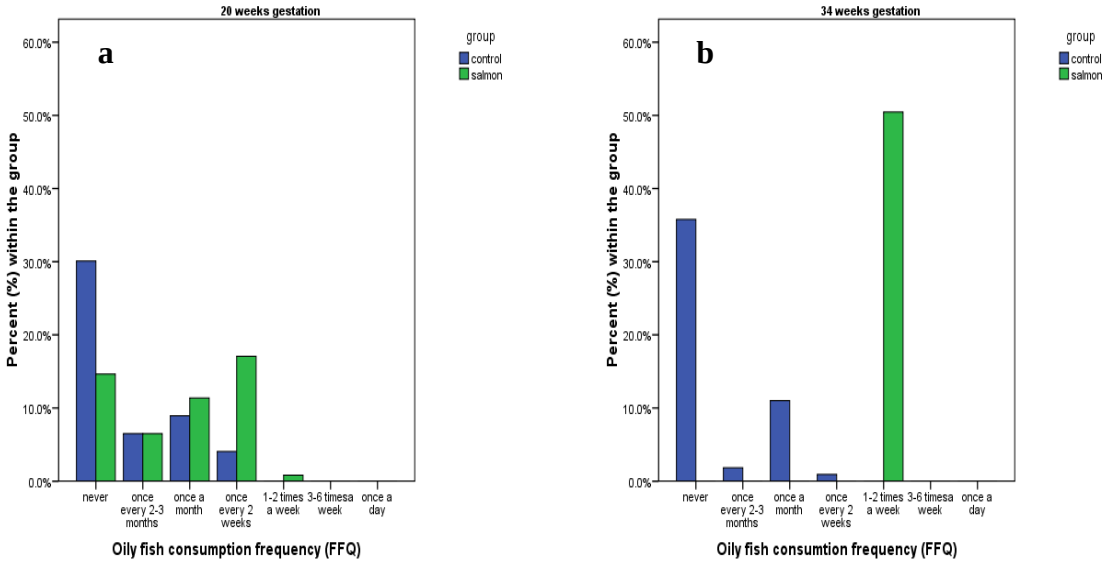


Figure 3.2: Distribution of oily fish consumption into FFQ categories at a) 20 weeks gestation, b) 34 weeks gestation

In order to compare the changes in intake associated with the intervention between the two study groups, the changes in consumption frequencies for all food groups were calculated (i.e. 34 weeks minus 20 weeks gestation) for the control and the study group and these are presented in Table 3.12. The changes in intake of cheese and sugar were significantly smaller for the salmon group compared to the control group, whereas the change in fish intake was significantly greater in the salmon group than in the control group as expected after the intervention.

Because of the baseline differences in fish intake between the groups, changes in fish consumption frequencies were calculated (i.e. 34 weeks minus 20 weeks gestation) (Table 3.13). Based upon the FFQ data and categories, the increase in the oily fish consumption observed in the salmon group was 1.21 times per week from 20 to 34 weeks gestation, whereas the oily fish consumption in the control group remained almost unchanged (0.04 times per week less), and this change was different between the groups ($p<0.001$) (Figure 3.3). Also the increase in total fish consumption in the salmon group (0.85 portions per week) was significantly different from the almost unchanged total fish consumption frequency in the control group (0.05 times per week less) ($p<0.001$) (Figure 3.4). Changes in consumption frequencies of all 48 food groups are described in Table 3.12 Changes in consumption frequencies of the different categories of meat were not different between control and salmon groups (Table 3.13).

Table 3.12: Changes in weekly consumption frequencies for all 48 food groups - between group comparisons

Food group	Change in consumption : 34-20 weeks gestation		
	Control group	Salmon group	p
Rice and pasta	-0.45 (2.01)	-0.14 (1.64)	0.385
White bread	-1.16 (9.89)	-0.95 (5.81)	0.895
Whole-meal bread	-0.18 (3.74)	-0.33 (8.82)	0.905
Quiche and pizza	-0.02 (0.47)	-0.11 (0.49)	0.329
Savory pancakes (incl. Yorkshire pudding)	-0.18 (0.98)	-0.01 (0.31)	0.234
Breakfast cereals	0.43 (3.09)	0.18 (2.97)	0.674
Cakes and biscuits	-0.53 (6.26)	1.48 (5.70)	0.081
Puddings	0.06 (2.14)	0.23 (1.80)	0.649
Cream	-0.05 (0.31)	-0.04 (1.14)	0.905
Full-fat milk (pt/day)	0.01 (0.23)	-0.05 (0.32)	0.303
Reduced-fat milk (pt/day)	0.11 (0.52)	0.09 (0.39)	0.877
Yogurt	-0.34 (2.58)	-0.33 (2.18)	0.983
Cheese and cottage cheese	0.07 (1.72)	-0.71 (2.40)	**0.055**
Eggs and egg dishes	-0.40 (1.29)	0.01 (1.10)	0.075
Full-fat spread	-0.04 (5.49)	1.23 (6.45)	0.272
Reduced-fat spread	0.61 (3.19)	-0.60 (5.17)	0.144
Cooking fats and salad oils	0.18 (2.54)	0.20 (3.04)	0.966
Red meat	0.11 (2.20)	-0.20 (1.85)	0.424
Chicken and turkey	0.13 (1.73)	-0.32 (0.96)	0.094
Liver, liver pate, and kidney	-0.04 (0.26)	-0.01 (0.16)	0.390
Processed meat	-0.87 (2.68)	-0.30 (2.16)	0.224
Total fish and shellfish	-0.05 (1.07)	0.85 (0.85)	**<0.001**
Salad vegetables	-0.62 (4.46)	-1.15 (3.61)	0.497
Green vegetables	-0.29 (3.22)	0.17 (3.27)	0.459
Root vegetables	-0.20 (1.90)	-0.20 (2.03)	0.990
Other vegetables	-0.28 (2.63)	-0.43 (2.36)	0.757
Tinned vegetables	0.06 (0.90)	0.35 (1.38)	0.201
Vegetable dishes	-0.05 (0.89)	-0.26 (0.77)	0.191
Beans and pulses	-0.14 (1.45)	-0.20 (1.37)	0.811
Chips and roast potatoes	0.07 (4.21)	-0.68 (3.17)	0.292

Boiled potatoes	-1.38 (6.99)	1.90 (8.16)	**0.026**
Crisps	-0.39 (1.97)	-0.30 (2.42)	0.821
Crackers	-0.01 (1.79)	-0.41 (1.70)	0.239
Citrus fruit and fruit juices	0.22 (5.64)	-0.22 (7.17)	0.721
Other fruit	-2.05 (7.20)	-0.12 (6.60)	0.148
Other fruit juices	-0.02 (3.66)	-1.36 (3.08)	**0.041**
Dried fruit	-0.20 (1.93)	-0.23 (1.47)	0.923
Cooked and tinned fruit	-0.21 (1.14)	0.28 (1.06)	**0.024**
Nuts	0.33 (1.41)	-0.12 (1.50)	0.114
Sugar (tsp/day)	0.43 (1.74)	-0.13 (0.84)	**0.037**
Sweet spreads and jam	0.34 (1.78)	0.22 (2.01)	0.749
Sweets and chocolate	0.32 (5.00)	0.99 (4.02)	0.442
High-energy soft drinks	1.18 (15.70)	0.10 (12.12)	0.689
Diet cola	-0.94 (4.36)	0.37 (4.70)	0.135
Tea and coffee	1.49 (10.69)	1.55 (5.20)	0.970
Decaffeinated tea and coffee	-0.06 (5.11)	-0.19 (5.90)	0.898
Hot chocolate drinks	-0.02 (2.23)	0.02 (2.83)	0.927
Miscellaneous	-0.32 (4.05)	-0.32 (3.29)	0.993

Values are mean (±standard deviation)number of times per week; independent samples t-test p values

Table 3.13: Changes in fish and meat weekly consumption frequencies - between group comparisons

Food category	**Change in consumption: 34-20 weeks gestation**		
	Control group	Salmon group	**p**
Non-oily fish	0.02 (0.96)	-0.25 (0.7)	0.104
Fish fingers and fish dishes	0.01 (0.38)	-0.11 (0.43)	0.128
Oily fish	-0.04 (0.14)	1.21 (0.26)	**<0.001**
Shellfish	-0.04 (0.27)	-0.25 (0.7)	0.298
Total fish	-0.05 (1.07)	0.85 (0.085)	**<0.001**
Pork	0.08 (0.91)	0 (0.12)	0.149
Chicken	0.13 (1.73)	-0.12 (0.46)	0.094
Lamb	0.08 (0.69)	-0.32 (0.96)	0.320
Beef	-0.04 (1.38)	-0.03 (0.37)	0.673
Total red meat	0.11 (2.2)	-0.2 (1.85)	0.424
Total meat	-0.67 (4.47)	-0.83 (3.24)	0.831

Values are mean (±standard deviation)number of times per week; independent samples t-test p values

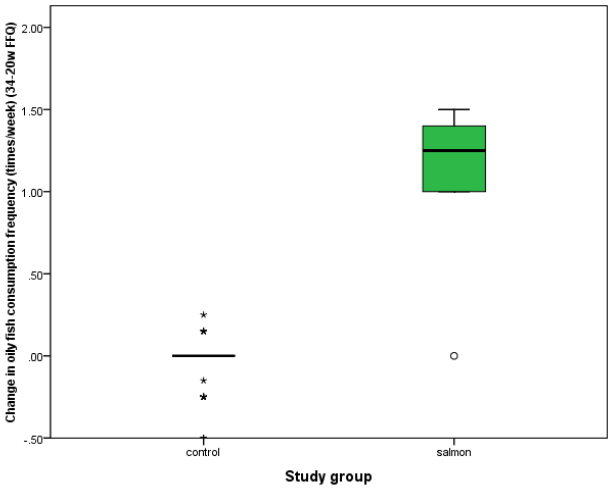


Figure 3.3: Change in oily fish consumption according to study group (times per week) (p<0.001)

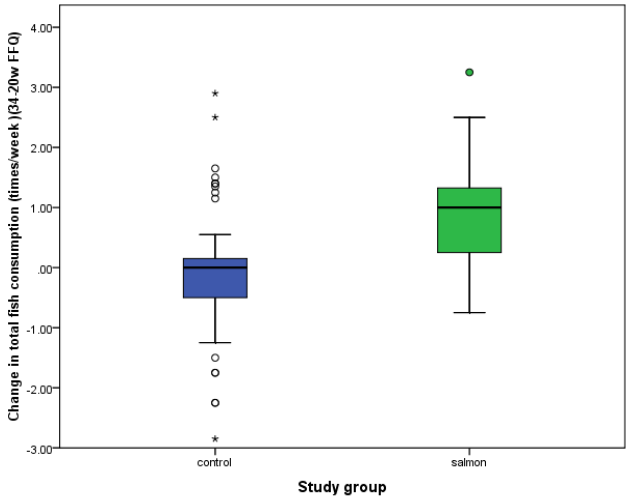


Figure 3.4: Change in total fish consumption according to study group (times per week) (p<0.001)

3.3.2 Nutrient intakes based on FFQ

Table 3.14 describes the between group comparisons for total dietary intake of nutrients derived from the FFQ at baseline (20 weeks gestation) and during intervention (34 weeks gestation). At baseline the only differences noted were the significantly higher intake of EPA, DHA, and EPA plus DHA in the salmon group (all p<0.001). During intervention intakes of EPA, DHA, EPA plus DHA were significantly higher in the salmon group compared to the control group (all p<0.001). It must be noted that the determination of EPA and DHA intakes from the FFQ is not accurate, and these must be considered to be crude estimates. Since fish are a major source of LC n-3 PUFAs (none of the subjects consumed fish oil supplements), the intake of those nutrients from the fish categories was explored as described in Table 3.15, Table 3.16, Table 3.17, Table 3.18, Table 3.19 and Table 3.20. At 34 weeks gestation (but not at baseline), the intake of LC n-3 PUFAs was higher in the salmon group (p<0.001). Also at 34 weeks there were a number of significant differences in intake of specific micronutrients between the two groups. Specifically, the salmon group had a higher intake of vitamin B12, selenium, and vitamin D than the control group (Table 3.14).

Table 3.14: Between group comparisons of daily intake of selected nutrients from total diet calculated from FFQ at 20 weeks and 34 weeks gestation

Nutrient	20 weeks gestation			34 weeks gestation		
	Control group	Salmon group	p	Control group	Salmon group	p
Energy (Kcal) [1]	2231 (±671)	2169 (±569)	0.583	2229 (±543)	2184 (±533)	0.668
Energy (KJ) [1]	9393 (±2822)	9133 (±2393)	0.582	9388 (±2289)	9197 (±2240)	0.661
Carbohydrates (g) [1]	297 (±94)	279 (±79)	0.260	297 (±82)	280 (±77)	0.276
Protein (g) [1]	81 (±24)	80 (±21)	0.968	80 (±22)	81 (±19)	0.948
Total fat (g) [1]	88 (±29)	89 (±26)	0.905	88 (±24)	90 (±24)	0.635
SFA (g) [1]	34 (±13)	34 (±12)	0.810	34 (±10)	35 (±11)	0.604
MUFA (g) [1]	31 (±10)	32 (±9)	0.573	32 (±9)	33 (±8)	0.514
PUFA (g) [1]	15.5 (±5.7)	15.5 (±4.7)	0.994	15.6 (±4.9)	15.3 (±4.7)	0.730
Total n-3 PUFAs (g) [1]	1.8 (±0.6)	1.9 (±0.5)	0.900	1.9 (±0.6)	2.5 (±0.5)	**<0.001**
Total n-6 PUFAs (g) [1]	13.5 (±5.1)	13.4 (±4.3)	0.902	13.6 (±4.4)	12.9 (±4.3)	0.445
Dietary fibre (g) (Englyst method) [1]	17.5 (±5.7)	17.8 (±5.9)	0.730	16.2 (±4.8)	17.4 (±5.4)	0.215
Sodium (g) [1]	3212 (±906)	3110 (±804)	0.508	3057 (±768)	2989 (±868)	0.669
Potassium (g) [1]	3470 (±1002)	3526 (±1051)	0.762	3439 (±926)	3530 (±895)	0.604
Calcium (mg) [2]	1135 (±432)	1167 (±517)	0.711	1199 (±429)	1200 (±404)	0.990

Magnesium (mg) [2]	348 (±105)	355 (±101)	0.722	342 (±102)	355 (±96)	0.475
Phosphorus mg) [2]	1485 (±452)	1504 (±469)	0.816	1514 (±428)	1534 (±391)	0.797
Zinc (mg) [2]	12.8 (±5.5)	12.8 (±5.6)	0.942	12 (±5.4)	12.3 (±5.4)	0.796
Retinol (µg) [2]	447 (±242)	451 (±234)	0.940	430 (±172)	417.5 (±155)	0.693
Retinol equivalents (µg) [1]	361 (±200)	357 (±190)	0.915	355 (±168)	327 (±132)	0.334
Carotene (µg) [2]	3120 (±1254)	3230 (±1432)	0.650	2847 (±1195)	3008 (±1216)	0.486
Thiamin (B1) (mg) [2]	2.49 (±1.18)	2.43 (±1.05)	0.789	2.23 (±1.08)	2.29 (±1.05)	0.768
Riboflavin (B2) (mg)[2]	2.64 (±1.13)	2.68 (±1.08)	0.861	2.64 (±1.13)	2.68 (±0.96)	0.821
Niacin (mg) [2]	25.5 (±10.8)	26.2 (±9)	0.685	24.8 (±10.5)	26 (±10.6)	0.554
Vitamin B6 (mg) [2]	3.96 (±2.92)	4.02 (±2.98)	0.913	3.50 (±2.72)	3.74 (±2.73)	0.649
Vitamin B12 (µg) [2]	5.84 (±2.44)	5.94 (±2.46)	0.823	5.91 (±2.65)	7.15 (±2.17)	**0.009**
Folate (µg) [2]	564.9 (±591)	556 (±632)	0.935	399 (±199)	409 (±197)	0.784
Vitamin C (mg) [2]	164 (±80)	173 (±82)	0.508	177 (±160)	179 (±106)	0.902
Selenium (µg) [2]	52.4 (±20.4)	56.4 (±16.8)	0.244	49.0 (±17.0)	58.2 (±16.7)	**0.005**
Vitamin E (mg) [3]	15.7 (±7.1)	15.5 (±6.8)	0.934	14.3 (±6.7)	15 (±7.2)	0.592
Iron (mg) [3]	15.7 (12.6, 25.8)	15.4 (12.1, 24.3)	0.495	20.3 (11.0, 30.9)	15.5 (11.2, 27.2)	0.641
Vitamin D (µg) [3]	4.1 (2.5, 10.1)	3.6 (2.8, 9.9)	0.590	2.9 (2.0, 12.4)	5.8 (5, 9.5)	**<0.001**
EPA (mg) [3]	17.6 (5.1, 36.3)	42.6 (23.8, 66.7)	**<0.001**	11.9 (3, 34.8)	133.7 (127.7, 145.9)	**<0.001**
DHA (mg) [3]	29.4 (9.1, 65.3)	77.6 (45.6, 121.9)	**<0.001**	19.7 (6.2, 67)	269 (260.7, 298.1)	**<0.001**
Total EPA+DHA (mg) [3]	46.4 (13.7, 100)	124.6 (69.4, 184.7)	**<0.001**	30.1 (9.1, 103)	402.6 (309.0, 444)	**<0.001**

[1] Nutrient intake only from diet (supplements did not contribute to the intake of these nutrients). Values are mean (±standard deviation), independent samples t-test p-values

[2] Nutrient for which supplements contributed to intake (diet plus supplement intake). Values are mean (±standard deviation), independent samples t-test p-values

[3] Nutrient for which supplements contributed to intake (diet plus supplement intake). Values are medians (interquartile range), Mann Witney test p-values

Note: EPA and DHA: the FFQ cannot provide reliable data on intake from the whole diet (incomplete values for some foods); Selenium: values completed for all foods in the FFQ - however data are limited as the content of foods varies widely and depends on soil content of the region.

Table 3.15 to Table 3.20 show the daily intake of seven nutrients of interest (EPA, DHA, total n-3 PUFAs, total n-6 PUFAs, selenium, vitamins D and E) from non-oily, oily (including study salmon) and total fish (including oily, non-oily, shellfish and fish dishes), compared between and within groups for each fish category, at baseline (20 weeks) and during intervention (34 weeks). Table 3.15 and Table 3.16 show the daily intake of these seven nutrients from non-oily fish, before and after intervention. At 20 weeks, pregnant women in the salmon group had a higher intake of all the seven nutrients from white fish (p=0.005) compared to the pregnant women in the control group, revealing a baseline difference between the two groups. This was not apparent at 34 weeks (p=0.204). For the within groups comparison (Table 3.16), the effect of the intervention is obvious as the salmon group showed a significant difference for all the seven nutrients from the white fish (for all nutrients p=0.014). There is no such difference within the control group (p=0.736).

Table 3.15: Between group comparisons of daily nutrient intakes from non-oily (white) fish according to the FFQ

Nutrient	20 weeks gestation			34 weeks gestation		
	Control group	Salmon group	p	Control group	Salmon group	p
Total n-3 PUFAs (mg)	22.9 (22.9, 45.9)	45.9 (22.9, 137.6)		22.9 (6.9, 137.6)	45.9 (22.9, 137.6)	
Total n-6 PUFAs (mg)	36 (36, 72)	72 (36, 216)		36 (10.8, 216)	72 (36, 216)	
EPA (mg)	4 (4, 7.9)	7.9 (4, 23.8)		4 (1.2, 23.8)	7.9 (4, 23.8)	
DHA (mg)	8.3 (8.3, 16.5)	16.5 (8.3, 49.5)	**0.005**	8.3 (2.5, 49.5)	16.5 (8.3, 49.5)	0.204
Selenium (µg)	2.79 (2.79, 5.57)	5.57 (2.79, 16.71)		2.79 (0.84, 16.71)	5.57 (2.79, 16.71)	
Vitamin D (µg)	0.071 (0.071, 0.141)	0.141 (0.071, 0.424)		0.071 (0.021, 0.424)	0.141 (0.071, 0.424)	
Vitamin E (mg)	0.044 (0.044, 0.089)	0.089 (0.044, 0.267)		0.044 (0.013, 0.267)	0.089 (0.044, 0.267)	

Values are medians (interquartile range), Mann Witney test p-values

Table 3.16: Within group comparisons of daily nutrient intakes from non-oily (white) fish according to the FFQ

Nutrient	Control group (n=55)			Salmon group (n=54)		
	20 weeks	**34 weeks**	**p**	**20 weeks**	**34 weeks**	**p**
Total n-3 PUFAs (mg)	22.9 (22.9, 45.9)	22.9 (6.9, 137.6)		45.9 (22.9, 137.6)	45.9 (22.9, 137.6)	
Total n-6 PUFAs (mg)	36 (36, 72)	36 (10.8, 216)		72 (36, 216)	72 (36, 216)	
EPA (mg)	4 (4, 7.9)	4 (1.2, 23.8)		7.9 (4, 23.8)	7.9 (4, 23.8)	
DHA (mg)	8.3 (8.3, 16.5)	8.3 (2.5, 49.5)	0.736	16.5 (8.3, 49.5)	16.5 (8.3, 49.5)	**0.014**
Selenium (µg)	2.79 (2.79, 5.57)	2.79 (0.84, 16.71)		5.57 (2.79, 16.71)	5.57 (2.79, 16.71)	
Vitamin D (µg)	0.071 (0.071, 0.141)	0.071 (0.021, 0.424)		0.141 (0.071, 0.424)	0.141 (0.071, 0.424)	
Vitamin E (mg)	0.044 (0.044, 0.089)	0.044 (0.013, 0.267)		0.089 (0.044, 0.267)	0.089 (0.044, 0.267)	

Values are medians (interquartile range), Wilcoxon test p-values

Table 3.17 and Table 3.18 show the daily intake of those seven nutrients from oily fish, including study salmon, at 20 and 34 week of pregnancy. At both 20 and 34 weeks pregnant women in the salmon group had a higher intake of all the seven nutrients (all $p<0.001$) in comparison to the control group. For the within groups comparison, the effect of the intervention is obvious for the salmon group, as expected, and in the control group as well. There is a significant difference within the salmon group for intake of all the seven nutrients from oily fish (for all $p<0.001$), and also within the control group ($p=0.025$).

Table 3.17: Between group comparisons of daily nutrient intakes from oily fish* according to the FFQ

Nutrient	20 weeks gestation			34 weeks gestation		
	Control group	Salmon group	p	Control group	Salmon group	p
Total n-3 PUFAs (mg)	0 (0, 106)	106 (0, 213)	**<0.001**	0 (0, 58)	763 (763, 763)	**<0.001**
Total n-6 PUFAs (mg)	0 (0, 21.3)	21.3 (0, 42.5)		0 (0, 11.7)	495 (495, 495)	
EPA (mg)	0 (0, 26.4)	26.4 (0, 52.9)		0 (0, 14.5)	122 (122, 122)	
DHA (mg)	0 (0, 48.4)	48.4 (0, 96.8)		0 (0, 26.6)	249 (249, 249)	
EPA+DHA from oily fish (mg)	0 (0, 74.8)	74.8 (0, 149.6)		0 (0, 41.2)	371 (371, 371)	
Selenium (µg)	0 (0, 1.36)	1.36 (0, 2.71)		0 (0, 0.75)	9.21 (9.21, 9.21)	
Vitamin D (µg)	0 (0, 0.482)	0.482 (0, 0.964)		0 (0, 0.265)	3 (3, 3)	
Vitamin E (mg)	0 (0, 0.053)	0.053 (0, 0.106)		0 (0, 0.029)	0.879 (0.879, 0.879)	

*Including study salmon. When consumption was 2 times/week, it was recorded in the FFQ category '1-2 times/week' which was interpreted as '1.5 times/week' for nutrient calculations.
Values are medians (interquartile range), Mann Witney test p-values

Table 3.18: Within group comparisons of daily nutrient intakes from oily fish* according to the FFQ

Nutrient	Control group (n=55)			Salmon group (n=54)		
	20 weeks	34 weeks	p	20 weeks	34 weeks	p
Total n-3 PUFAs (mg)	0 (0, 106.3)	0 (0, 58.4)	**0.025**	106 (0, 213)	763 (763, 763)	**<0.001**
Total n-6 PUFAs (mg)	0 (0, 21.3)	0 (0, 11.7)		21.3 (0, 42.5)	495 (495, 495)	
EPA (mg)	0 (0, 26.4)	0 (0, 14.5)		26.4 (0, 52.9)	122 (122, 122)	
DHA (mg)	0 (0, 48.4)	0 (0, 26.6)		48.4 (0, 96.8)	249 (249, 249)	
EPA+DHA from oily fish (mg)	0 (0, 74.8)	0 (0, 41.2)		74.8 (0, 149.6)	371 (371, 371)	
Selenium (µg)	0 (0, 1.36)	0 (0, 0.75)		1.36 (0, 2.71)	9.21 (9.21, 9.21)	
Vitamin D (µg)	0 (0, 0.482)	0 (0, 0.265)		0.482 (0, 0.964)	3 (3, 3)	
Vitamin E (mg)	0 (0, 0.053)	0 (0, 0.029)		0.053 (0, 0.106)	0.879 (0.879, 0.879)	

*Including study salmon. When consumption was 2 times/week, it was recorded in the FFQ category '1-2 times/week' which was interpreted as '1.5 times/week' for nutrient calculations.
Values are medians (interquartile range), Wilcoxon test p-values

The same pattern occurred for nutrient intake from all (i.e. total) fish (Table 3.19, Table 3.20). At 20 weeks gestation, pregnant women in the salmon group had a higher intake of all these nutrients from total fish, compared to the control ($p \leq 0.001$). This result reveals a baseline difference between the 2 groups. The intakes of all seven nutrients of interest from total fish, were higher in the salmon group compared with the control at 34 weeks ($p<0.001$). Within each group (Table 3.20), the salmon group had a higher intake of all seven nutrients during intervention (all $p<0.001$), whereas the control group's daily intake of those nutrients did not change significantly ($p>0.2$). As it shown in Figure 3.5, the

median daily intake of LC n-3 PUFAs for the salmon group from total fish during intervention at 34 weeks, was 133 mg EPA, 267 mg DHA, and 402 mg EPA plus DHA, according to FFQ fish consumption frequencies.

Table 3.19: Between group comparisons of daily nutrient intakes from total fish* according to the FFQ

Nutrient	20 weeks gestation			34 weeks gestation		
	Control group	Salmon group	p	Control group	Salmon group	p
Total n-3 PUFAs (mg)	70.7 (26.3, 165.3)	199.9 (131.3, 285.6)	**<0.001**	59.3 (16.8, 160.8)	817.6 (794.7, 900.4)	**<0.001**
Total n-6 PUFAs (mg)	90.8 (47.4, 252.9)	219.3 (105, 271.6)	**0.001**	91.5 (18.6, 237.7)	600.6 (551, 710)	
EPA (mg)	17.6 (4.6, 36.3)	42.6 (23.8, 66.2)	**<0.001**	11.9 (3, 34.4)	133.3 (127.7, 145.9)	
DHA (mg)	29.4 (8.9, 65.3)	74.5 (45.6, 117.7)	**<0.001**	19.7 (5.1, 63)	267.2 (259, 298.1)	
EPA+DHA from total fish (mg)	46.4 (13.7, 100)	118 (69.4, 182.7)	**<0.001**	30.1 (8.1, 96.7)	402.6 (386.7, 444)	
Selenium (µg)	5.87 (2.79, 12.83)	10.71 (6.3, 19.43)	**0.001**	5.46 (1.75, 17.42)	15.54 (12.75, 25.93)	
Vitamin D (µg)	0.141 (0.071, 0.617)	0.624 (0.374, 1.106)	**<0.001**	0.141 (0.021, 0.569)	3.141 (3.071, 3.424)	
Vitamin E (mg)	0.089 (0.044, 0.259)	0.214 (0.095, 0.326)	**<0.001**	0.089 (0.018, 0.267)	0.968 (0.923, 1.145)	

*Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.
Values are medians (interquartile range), Mann Witney test p-values

Table 3.20: Within group comparisons of daily nutrient intake from total fish* according to the FFQ

Nutrient	Control group (n=55)			Salmon group (n=54)		
	20 weeks	34 weeks	p *	20 weeks	34 weeks	p *
Total n-3 PUFAs (mg)	70.7 (26.3, 165.3)	59.3 (16.8, 160.8)	0.456	199.9 (131.3, 285.6)	817.6 (794.7, 900.4)	**<0.001**
Total n-6 PUFAs (mg)	90.8 (47.4, 252.9)	91.5 (18.6, 237.7)	0.582	219.3 (105, 271.6)	600.6 (551, 710)	
EPA (mg)	17.6 (4.6, 36.3)	11.9 (3, 34.4)	0.262	42.6 (23.8, 66.2)	133.3 (127.7, 145.9)	
DHA (mg)	29.4 (8.9, 65.3)	19.7 (5.1, 63)	0.334	74.5 (45.6, 117.7)	267.2 (259, 298.1)	
EPA+DHA from total fish (mg)	46.4 (13.7, 100)	30.1 (8.1, 96.7)	0.322	118 (69.4, 182.7)	402.6 (386.7, 444)	
Selenium (µg)	5.87 (2.79, 12.83)	5.46 (1.75, 17.42)	0.761	10.71 (6.3, 19.43)	15.54 (12.75, 25.93)	
Vitamin D (µg)	0.141 (0.071, 0.617)	0.141 (0.021, 0.569)	0.326	0.624 (0.374, 1.106)	3.141 (3.071, 3.424)	
Vitamin E (mg)	0.089 (0.044, 0.259)	0.089 (0.018, 0.267)	0.694	0.214 (0.095, 0.326)	0.968 (0.923, 1.145)	

*Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.
Values are medians (interquartile range), Wilcoxon test p-values

Because the two groups had a significantly different baseline intake of the seven nutrients of interest from fish, the change in daily intake of these nutrients from total fish was calculated (Table 3.21). The mean daily intake of EPA plus DHA from total fish increased by 269 mg in the salmon group and decreased by 12 mg in the control group ($p<0.001$). Also the change of intakes of EPA, DHA, total n-3 PUFAs, total n-6 PUFAs, selenium, vitamin D and vitamin E from total fish were different between groups (all $p<0.001$ apart from selenium $p=0.011$).

Table 3.21: Changes in daily nutrient intakes from total fish* according to the FFQ

Nutrient	Change in intake: 34-20 weeks gestation		
	Control group	Salmon group	**p**
Total n-3 PUFAs (mg)	-16.5 (120.6)	611.8 (110.6)	**<0.001**
Total n-6 PUFAs (mg)	0.2 (145.0)	420.0 (119.8)	**<0.001**
EPA (mg)	-4.8 (25.4)	86.5 (26.7)	**<0.001**
DHA (mg)	-7.5 (47.7)	182.8 (49.0)	**<0.001**
EPA+DHA(mg)	-12.2 (72.8)	269.3 (75.6)	**<0.001**
Selenium (µg)	-0.18 (10.92)	4.53 (7.86)	**0.011**
Vitamin D (µg)	-0.07 (0.44)	2.37 (0.45)	**<0.001**
Vitamin E (mg)	-0.01 (0.19)	0.77 (0.12)	**<0.001**

*Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.
Values are mean (± standard deviation) nutrient units/day; independent samples t-test p-values

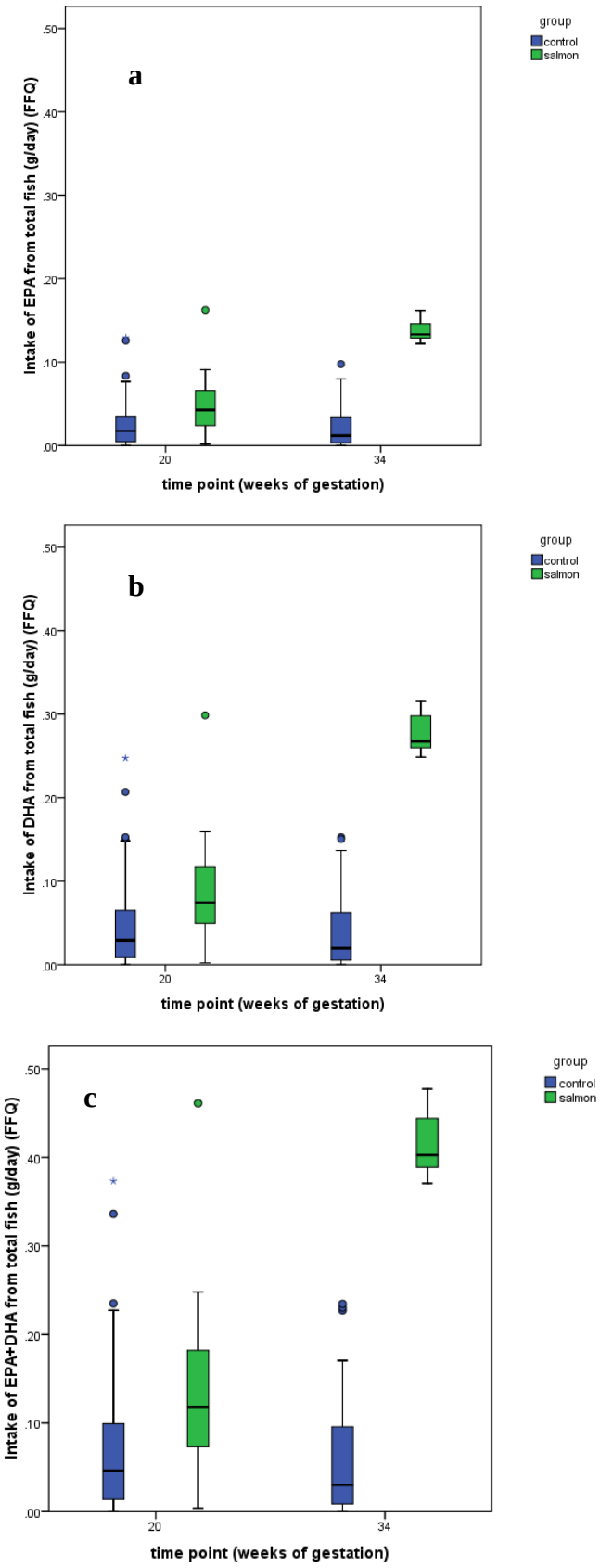


Figure 3.5: Daily intake of a) EPA, b) DHA and c) EPA plus DHA from total fish at 20 and 34 weeks gestation according to the FFQ
p≤0.002 between groups (Mann-Whitney test)

3.3.3 Consumption frequencies and nutrient intakes based on fish diaries

The results presented represent a subset of the data, as not all of the subjects returned completed fish diaries, although the majority did (salmon group n=49; control group n=47). The weekly consumption of study salmon by the salmon group during weeks 21 to 38 of pregnancy is shown in Figure 3.6.

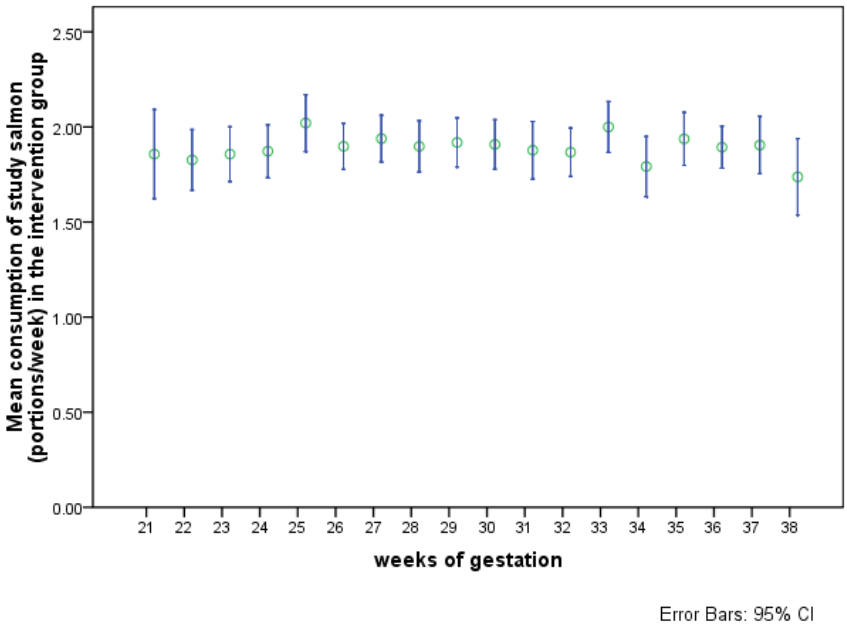
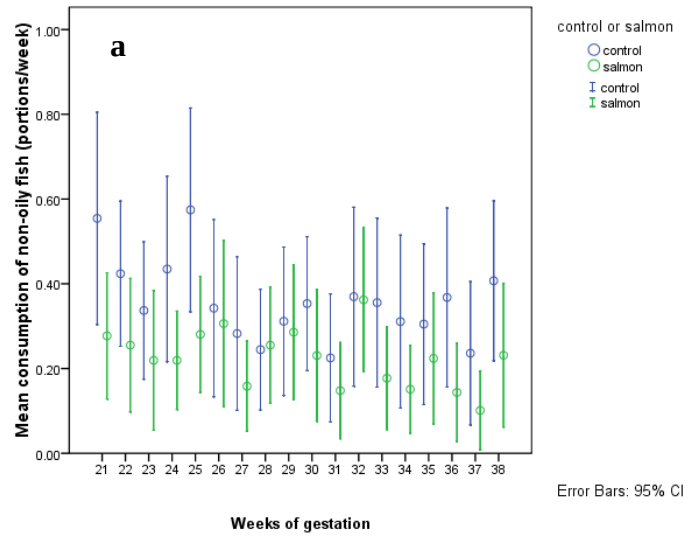
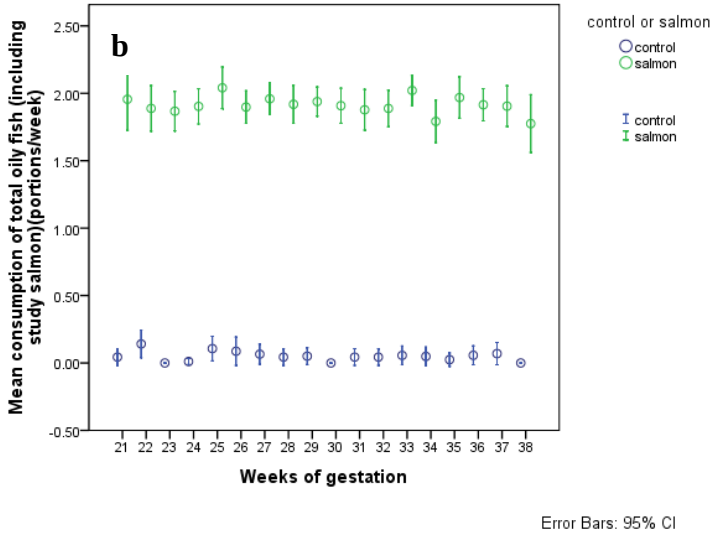


Figure 3.6: Weekly consumption of study salmon by the salmon group throughout pregnancy

Figure 3.7 describes the weekly consumption of non oily, oily and total fish in both groups during the intervention period.





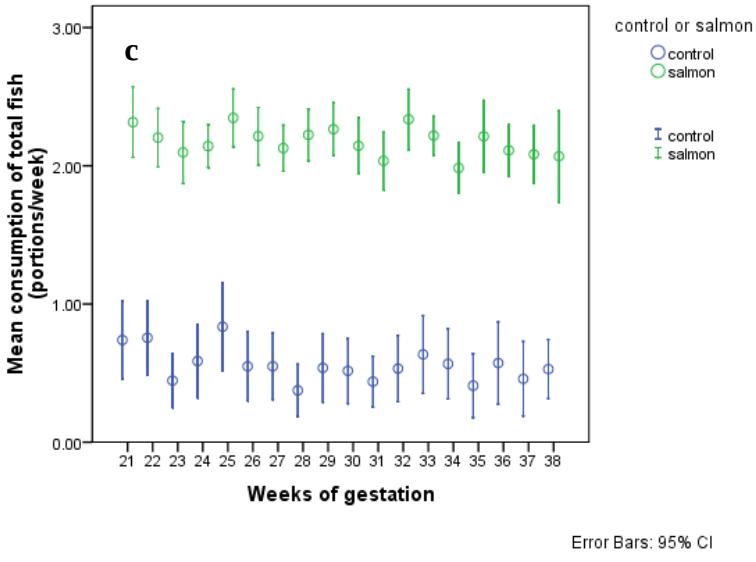


Figure 3.7: Weekly consumption of a) non-oily, b) oily, c) total fish according to study group

Table 3.22 summarises the mean weekly consumption frequency of study salmon and other fish categories (similar to the FFQ categorisation) consumed by the two groups during intervention (21 to 38 weeks of gestation). According to the fish diaries, the salmon group was consuming 1.94 portions of study salmon per week whereas the control group was consuming 0 portions of oily fish per week (p<0.001). The salmon group was consuming fewer portions of white fish than the control group (0.17 and 0.33 portions per week respectively, p=0.036). Therefore, total fish consumption was higher in the salmon group (2.11 portions per week) in comparison to the control group (0.47 portions per week) (p<0.001).

Figure 3.8 shows the mean weekly consumption of study salmon in the salmon group during intervention, which was 1.94 portions per week.

Table 3.22: Between group comparison of mean weekly consumption frequencies of fish categories as recorded in the fish diaries (data on 21-38 weeks gestation)

Fish category	21 to 38 weeks gestation		
	Control group (n=47)	Salmon group (n=49)	p
Study salmon	0 (0, 0)	1.94 (1.83, 2.00)	**<0.001**
White fish	0.33 (0.06, 0.56)	0.17 (0.06, 0.33)	**0.036**
Oily fish*	0 (0, 0.07)	1.94 (1.87, 2.00)	**<0.001**
Fish fingers and fish dishes	0 (0, 0.11)	0 (0, 0)	**0.002**
Shellfish	0 (0, 0)	0 (0, 0)	0.507
Total fish**	0.47 (0.17, 0.8)	2.11 (2, 2.35)	**<0.001**

*Including study salmon

**Total fish including non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.

Values are median (interquartile range) number of portions/week. Mann Whitney test p-values

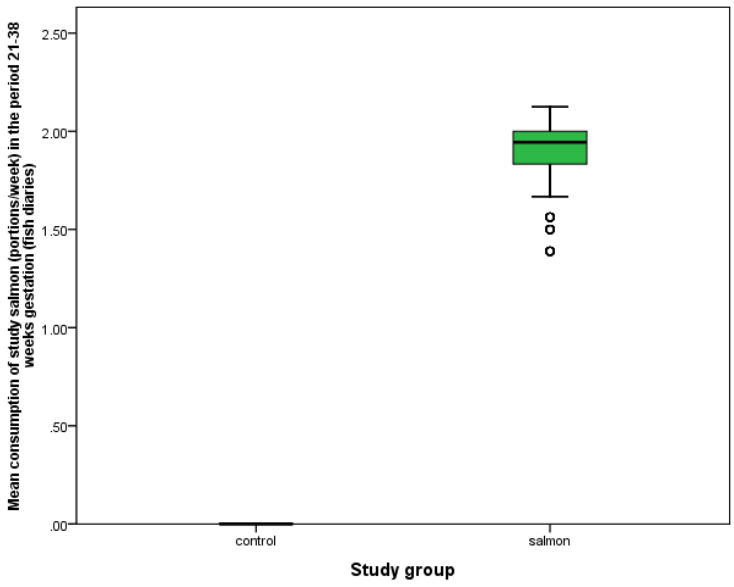


Figure 3.8: Mean weekly consumption of study salmon by the salmon group during the 21st-38th week of pregnancy (p<0.001 between groups (Mann Whitney test))

Table 3.23, Table 3.24 and Table 3.25 show the daily nutrient intakes of the seven nutrients of interest from non-oily, oily and total fish for both groups during intervention, based upon the information recorded in the fish diaries. The salmon group had a lower intake of those nutrients from white fish in comparison to the control group (p=0.036) (Table 3.23). In contrast, the intake of the seven nutrients from oily fish (including study salmon) and total fish was higher in the salmon group compared to the control (for both oily and total fish p<0.001) (Table 3.24, Table 3.25). From total fish, the daily intake of EPA, DHA, and EPA plus DHA was 162 mg, 326 mg, and 490 mg for the salmon group and 10 mg, 16 mg, 24 mg for the control group, respectively.

Table 3.23: Between group comparisons of mean daily nutrient intake from non-oily (white) fish as recorded in the fish diaries

Nutrient	21 to 38 weeks gestation		
	Control group (n=47)	Salmon group (n=49)	p
EPA (mg)	5.2 (0.9, 8.7)	2.6 (0.9, 5.2)	**0.036**
DHA (mg)	11.0 (1.8, 18.3)	5.5 (1.8, 11)	
Total n-3 PUFAs (mg)	30.6 (5.1, 51)	15.3 (5.1, 30.6)	
Total n-6 PUFAs (mg)	48 (8, 80)	24 (8, 48)	
Selenium (µg)	3.71 (0.62, 6.19)	1.86 (0.62, 3.7)	
Vitamin D (µg)	0.094 (0.016, 0.157)	0.047 (0.016, 0.094)	
Vitamin E (mg)	0.056 (0, 0.01)	0.03 (0.01, 0.059)	

Values are median (interquartile range) nutrient unit/day. Mann Whitney test p-values

Table 3.24: Between group comparisons of mean daily nutrient intake from oily fish* as recorded in the fish diaries

Nutrient	21 to 38 weeks gestation		
	Control group (n=47)	Salmon group (n=49)	p
EPA (mg)	0 (0, 7.6)	158 (153, 164)	**<0.001**
DHA (mg)	0 (0, 14)	322 (311, 333)	
Total n-3 PUFAs (mg)	0 (0, 30.7)	989 (950, 1017)	
Total n-6 PUFAs (mg)	0 (0, 6.1)	642 (609, 660)	
Selenium (µg)	0 (0, 0.392)	11.9 (11.5, 12.3)	
Vitamin D (µg)	0 (0, 0.14)	3.89 (3.74, 4)	
Vitamin E (mg)	0 (0, 0.015)	1.14 (1.08, 1.17)	

*Oily fish includes study salmon

Values are median (interquartile range) nutrient unit/day. Mann Whitney test p-values

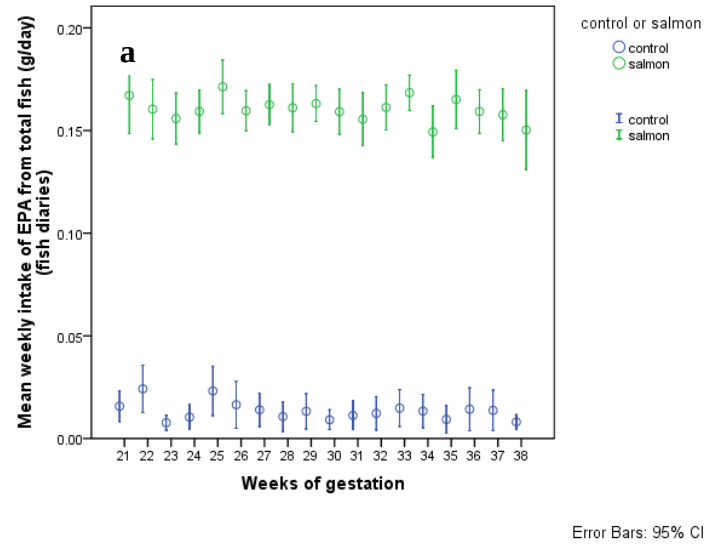
Table 3.25: Between group comparisons of mean daily nutrient intake from total fish* as recorded in the fish diaries

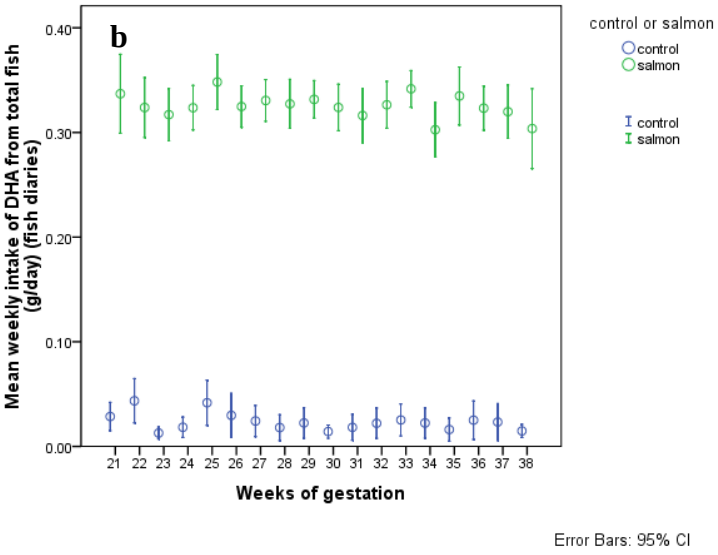
Nutrient	21 to 38 weeks gestation		
	Control group (n=47)	Salmon group (n=49)	p
EPA (mg)	**10 (4.5, 18.3)**	**162 (156, 167)**	**<0.001**
DHA (mg)	**16.2 (7.6, 37.2)**	**326 (318, 339)**	**<0.001**
Total n-3 PUFAs (mg)	43.4 (23.2, 98.8)	996 (974, 1035)	**<0.001**
Total n-6 PUFAs (mg)	60.3 (16.4, 106.9)	660 (642, 691)	**<0.001**
Total fish EPA+DHA (mg)	**24.4 (12.7, 56.7)**	**491 (475, 506)**	**<0.001**
Selenium (µg)	4.33 (1.51, 6.96)	13.49 (12.27, 15.73)	**<0.001**
Vitamin D (µg)	0.123 (0.047, 0.311)	3.9 (3.8, 4.1)	**<0.001**
Vitamin E (mg)	0.069 (0.023, 0.130)	1.16 (1.13, 1.19)	**<0.001**

*Total fish includes non-oily fish, oily fish (and study salmon), fish fingers and fish dishes, shellfish.
Values are median (interquartile range) nutrient unit/day. Mann Whitney test p-values

Figure 3.9 and Figure 3.10 describe the daily intake (for each week of pregnancy) and mean daily intake (for all pregnancy weeks) respectively of EPA, DHA and EPA plus DHA, from non-oily, oily and total fish, in both study groups.

Finally Figure 3.11 represents the compliance of the salmon group to consuming two portions of study salmon weekly. The fish diaries data subset showed that 79.6% of the volunteers in the salmon group were having two or more portions of study salmon per week for 81.25% of the weeks during intervention. The data used covers the period between 21 to 38 weeks of pregnancy. Also 24.5% of the volunteers in the salmon group had two or more portions per week of study salmon every week from gestation week 21 to 38. So, the salmon group complied well with consuming the salmon as requested for the intervention (2 portions per week).





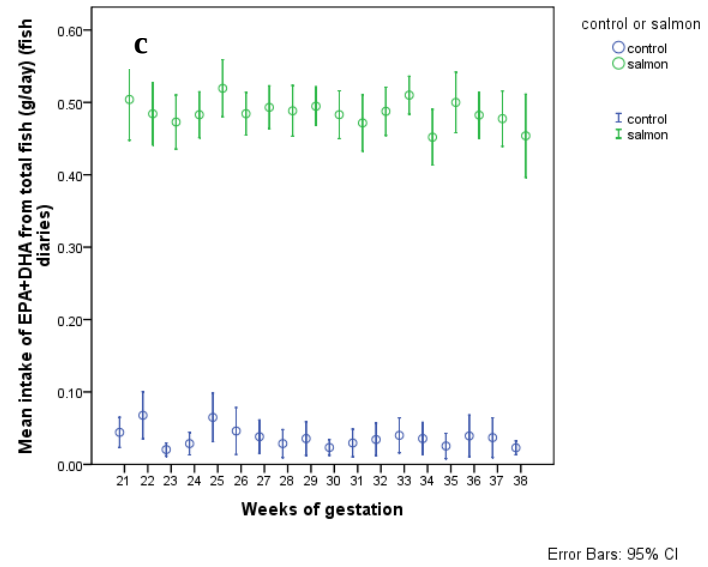


Figure 3.9: Daily intake of a) EPA, b) DHA, c) EPA plus DHA, from total fish during the 21st-38th week of pregnancy according to study group

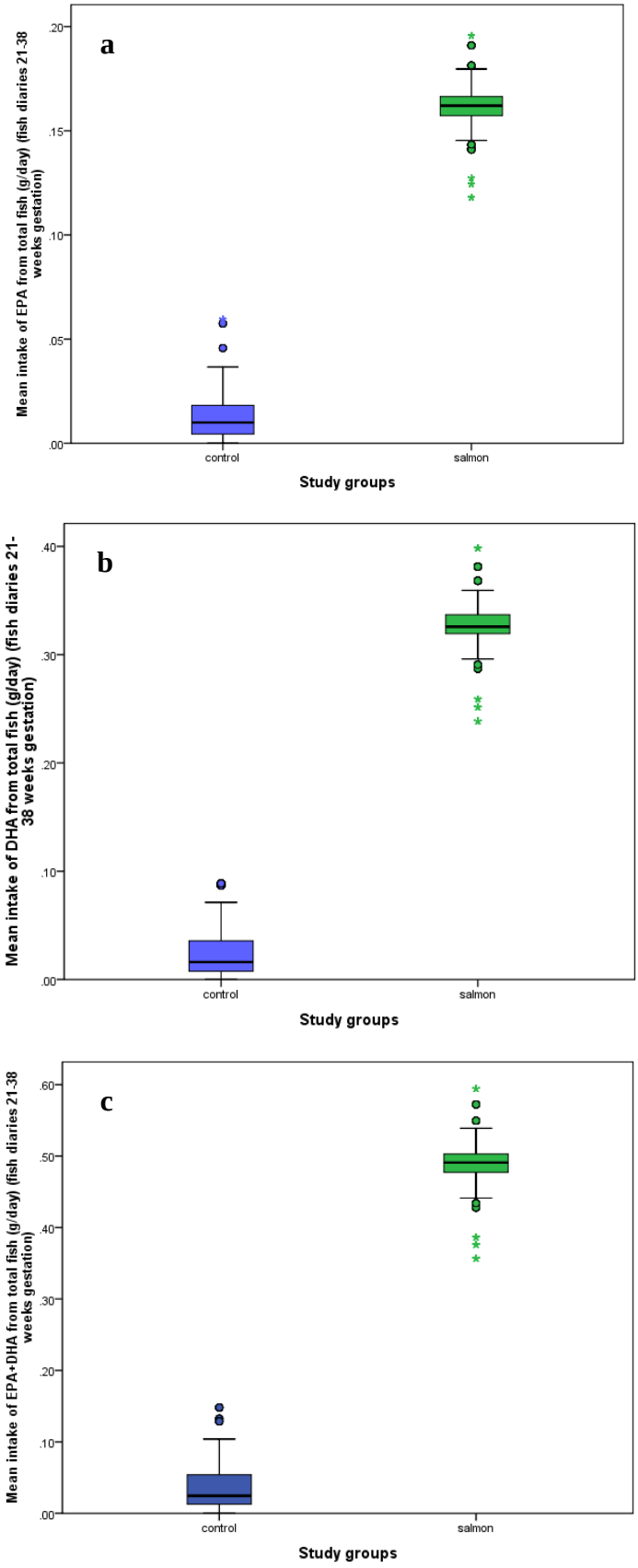


Figure 3.10: Daily intake of a) EPA, b) DHA, c) EPA plus DHA, from total fish during the 21st-38th week of pregnancy (all p<0.001 between groups (Mann Whitney))

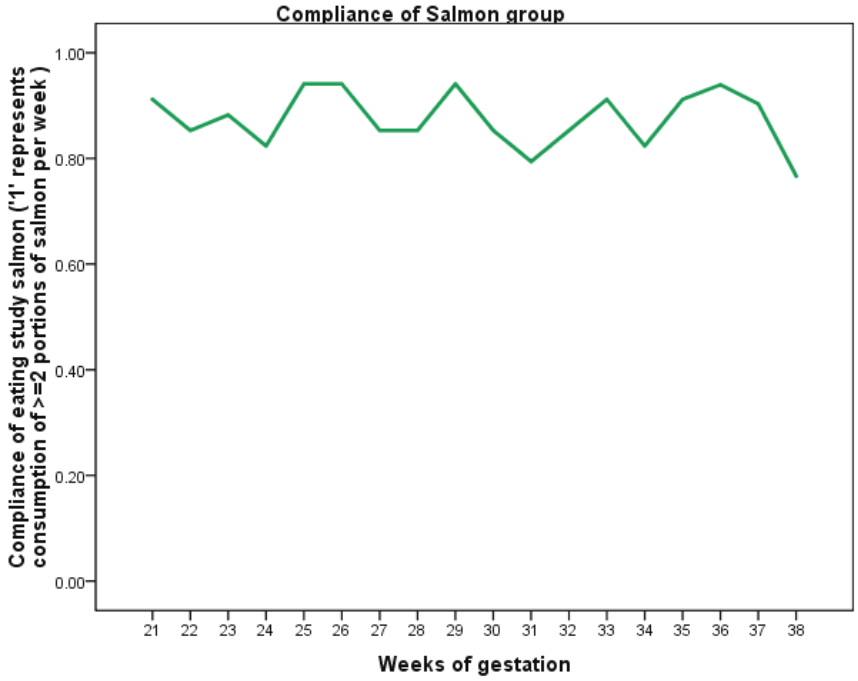


Figure 3.11: Compliance of the salmon group to consume ≥ 2 portions of study salmon from gestation week 21 to 38

3.4 Discussion

SIPS is an intervention study with oily fish in pregnant women. It is the first such study to be conducted. The intervention took women who did not normally eat oily fish up to the maximum level of consumption recommended by the UK government (two portions per week). According to information available from self-reporting fish diaries, the oily fish intervention was successful and compliance was high in both control and salmon groups.

The primary hypotheses of the work presented in this chapter were that the oily fish intervention will: a) increase oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/week) during pregnancy, in women whose offspring is at a high risk of developing atopic disease; and b) increase maternal LC n-3 PUFA intake during pregnancy, and that this intake will be higher in the salmon than in the control group. According to the results presented these hypotheses can be accepted.

The secondary hypotheses of the study were that the oily fish intervention will: a) change the dietary patterns for the salmon group; and b) increase the maternal intake of vitamin D and selenium, and that their intakes will be higher in the salmon than in the control group. According to the results presented these hypotheses can be accepted.

Thus, if women who do not normally consume much oily fish, increase their consumption to twice per week, in accordance with current UK recommendations, they will increase their intake of LC n-3 PUFAs, selenium and vitamin D.

Information gathered from the FFQs indicates that subjects in the salmon group complied to the intervention and at 34 weeks women in that group were all eating salmon corresponding to the category 1-2 times per week; this is consistent with information from the fish diaries that show that the women were eating 1.94 portions of study salmon per week during pregnancy (weeks 21 to 38 of gestation) indicating that women in this group were eating about 2 portions of salmon per week.

It is of importance to note that the FFQ most likely underestimates the intake of salmon, since an intake of 2 portions per week falls into the FFQ category 1-2 times per week, for which the multiplication factor for all calculations was 1.5. This is a limitation of the FFQ as a dietary assessment method, because it has an ordinal scale of consumption frequency

categories, rather than a scale providing a true measure. So, according to the FFQs at 34 weeks the salmon group consumed oily fish 1.5 times per week during the 22nd to 34th week of gestation, whereas according to fish diaries the salmon group consumed 1.94 portions per week from week 21 to 38 of gestation. This shows that the fish diaries were important not only to assess and demonstrate compliance to the intervention but also as a precise measure of consumption frequency and subsequently nutrient intake as will be discussed further later on. The use of the diaries can be considered as a strength of SIPS.

Moreover, the FFQs suggested that the control group was keeping its consumption of oily fish at low levels, with the majority eating oily fish 'never' or 'once per month'. Again this is consistent with information from the fish diaries, which show that the control group had 0 (median) weekly consumption frequency.

From between group comparisons of consumption frequencies of FFQ fish groups, at baseline (20 weeks gestation), there was a higher consumption of total fish in the salmon group (1.75 times/week) in comparison to the control group (0.75 times/week). This is an existing baseline difference between the two groups, which cannot be explained. When looking at the weekly frequencies of fish consumption according to the four fish categories of the FFQ, at study entry the salmon group had higher oily fish consumption (0.25 times/week equal to twice per month vs. 0 times/week) as well as white fish consumption (0.5 times/week vs. 0.25 times/week) than the control group. There is no baseline data from the fish diaries, as the subjects started completing them at the beginning of the intervention. In this way, the fish diaries can only offer dietary information during the study period, and not prior to it, whereas the FFQs provide dietary data for the 3 months prior to the FFQ administration. This is considered as an advantage of the FFQ, reflecting the consumption frequency over a long period of time in the past.

Another advantage of the FFQ is that it is validated on the Southampton population for use in pregnant women (228) and it includes distinct fish categories. The FFQ method has also been used in large epidemiological studies testing similar hypotheses to SIPS i.e. the maternal fish intake during pregnancy in relation to infant atopic outcomes (203), a fact enhancing the choice of FFQ as the main dietary assessment method in SIPS.

Between groups, during intervention (i.e. at 34 weeks) there was a higher consumption frequency of total fish in the salmon group compared to the control (2.35 times/week vs. 0.75 times/week), as expected, since this period is reflecting the intervention period (22

until 34 weeks of gestation). Breaking down the total fish frequency of consumption into the four fish FFQ categories, only oily fish consumption (mainly study salmon in the salmon group) was significantly increased in the salmon group to 1.5 times/week compared to the control group of which the weekly consumption frequency remained 0. However, the fish diaries showed that the salmon group increased total and oily fish consumption (agreement with FFQ data) but also the salmon group had a lower consumption frequency of white fish and fish fingers/fish dishes (FFFD), compared to the control group. These latter observations were not revealed from the FFQ data. This could mean that because of the categorisation of the consumption frequencies the FFQ is not very sensitive in picking up such differences. Overall, from the fish diaries, the salmon group was consuming more than 2 portions of oily fish per week and the control group was eating less than 1 portion of total fish per week during pregnancy.

The FFQ identified that white fish consumption decreased in the salmon group, most probably because the salmon replaced some white fish in the diet. Furthermore, red meat consumption decreased in the salmon group, probably identifying its replacement by study salmon. The within group comparisons also revealed that in the salmon group, chicken and turkey consumption decreased. Thus, salmon appears to be incorporated into the diet by partly replacing white fish, red meat, chicken and turkey.

The average total fish consumption frequency and portion of total fish consumed at baseline was for the control group 0.75 times/week x 140 g/portion = 105 g/week and per day the total fish consumed was 105/7 = 15 g/day. For the salmon group the quantity of total fish consumed at baseline was 1.75 times/week x 140 g/portion = 245 g/week and per day the quantity of total fish consumed was 245/7 = 35 g/d. The SIPS salmon group total fish intake (35 g/day) is very close to the intake reported at the latest NDNS (200) intake of total fish (33 g/day), but the control group has a much lower intake (15 g/day), being less than half of the average reported in the NDNS (200). This reveals that both groups are habitually low consumers of seafood.

Overall, from the changes calculated in fish and meat weekly consumption frequencies, it is obvious that the oily fish and total fish consumption frequencies were increased in the salmon group (1.21 times/week and 0.85 times/week, respectively), whereas they dropped slightly in the control group (0.04 times/week and 0.05 times/week, respectively).

Regarding nutrient intakes, FFQs might not be the ideal tool to investigate nutrient-disease associations (222). However, the SWS FFQ used by SIPS has been validated for calculating nutrients compared to a 4 day prospective food diary (222). In accordance with the decrease in oily fish consumption frequency in the control group, the intake of the seven nutrients of interest obtained from oily fish (EPA, DHA, n-3 PUFAs, n-6 PUFAs, Se, vitamin D and vitamin E) decreased in that group at 34 weeks. However, within the salmon group, intake of each of these nutrients, increased significantly (within group comparisons) not only from oily fish but also from non-oily and total fish in comparison to the control. Nevertheless, in the same way that there was a baseline (i.e. 20 weeks of gestation) difference of consumption frequency in non-oily, oily fish and total fish between the two study groups, there was a difference in intake of all seven nutrients from non-oily, oily and total fish between groups (intakes were higher in the salmon group). However, importantly, the intake of all seven nutrients of interest was increased in the salmon group during the intervention, although the consumption of salmon twice per week was recorded in the FFQ as consumption 1-2 times per week and was interpreted as 1.5 times per week for calculations.

Also, a limitation of this FFQ is that it has not been validated to calculate total diet EPA and DHA intake. However the main sources of EPA and DHA are fish and fish oil supplements or functional foods. The FFQ indicated that no subject was consuming fish oil supplements during pregnancy (this was an exlusion criterion) and only a minority of subjects was consuming functional foods that contained fish oils. This consumption did not differ significantly between the two groups. The calculations of 'total diet' (Table 3.14) EPA and DHA intake were including the functional foods and between the two groups no substantial difference was observed in EPA and DHA intake from 'total fish' (Table 3.19). So, it seems that the intake of EPA and DHA from total fish is a good estimation of EPA and DHA from total diet. There are recent reviews of the use of FFQs as a validated tool to estimate LC n-3 PUFA and micronutrient intake during pregnancy (229;230).

The increase in intake of key nutrients other than LC n-3 PUFAs in the salmon group clearly indicates that salmon delivers more than just EPA and DHA and in this respect salmon may be superior to supplementation with fish oil capsules. From the calculation offered in Table 3.26, it is clear that the minimum UK recommendation for intake of EPA and DHA (450 mg/day) can be met, from two portions of study salmon and that the salmon can make a major contribution in the intake of vitamin D (40 % of the recommended intake) and Se (20 % of the recommended intake).

Table 3.26: Calculated contribution of two portions of study salmon to EPA+DHA, vitamin D and Se intakes in the salmon group

	UK recommendation per day	UK recommendation per week	Amount per portion of study salmon	Amount provided from two portions per week	Contribution of study salmon to recommended intake	Average UK intake per day
EPA+DHA (23)	> 450 mg	> 3150 mg	1730 mg	3460 mg	109%	< 200mg
Vitamin D [1]	10 µg	70 µg	14 µg	28 µg	40%	2.7 µg [3]
Selenium [2]	60 µg	420 µg	43 µg	86 µg	20%	39 µg [3]

[1] Vitamin D Reference Nutrient Intake (RNI) for pregnant and lactating women (231)
[2] Selenium Reference Nutrient Intake (RNI) for 19-50 year old women (231)
[3] According to NDNS 2008/2009 (200)

The fish diaries cover most of the intervention period (21-38 weeks of gestation) and the nutrient intake results obtained from these can be compared to the results of the FFQ analysis at 34 weeks (during intervention). The intake of all the 7 nutrients from oily and total fish was higher in the salmon group, in agreement with the FFQ data. However, the fish diaries show a difference for the intake of the seven nutrients from non-oily fish which was not seen using the FFQ. This could be another disadvantage of FFQs; they may not be able to detect small differences in nutrient intake. In contrast, the fish diaries provided detailed information on the quantities and types of fish consumed. Also, as said before, the consumption frequencies were not categorised, but measured in an exact and precise way. The volunteers had to complete the diaries on a daily basis, so that the recall bias would be much less than for the FFQ. However, the fish diaries are not validated, as the FFQs are, and also they are influenced by individual variability in the way in which they are completed (enthusiasm in filling the diaries, psychological/mood fluctuation because of pregnancy, over/under estimation of portion sizes). Furthermore, the fish diaries were returned by a subset of subjects while the FFQs were completed by all subjects.

Dietary patterns can be explored using the FFQ data, but the fish diaries are not useful for this because they focus only on seafood. However, the feedback from SIPS was that the length and time consuming administration of the FFQ was a burden for the subjects, especially at 34 weeks (the second time), since they had already done it at baseline. An alternative option, would be the use of a shorter FFQ, more focused on fish consumption and LC n-3 PUFA intake, as Crozier *et al*. have done to summarise the diets of young women in Southampton (232).

The increased intake of EPA, DHA, and LC n-3 PUFAs in the salmon group at 34 weeks is a very important demonstration of SIPS. According to the FFQ, EPA plus DHA intake together reached a median of 402 mg/day from total fish in the salmon group and 30 mg/day in the control group, whereas the recommendation for adults is to consume a minimum of 450 mg /day from the diet (23). According to the fish diaries, which are more accurate in this regard, EPA plus DHA intake reached a median of 490 mg/day from total fish in the salmon group and 24 mg/day in the control group. The data from the fish diaries indicate that almost all of the women in the salmon group were consuming more than the minimum recommended amount of EPA plus DHA (450 mg/day) during the intervention. Thus SIPS demonstrates for the first time that consuming two portions of oily fish per week enables pregnant women to reach the recommended UK minimum intake of EPA plus DHA.

There are also specific recommendations for DHA intake by pregnant women to be 200 mg/day as suggested by Koletzko *et al.* in 2007 (233). Data from the fish diaries indicates median DHA consumption of about 360 mg/day in the salmon group, and further that every woman in that group easily met this recommendation.

The finding that eating two portions of farmed salmon per week allows pregnant women to achieve recommended intakes for DHA and for LC n-3 PUFAs and to increase their intake of vitamin D and selenium may have health implications and might contribute to the reduction of the atopic risk. Since the constraint of eating 2 portions of oily fish per week exists because of the contaminants risk, the solution of eating farmed oily fish twice a week, might be ideal both for safe consumption of oily fish and for optimal nutrient intake.

There are no studies in the literature of fish intervention during pregnancy. Therefore the findings from SIPS cannot be directly compared with similar existing studies. However, it is worth noting that there has been considerable recent interest in assessing the effect of increased fish intake on various health outcomes in adults including blood lipid concentrations (234), platelet-monocyte aggregation (235), oxidative stress, glucose metabolism and insulin sensitivity (236), blood pressure (48), markers of colorectal cancer risk (237;238) and markers of inflammation (47;48).

3.5 Conclusion

SIPS successfully increased oily fish intake in the salmon group to the target of 2 portions of study salmon per week. This was reported both in the FFQs and in the fish diaries although there were differences in the reporting of the intakes of the seven nutrients of interest (EPA, DHA, total n-3 PUFAs, total n-6 PUFAs, vitamin D, vitamin E and selenium) between the two methods. According to the FFQ (at 34 weeks gestation) all women in the salmon group ate salmon 1-2 times per week while according to the fish diaries (between 21-38 weeks gestation) the median intake was 1.94 portions (of study salmon) per week. According to the FFQ, median daily intake of EPA plus DHA was 403 mg for the salmon group and 30 mg for the control group. The fish diary results showed higher levels of intake for the salmon group (490 mg/day EPA plus DHA) and similar for the control group (24 mg/day EPA plus DHA) compared to the FFQ. In addition, increased salmon intake increased total diet intake of selenium, vitamin D and total n-3 PUFAs. Increased salmon consumption was compensated by decreased consumption of other types of seafood (non-oily fish and fish fingers/fish dishes) and of red meat, chichen, and total meat. SIPS demonstrated that eating two portions of salmon per week enables pregnant women to achieve existing recommendations for intake of DHA and of LC n-3 PUFAs and to increase their intake of other key nutrients like vitamin D and selenium. Thus, if women who do not normally consume much oily fish, increase their consumption to twice per week, in accordance with current UK recommendations, they will increase their intake of LC n-3 PUFAs (meeting current recommendations), selenium and vitamin D.

4 Salmon in pregnancy study (SIPS): the effects of pregnancy and salmon intervention on maternal and infant fatty acid status

4.1 Introduction

A detailed description of fatty acids (terminology, structure, essential fatty acids, dietary sources, fatty acid metabolism, and recommendations on intake of long-chain (LC) n-3 polyunsaturated fatty acids (PUFAs)) was provided in Section 1.1. This chapter describes the effects of pregnancy and of the salmon intervention described in Chapter 2 on maternal fatty acid status and the effect of the salmon intervention on neonatal fatty acid status. By way of introducing this work, the essential fatty acids and the major role that they play in fetal development and later infant health status, maternal LC n-3 PUFA provision to the fetus and the factors that determine this transfer, and related aspects are described.

4.1.1 Essential fatty acids

Humans can synthesize saturated and monounsaturated fatty acids, but they cannot synthesize the n-3 and the n-6 families of PUFAs. The parent fatty acids of these families, alpha-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LA) are essential fatty acids and must be present in the diet. The fatty acids of these families cannot be interconverted, so both n-3 and n-6 fatty acids are essential. ALA and LA are converted to longer chain, more highly unsaturated fatty acids through enzymatic chain elongation and desaturation. ALA is converted to eicosapentaenoic acid (20:5n-3, EPA), then on to docosahexaenoic acid (22:6n-3, DHA), whereas LA is converted to arachidonic acid (20:4n-6, ARA). DHA is a critical component of cell membranes, especially in the brain and the retina. ARA is both a membrane component and a precursor to potent signaling molecules, the prostaglandins, leukotrienes, thromboxanes. The chain elongation/desaturation enzymes are shared by both n-3 and n-6 fatty acids with competition between substrates for these enzymes (239). More detailed information is provided in section 1.1.5.

4.1.2 Fetal and infant LC PUFA accumulation and health

The supply of essential fatty acids and LC PUFAs is critical and central to the synthesis of structural lipids and hence, to normal development of the fetus (10;34;240). Brain accumulation of DHA starts *in utero*, with quantitatively marked deposition in the second half of gestation. DHA accumulation in the brain continues after birth, total brain DHA deposition reaching about 4 g between two and four years of age (34). DHA is also an

important structural component of retina lipids, comprising as much as 50% of total fatty acids of rod and cone outer segments (241;242). Unlike DHA, other LC n-3 PUFAs, including EPA, do not accumulate to any appreciable extent in the growing brain and eye. However, brain accumulation of the n-6 PUFA ARA also occurs during pre- and post-natal development. Elongation and desaturation enzymes for PUFA conversion are present in the fetal liver early in gestation, but activity appears to be low before birth (242;243). Therefore, the LC PUFAs that the fetus accumulates *in utero* are derived predominantly from the mother through placental transfer, with the amounts in cord blood influenced by the maternal diet (177). Both preterm and term infants are capable of synthesizing DHA and ARA (244); conversion rates of LA to ARA and of ALA to EPA and on to DHA are influenced by hormones, genetics, gender, and the amount of precursor fatty acids available in the diet (10;245). Human milk always contains both ARA and DHA (246). In the past infant formulae did not contain these fatty acids. Synthesis rates of LC PUFAs from their essential precursors are insufficient to maintain stable plasma and red blood cell LC PUFA levels in infants receiving unsupplemented formula, and LC PUFA levels decline in infants fed unsupplemented formula compared to those fed human milk (242).

Animal experiments indicate that a lack of n-3 PUFAs in the diet results in poor visual development and in learning and behavioural abnormalities (18). There is also evidence that preterm infants, in whom this early supply is interrupted, have poorer visual and cognitive development than term infants, although the provision of breast milk or DHA-supplemented formulas improves these outcomes (10;34;247).

A recent international consensus statement proposed an average DHA intake of at least 200 mg/day during both pregnancy and lactation to support infant growth and developmental outcomes (233;242) Women of child bearing age can meet the recommended intake of DHA by consuming one to two portions of fish per week, including oily fish, which is a good source of LC n-3 PUFAs; this was confirmed by the findings presented in Chapter 3. Consumption of this amount of fish does not generally exceed the tolerable intake levels of environmental contaminants. Other sources of LC n-3 PUFAs include enriched foods and dietary supplements. Dietary ALA, the precursor to DHA, is much less effective in promoting optimal DHA status than consumption of preformed DHA and is insufficient to promote desirable levels of DHA deposition in the fetal brain (233). A number of studies have also demonstrated that intake of fish or fish oils during pregnancy results in slightly longer gestation, marginally higher birth weight, and reduced risk of preterm delivery (38;39;248), creating an even higher demand for maternal LC PUFAs (249).

LC n-3 PUFAs have also been shown to influence immune function and it is now considered that supply of EPA and DHA to the fetus might be important for appropriate immune development (48;131). This has been studied mainly in regard to the potential to reduce the risk of developing atopic disease in infancy and children (35;132;180;181). There is evidence from epidemiological studies that maternal intake of fish or fish oils during pregnancy is associated with reduced risk of allergy, asthma and atopy in the offspring (Section 1.5; (203)).

4.1.2.1 Lipid 'pools'

Fatty acids are insoluble in aqueous solution and thus need to be bound to other molecules for transportation. In plasma, fatty acids are either esterified to form more complex lipids such as triacylglycerols (triglycerides, TAGs), phospholipids and cholesteryl esters (CEs), or bound non-covalently to albumin in the form of non-esterified fatty acids (NEFAs). Fatty acids are bound by an ester bond to either the glycerol backbone of TAGs or the fourth ring of cholesterol in CEs (Figure 4.1). In phospholipids, fatty acids can be attached to the glycerol backbone by either ester, alkyl or alkenyl bonds. Phospholipids containing fatty acids with alkyl or alkenyl bonds are found in only small amounts in plasma, but are important components of cell membranes, in particular in erythrocytes, leukocytes, brain and liver. Phospholipids can be classified according to the head group attached to the third carbon of which most frequent and major in the plasma and cells is phosphatidycholine (PC) (choline as head group), located at the outer leaflet of cell membranes and as a monolayer enclosing plasma lipoproteins (Figure 4.1). TAGs, PC and CEs represent transport pools of fatty acids typically carrying fatty acids from the gut or liver to peripheral tissues and back. PC is major component of cell membranes along with other phospholipids. The fatty acid composition of plasma PC is similar to the PC composition of cell membranes, and in this way plasma PC could crudely reflect the functional fatty acid pool (functionality of cells). The composition of plasma NEFAs in the fasting state reflects the storage fatty acid pool, because adipose tissue fatty acids are released after TAG hydrolysis during fasting.

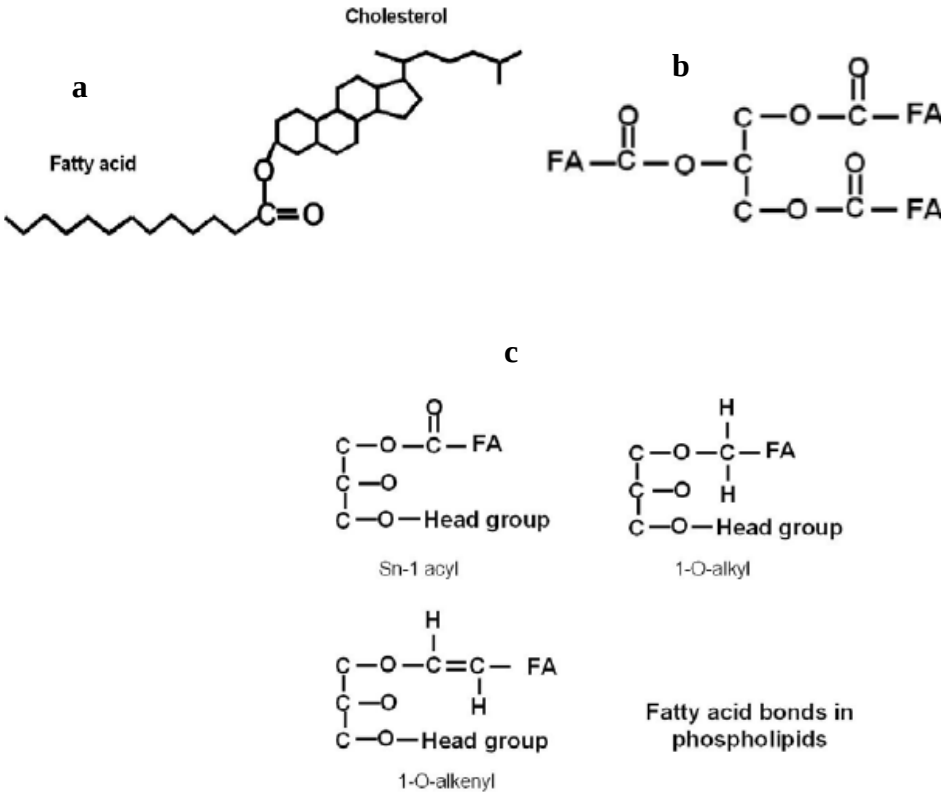


Figure 4.1: Schematic structures of: a) cholesteryl esters (CEs), b) triglycerides (TAGs), and c) phospholipids

4.1.3 Pregnancy and placental transport of LC-PUFA

During early pregnancy, LC PUFAs may accumulate in maternal fat stores and become available for placental transfer during late pregnancy (250), when the fetal growth rate is maximal and fetal requirements for LC PUFAs are greatly enhanced. During this late part of gestation, enhanced lipolytic activity in maternal adipose tissue contributes to the development of maternal hyperlipidaemia (250); there is an increase in plasma TAG concentrations, with smaller rises in phospholipid and cholesterol concentrations (250). Besides the increase in plasma very low density lipoprotein (VLDL), there is a proportional enrichment of TAGs in both low density lipoproteins (LDLs) and high density lipoproteins (HDLs) (250). These lipoproteins transport LC PUFAs in the maternal circulation. The presence of lipoprotein receptors in the placenta allows their placental uptake, where they are hydrolysed by lipoprotein lipase, phospholipase A_2, and intracellular lipase (250). The fatty acids that are released can be metabolized and diffuse into the fetal circulation. Although present in smaller proportions, maternal plasma NEFAs are also a source of LC PUFAs for the fetus, their placental transfer being facilitated by the presence of a membrane fatty acid binding protein (250). The demands for cholesterol in the fetus are high as it is one of the main elements of the cell membrane structures. Although maternal cholesterol substantially contributes to fetal cholesterol during early

pregnancy, during late gestation fetal cholesterol biosynthesis (rather than cholesterol transfer from maternal lipoproteins) seems to be the main mechanism for satisfying fetal requirements (240;250;251).

4.1.4 Fetal selectivity and studies relating maternal and neonatal fatty acid status with or without maternal dietary intake

The presence of membrane fatty acid binding protein in human placenta results in selective transfer of certain fatty acids to the fetus (252). Comparison of the maternal and fetal concentrations of two of the most important fatty acids for fetal development (ARA and DHA) indicates generally higher concentration of these LC PUFAs in all the major lipid classes in fetal blood and tissues (253). Figure 4.2 demonstrates the proportions of ARA and DHA in all lipid fractions in maternal circulation, placenta and fetal circulation as well as in adipose tissue and brain (253). The selective increase in the concentration of LC PUFAs, and of DHA and ARA in particular, in the fetal circulation and tissues has been termed biomagnification (253). The time at which this gradient manifests also appears to be synchronized with the maximum fetal demand (252;253).

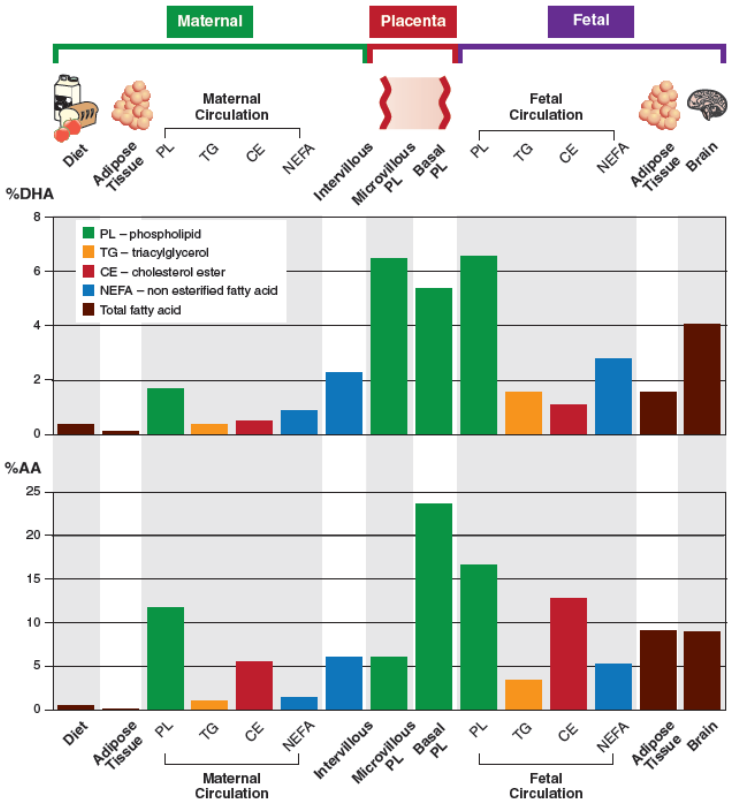


Figure 4.2: ARA and DHA as a percentage (%) of total fatty acids in the diet of pregnant mothers, the adipose tissue, maternal and cord blood plasma phospholipids, triglycerides, cholesteryl esters and non-esterified fatty acids, the placental microvillous and basal membranes and adipose tissue and brain at birth (taken from (253)).

Berghaus *et al*. showed that LC PUFAs were preferentially incorporated into PC and CEs of both maternal and cord plasma. EPA and DHA were found in higher proportions in cord than in maternal blood compared to their precursors (ALA and LA), which may indicate a selective placental transport for certain physiologically important LC PUFAs (254).

De Vriese *et al*. found that the fatty acid profile of cord blood plasma was different from that of maternal plasma at delivery. LA and ALA were lower in cord plasma CEs than in maternal plasma CEs whereas ARA and DHA were twice as high in umbilical cord plasma CEs. Cord plasma PC had a higher content of LC PUFAs than maternal plasma PC at delivery. The FA profile of umbilical plasma at birth showed preferential accumulation of ARA and DHA in CE and PC lipid fractions, indicating a preferential supply of the fetus with LC PUFAs needed for development. Also they showed that maternal plasma PC EPA and DHA percentages were positively associated with maternal dietary intake of these fatty acids during pregnancy (255;256).

Hornstra and colleagues have conducted several studies in this field and have shown that the total amounts of fatty acids in maternal plasma increased significantly during pregnancy (257-259). The pattern of change was similar for the individual fatty acids and fatty acid families. The relative amount (i.e. expressed as a percentage of total fatty acids) of LA did not change during pregnancy, whereas the relative amount of ARA decreased. However, the cord ARA (%) was significantly higher than maternal ARA (%). Despite maternal mobilization of DHA, suggested by a temporary increase in maternal blood DHA status until 18 weeks gestation, maternal blood DHA status steadily declined thereafter (258). In another study by Hornstra's group, women of different countries and origins were assessed and it was shown that the reduction in maternal fatty acid status during pregnancy was generalisable, and independent of differences in dietary habits and ethnic origin (258;259). Van Houwelingen *et al.* found that maternal plasma PC fatty acids expressed as a percentage were highly significantly correlated with fetal plasma PC fatty acids (260).

Donahue *et al.* recently published that mean maternal erythrocyte and cord plasma PUFA concentrations were 7.0 % and 5.2% (total n-3 PUFAs), 5.0 % and 4.6% (DHA+EPA), and 27.9% and 31.4% (total n-6 PUFAs). Mid-pregnancy diet to blood and blood to blood correlations were strongest for DHA+EPA (r=0.38 for diet to maternal blood, r = 0.34 for diet to cord blood, r=0.36 for maternal blood with cord blood), and less strong for n-6 PUFAs.

A recent study has related fatty acids in maternal plasma and erythrocytes with cord plasma and erythrocytes and breastmilk in India. Kilari *et al*. found that levels of DHA and ARA in both plasma and erythrocytes were higher in cord blood compared to maternal blood. Maternal plasma and erythrocyte DHA levels had a positive association with the respective levels in cord blood. However, such an association was not seen for ARA. Maternal plasma n-3 and n-6 fatty acids were positively associated with the respective milk fatty acids. These results showed that milk LC PUFA status reflects the concentrations of maternal LC PUFA in women delivering babies at term. Improving the maternal LC PUFA status throughout pregnancy and lactation might improve the milk LC PUFA status and benefit the offspring.

Tracer studies in vivo using ^{13}C-labeled fatty acids administered to the mother immediately before caesarean section have also demonstrated selective channeling of individual fatty acids to the fetal circulation (253). Larque *et al.* have shown that ^{13}C-enrichment of fatty acids in the NEFAs of cord plasma tended to be higher than in NEFAs of placenta, with a significant difference for the non-esterified DHA. These results suggested that only a part of the placental NEFAs participated in fatty acid transfer, and that the placenta showed a preferential accretion of DHA relative to the other fatty acids. More recently, Gil-Sanchez *et al*. found a significantly higher ratio of ^{13}C-DHA concentrations in cord plasma than in maternal plasma, which was higher than that for the other studied fatty acids. ^{13}C-DHA was predominantly esterified into phospholipids and TAGs in maternal plasma, which might enhance its placental uptake and transfer.

These studies show the direct relation of maternal and fetal fatty acid status through the placenta especially for LC PUFAs such as DHA and ARA. The importance of maternal dietary fatty acids in controlling the availability of LC PUFAs to the fetus and newborn has also been demonstrated. In fact, since it is considered that the developing fetus depends mainly, or completely, on the maternal supply of LC PUFAs, supplementation of the maternal diet with fish oils or the intake of oily fish (at least once per week) during pregnancy to increase levels in the fetus and in neonates, has been advised (23;201). A higher consumption of oily fish during pregnancy is related with the provision of several nutrients (LC n-3 PUFAs, vitamin D, antioxidants such as Se and vitamin E) to the fetus and this may be associated with improved health in infancy and childhood. There is evidence of the protective effect of these nutrients based on intervention trials (132;178;207-209). However, there are no published intervention trials with fish during pregnancy. Since eating fish is advised, but fish are also a source of contaminants,

aquaculture of fish to have low contaminant levels could be an ideal solution to enable oily fish consumption by pregnant women and possibly the intake of the recommended amounts of LC n-3 PUFAs. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish in pregnant women. SIPS focuses on pregnant women whose offspring have increased risk of developing atopic disease, aiming to identify whether there is an effect on atopy outcomes in the offspring. Also, consumption of tailor-made salmon by pregnant women could prevent the depletion of the mother and could improve the status of the newborn in EPA and DHA. A total of 123 women were randomly assigned to consume either 2 portions of farmed salmon per week (from week 20 till delivery) or to continue their habitual diet low in oily fish (less or equal to two portions per month) (see Chapter 2).

The aim of this chapter is to describe the effect of the increased maternal oily fish consumption on the fatty acid composition of maternal plasma PC, cord plasma lipids and cord blood mononuclear cells (CBMCs) with an emphasis on LC n-3 PUFAs.

The hypotheses of the work described in this chapter are that the maternal oily fish intervention will:

1. result in higher amounts of EPA and DHA in maternal PC and in neonatal (cord) plasma lipids;
2. result in higher amounts of EPA and DHA in CBMCs.

4.2 Materials and methods

4.2.1 Study design and subjects

The study design, the subjects' characteristics, and the intervention have been described in Chapter 2. In summary, the population studied was pregnant women with high risk of having an atopic offspring. The study groups were two, of which the control group (n=61) was asked to keep their habitual diet unchanged during the pregnancy study period (from 20 weeks until delivery) and the salmon group (n=62) which was asked to consume 2 portions of farmed salmon per week from 20 weeks until delivery. Every portion of salmon provided 1.73 g of EPA plus DHA; the weekly intake of LC n-3 PUFAs from the study salmon was 3.46 g equivalent to a daily intake of 494 mg/day. The nutrient analysis of salmon is described in Chapter 2, Table 2.3. The consumption of the study salmon provided more EPA plus DHA than the minimum recommended intake in the UK for pregnant women (450 mg/day; (26)). Subjects of the salmon and the control groups came into the maternity hospital fasted at three time points during pregnancy (20, 34, 38 weeks gestation), and fasting blood was taken to enable fatty acid analysis of plasma lipids. At birth, umbilical cord blood was collected to enable fatty acid analysis of plasma lipids and of CBMCs and the measurement of immune markers (Chapter 5).

4.2.2 Maternal blood collection

4.2.2.1 Blood tubes

Plastic 6 mL lithium heparin (LH) vacutainer blood bottles, plastic 3.5 mL Serum Separator Tube (SST 11) vacutainer blood bottles, and plastic 2 mL ethylenediaminetetraacetic acid (EDTA) vacutainer blood bottles were obtained from NHS stores, Southampton General Hospital.

4.2.2.2 Procedure

Maternal blood collection was performed by an adequately trained nurse (Norma Diaper or a nurse from the SWS) or member of the SIPS team (Paul Noakes, Liz Miles). At 20, 34, and 38 weeks gestation, blood was collected into three LH bottles and stored at room temperature (for less than 8 hours) for further analysis, and into one EDTA bottle which was also stored at room temperature for haematology testing. At 20 weeks gestation blood

was also collected in a SST bottle and stored at room temperature for routine immunology testing.

4.2.3 Cord Blood collection

4.2.3.1 Blood Tubes

Plastic 6 mL lithium heparin (LH) vacutainer blood bottles, plastic 2 mL ethylenediaminetetraacetic acid (EDTA) vacutainer blood bottles, alcohol wipes– sterets, 19 gauge needles, sterile 50 ml tubes (Elkay Laboratories) and 50 ml syringes were obtained from NHS stores, Southampton General Hospital.

4.2.3.2 Procedure

Midwifery staff at Princess Anne Hospital assisted with all cord blood collections. Umbilical cord blood was only collected from normal full-term (> 37 weeks) deliveries with informed consent from the mothers. Cord blood was preferentially collected during the third phase of delivery while the placenta is still in the uterus, in order to ensure a good volume of collection. If this was not possible, cord blood was collected after delivery and the placenta had been expelled. In either case, the umbilical cord was clamped immediately and cleaned with 70% alcohol and/or iodine swab, this minimised maternal blood contamination. Whole cord blood was collected using a 16-19G needle on a 50 mL syringe. After umbilical cord vein puncture, the cord blood was collected into the 50 mL syringe. The volume collected ranged from 10 to 30 ml depending on the condition of the placenta. Cord blood samples were collected into 2 LH tubes and 2 EDTA tubes, one of each was kept at room temperature and the other in the fridge (4°C) for less than 8 hours for further analysis. The EDTA bottle which was stored at room temperature was sent for haematology testing. The cord blood samples were then either stored at room temperature or in fridge and within the next 8-12 hours blood was separated into plasma, mononuclear cells and erythrocytes by gradient centrifugation.

4.2.4 Preparation of plasma and mononuclear cells from maternal and cord blood

4.2.4.1 Materials

Histopaque (density 1.077 g/L, containing polysucrose 5.7 g/dL, and sodium diatrizoate 9.0 g/dL), L-glutamine (200 mM), antibiotics (penicillin, streptomycin), ethylenediaminetetraacetic acid (EDTA), potassium bicarbonate, and ammonium chloride, crystal violet were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid. RPMI medium (without L-glutamine and antibiotics) and

heat-inactivated fetal bovine serum (HI-FBS) were purchased from PAA Laboratories. Dimethyl sulphoxide (DMSO) and acetic acid were purchased from Fisher Scientific. Saline (0.9% sodium chloride) was purchased from SUHT Pharmacy, Southampton General Hospital.

White cell counting fluid was made as follows: 100 mL PBS, 2 mL acetic acid, approximately 5 g crystal violet. The solution was millipore filtered before use.

4.2.4.2 Procedures

Heparinised whole blood was layered onto Histopaque (ratio of blood to Histopaque: 2:1) and centrifuged for 15 min at 800 × *g* (2000 rpm, R = 18 cm) at room temperature. Plasma was collected from the top layer and frozen at -80˚C for fatty acid and cytokine analysis (Chapter 5), as well as to be used for culturing CBMCs.

The CBMCs were collected from the interphase and washed once for 10 min at 450 × *g* (1500 rpm, R = 18 cm) with RPMI medium containing 0.75 mmol/L glutamine and antibiotics (penicillin and streptomycin). After being resuspended in 1 mL RPMI medium, the cells were counted with a haemocytometer using white cell counting fluid (crystal violet). After counting total CBMCs, 2×10^6 cells were removed and frozen at -80˚C for fatty acid analysis.

The remaining CBMCs were washed once (10 min, 450 × *g*) with RPMI medium containing 2% HI-FBS. The CBMCs were resuspended in RPMI medium containing 2% HI-FBS and were cryopreserved with 15% DMSO in HI-FBS (freezing medium). The freezing medium was added to the resuspended CBMCs drop-wise on ice (ratio of resuspended cells in RPMI medium containing 2% HI-FBS to freezing medium: 1:1). The CBMC concentrations were adjusted to 12-20 × 10^6 cells/ml/cryovial and were frozen at -80˚C in a Nalgene Cryo 1˚C freezing container (filled with 250 mL isopropanol). After at least 4 hours of freezing, cryovials containing the CBMCs were stored in liquid nitrogen for later thawing and culturing (Chapter 5).

4.2.5 Fatty acid analysis of maternal and cord plasma lipids and cord blood mononuclear cells by gas chromatography

4.2.5.1 Materials

Sodium chloride (NaCl), butyrated hydroxytoluene (BHT), potassium bicarbonate ($KHCO_3$), potassium carbonate (K_2CO_3), heneicosanoin acid, cholesteryl heptadecanoate, tripentadecanoin, and menhaden oil standard fatty acid methyl ester mix were all

purchased by Sigma. Dipentadecanoyl PC was purchased from Avanti Polar Lipids. Chloroform, methanol, hexane, ethyl acetate, toluene, glacial acid, sulphuric acid were purchased from Fisher Scientific. Aminopropylsilica solid phase extraction (SPE) cartridges (NH2) were purchased from Varian. Institute of Human Nutrition (IHN) oil standard mix was an in house preparation using Sigma and Nuchek fatty acid standards.

4.2.5.2 Procedure

For plasma samples, quantitative analysis of fatty acids was performed, and a known amount of plasma was used for analysis (0.5 ml). Internal standards of known concentration and volume were added to plasma samples prior to lipid extraction (Table 4.1). To the plasma with the added standards was also added 0.9% NaCl so as to reach a final volume of 0.8 ml.

Table 4.1: Internal standards used for quantitative fatty acid analysis of plasma lipid classes

Lipid class	Numerical formula of standard fatty acid	Systemic name of standard fatty acid	Concentration of standard solution in hexane	Volume of standard solution added per sample	Amount of standard added per sample
Phosphatidylcholine (PC)	15:0	Dipentadecanoyl PC	1mg/mL	100 μL	100 μg
Non-esterified fatty acids (NEFAs)	21:0	Heneicosanoic acid	0.5 mg/mL	25 μL	12.5 μg
Cholesteryl esters (CEs)	17:0	Cholesteryl heptadecanoate	3 mg/mL	50 μL	150 μL
Tryglecerides (TAGs)	15:0	Tripentadecanoin	1mg/mL	50 μL	50 μg

For the CBMCs that had been stored at -80°C for fatty acid analysis (2 x 10^6 cells), the whole sample was used after defrosting, and 0.9% NaCl was added so as the samples to reach the volume 0.8 ml. These samples were not analysed quantitatively so no internal standards were added.

Total lipid was extracted from plasma and CBMCs with chloroform:methanol (2:1; vol/vol) containing butylated hydroxytoluene (BHT) as antioxidant. After adding NaCl (1 M), the sample was centrifuged and the lower non-aqueous phase collected. The sample was dried under nitrogen gas at 40°C. Plasma lipid classes (TAGs, PC, CEs, NEFAs) were separated for plasma samples but total lipids were analysed for CBMCs.

Separation of plasma lipid classes was performed by solid phase extraction (SPE). An aminopropylsilica SPE cartridge was placed on the SPE tank. Total lipid extracts were dissolved in chloroform and applied to the column. The sample was allowed to drip

through under gravity and the remaining liquid was collected under vacuum. The column was washed twice with chloroform under vacuum. The washes contained TAGs and CEs and were dried under nitrogen. PC was eluted by chloroform:methanol (60:40; vol/vol) under vacuum until dry. The washes containing PC were dried under nitrogen gas at 40°C. NEFAs were eluted by chloroform:methanol:glacial acetic acid (100:2:2; vol/vol/vol) under vacuum until dry. The fractions were dried under nitrogen. A new aminopropylsilica SPE cartridge was placed on the SPE tank, and the cartridge was washed four times with hexane. The TAG and CE fraction collected initially was dissolved in hexane and applied to the column. The sample was allowed to drip through under gravity and the remaining liquid was collected under vacuum. The column was washed twice with hexane under vacuum. The washes containing CEs were dried under nitrogen gas at 40°C. Then TAGs were eluted by hexane: chloroform: ethyl acetate (100:5:5; vol/vol/vol) under vacuum and the fraction was dried under nitrogen gas at 40°C.

The lipid classes from plasma and the total lipid extractions from CBMCs were diluted with toluene. Fatty acid methyl esters (FAMEs) were formed by adding methanol containing 2% (v/v) sulphuric acid and incubating at 50˚C for 2 hours. Neutralising solution ($KHCO_3$, K_2CO_3) and hexane were added to the sample. The sample was centrifuged and the upper phase containing the FAMEs collected and then dried under nitrogen. The sample was diluted with hexane and transferred to a gas chromatography (GC) vial. The sample was dried under nitrogen.

The FAMEs were separated and analysed by gas chromatography on a Hewlett-Packard (Hewlett-Packard; Avondale, PA, USA) 6890 gas chromatograph fitted with a Hewlett-Packard (HP 6890 Series GC) system autoinjector. A 30 m BPX-70 SGE capillary column was used. Helium was used as the carrier gas. The detector was a flame ionising detector (FID) held at 250°C. The hydrogen flame caused combustion of the FAMEs and thus generated an ion current proportional to the amount of FAME in the sample. However an experimentally determined correction needed to be made for the number of carbons in a FAME. The resulting chromatogram contained a series of peaks, each corresponding to a FAME. FAMEs were identified by comparison with standards (Institute of Human Nutrition fatty acid methyl esters mixture (IHN) and Menhaden oil fatty acid methyl esters) (Figure 4.3; Figure 4.4) run previously. The area under its peak is propotional to the mass of the FAME injected onto the column. Figure 4.5 to Figure 4.9 show a typical trace of fatty acids in PC, the major plasma phospholipid, NEFAs, CEs, and TAGs of plasma and a typical trace of fatty acids in CBMCs .

4.2.6 Fatty acid analysis

4.2.6.1 Proportion of FAs

Each peak is characteristic and unique for each fatty acid and it appears according to the order that each of the FAME is detected by the FID on the GC. The time that each FAME peak appears on the chromatogram is called retention time, and is characteristic for each fatty acid. Typically, FAMEs with shorter chains and fewer double bonds have a shorter retention time than FAMEs with longer hydrocarbon chains and more double bonds, and thus they appear first on the chromatogram. This is attributed to the physical properties of the FAMEs according to their structure. The identification of the specific fatty acids happens by comparison of the retention times of FAMEs within the standard mixtures (IHN, Menhaden) with those of FAMEs in the sample. After the identification of the fatty acid, the area under the peak is calculated and the proportion of each peak area to the sum of all peak areas is calculated. This represents the percentage of the fatty acid in the total fatty acids in the sample analysed: total lipids (for CBMCs) and lipid class (for plasma; PC, NEFAs, CEs, TAGs).

4.2.6.2 Absolute concentration of FAs

The addition of internal standards in the plasma sample at the lipid extraction phase, allows the determination of the absolute concentrations of each fatty acid in the chromatogram. The area under the internal standard peak refers to the known amount of standard fatty acid added to the sample. This piece of information along with the known initial amount of sample used for analysis, allows for calculations of the absolute concentration of each fatty acid in the sample.

IHN mixture

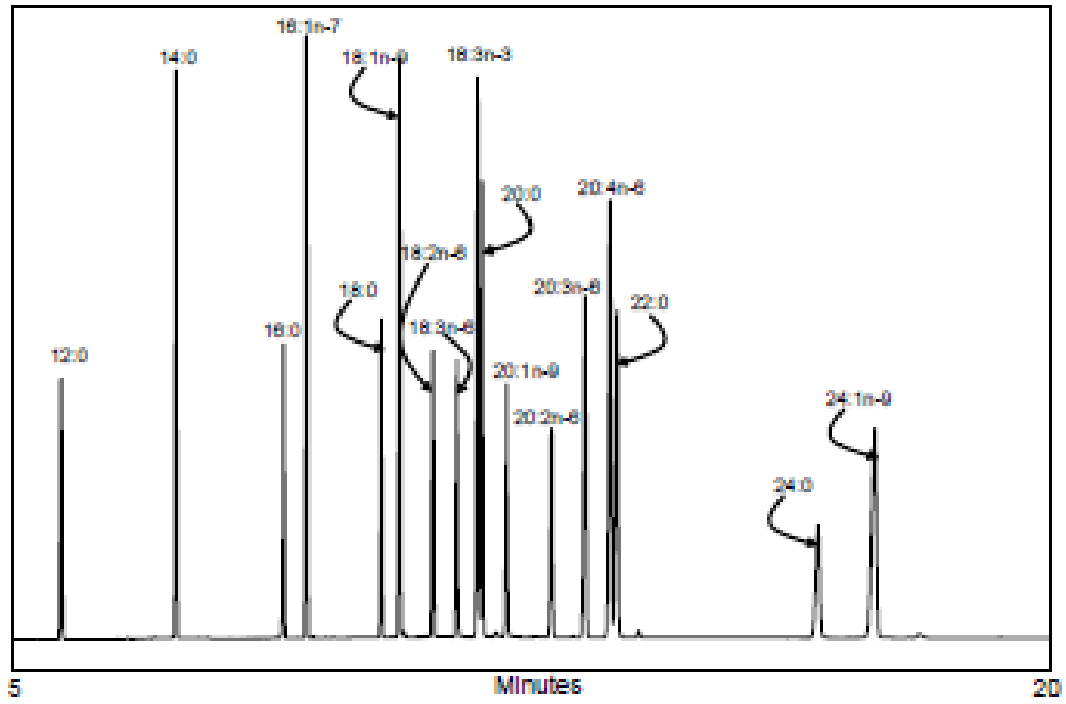


Figure 4.3: Typical GC profile of the Institute of Human Nutrition mixture standard mix

Menhaden oil

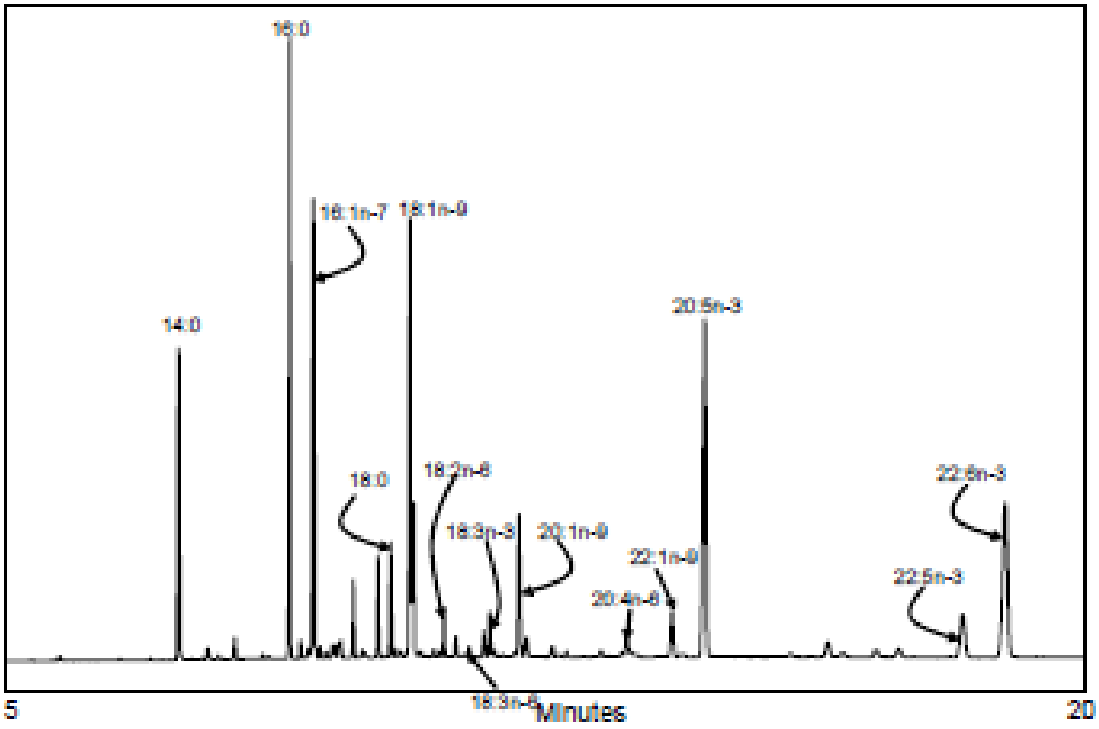


Figure 4.4: Typical GC profile of the Menhaden oil standard

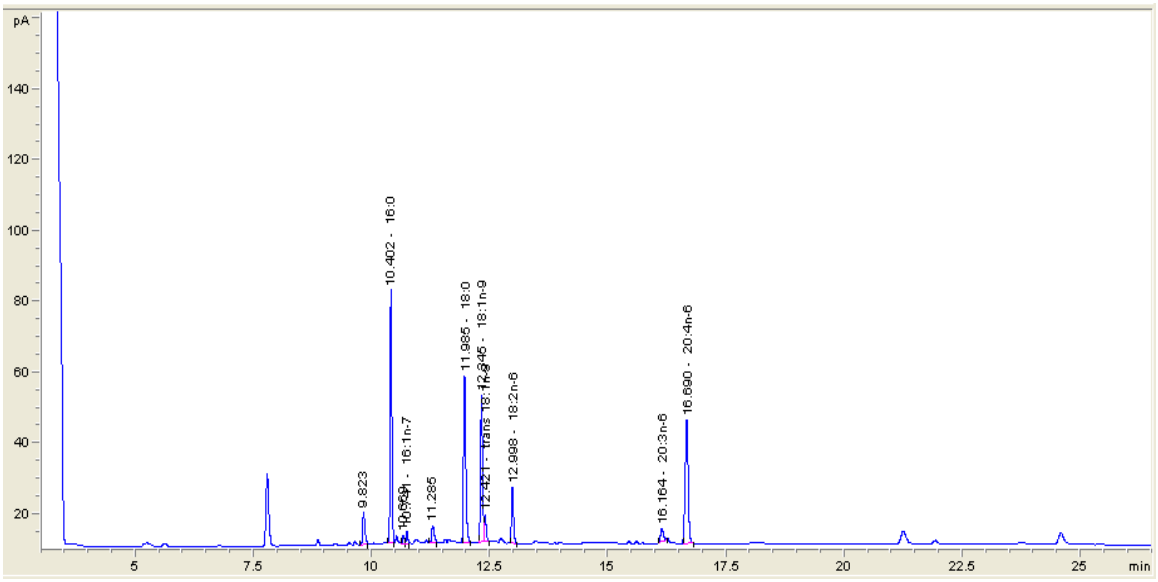


Figure 4.5: Typical trace of fatty acids in CBMCs

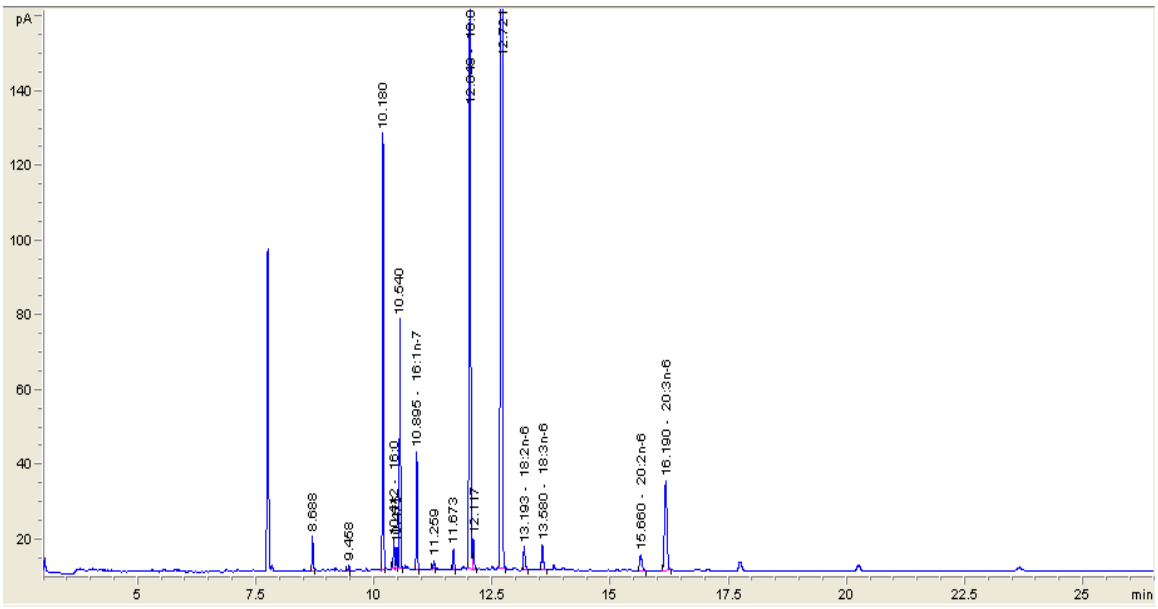


Figure 4.6: Typical trace of fatty acids in plasma CEs

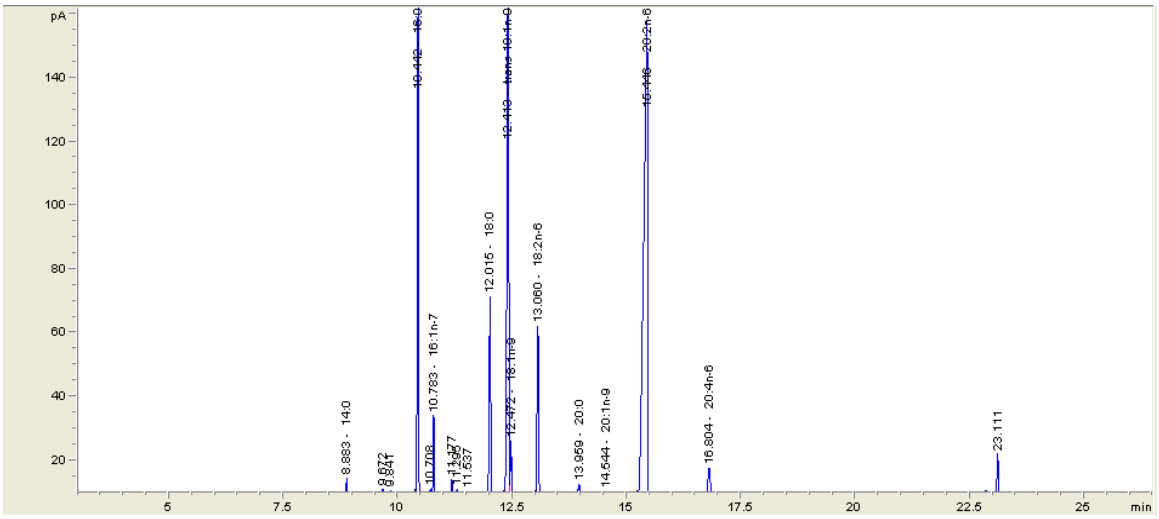


Figure 4.7: Typical trace of fatty acids in plasma NEFAs

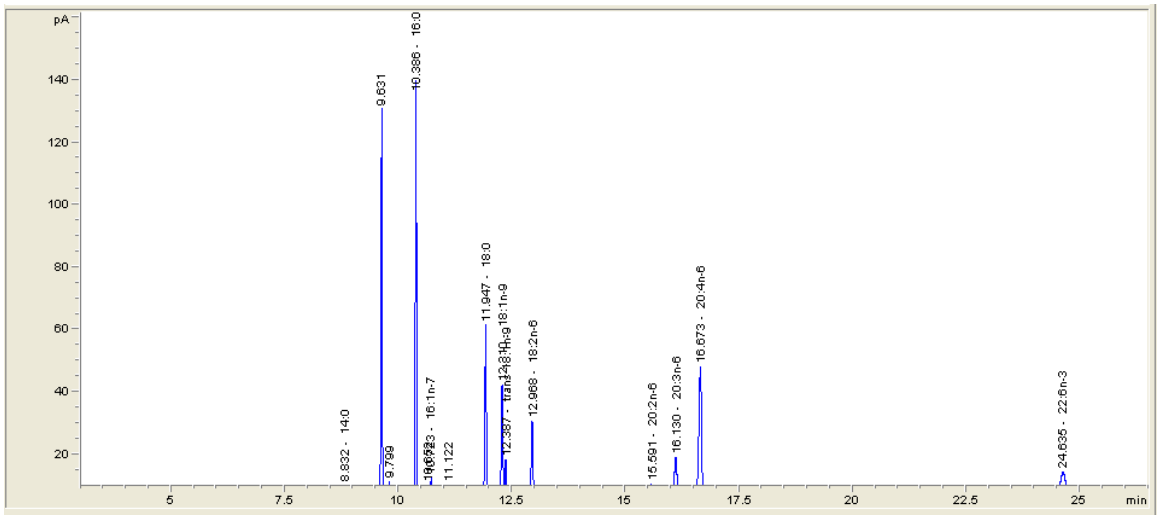


Figure 4.8: Typical trace of fatty acids in plasma PC

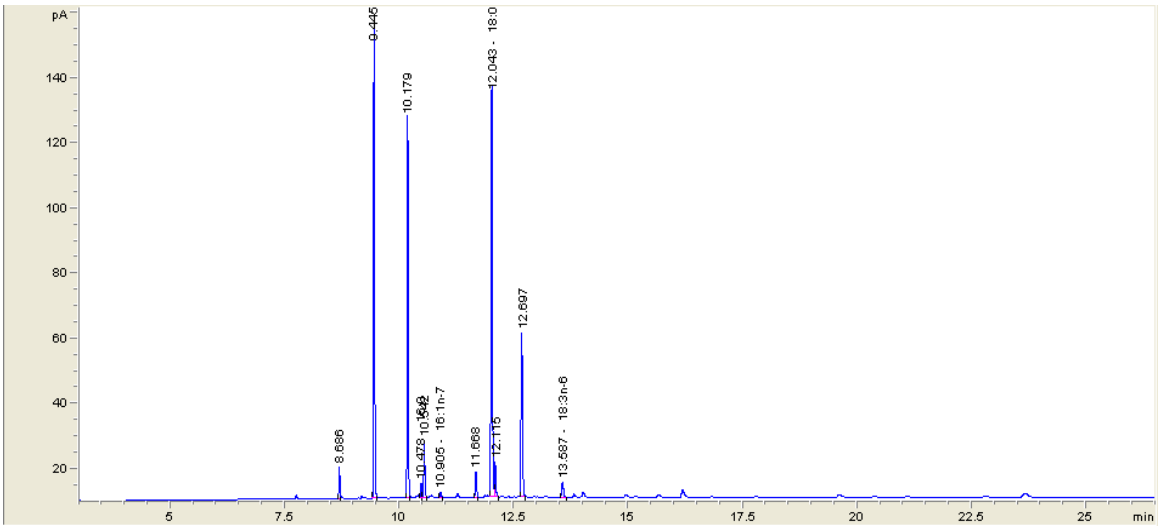


Figure 4.9: Typical trace of fatty acids in plasma TAGs

4.2.7 Statistical analysis

Fatty acid data were normally distributed and the statistical tests used to interpret these data were parametric. For between group comparisons during pregnancy (20, 34, 38 weeks of gestation) and in cord blood, independent samples t-test was used. The General Linear Model (GLM) of analysis, 3 repeated measures analysis of variance (ANOVA) was used to examine overtime changes during pregnancy related to the dietary intervention. Correlations were also made between maternal and cord PC percentages (%) of fatty acids and CBMC fatty acid composition. Also correlations between maternal diet and fatty acid status at birth were made as well as correlations between infant birth characteristics, gestational length and fatty acid status in cord blood. For all correlations, bivariate Spearman correlations were used. For all comparisons a p-value equal to or less that 0.05 ($p \leq 0.05$) was considered statistically significant.

4.3 Results

4.3.1 Maternal fatty acid status: Fatty acid composition of plasma phospholipids (PC) (% only)

PC is the major phospholipid in the circulation, typically contributing about 80% of the plasma phospholipid pool (261). PC is a component of all plasma lipoproteins. PC may be regarded as a transport pool of fatty acids, playing a role in delivery of fatty acids to cells and tissues. The fatty acid composition of plasma PC has a strong similarity to that of cell membrane PC (262). Table 4.2 describes the maternal plasma PC fatty acid composition expressed as the percentage contribution of each fatty acid (to total PC fatty acids), for the two groups during pregnancy (20, 34, and 38 weeks gestation). Repeated measures ANOVA identified significant effects of time on the percentages of most fatty acids and significant effects of group (i.e. salmon vs. control) on the percentages of elaidic acid (trans 18:1n-9), ALA, EPA, docosapentaenoic acid (DPA; 22:5n-3), DHA, total n-3 PUFAs, dihomo-γ-linolenic acid (DGLA; 20:3n-6), ARA and total n-6 PUFAs. There were also significant time x group interactions for EPA, DHA, total n-3 PUFAs, DGLA, ARA and total n-6 PUFAs. These were explored further by between group comparisons at each time point. At 20 weeks gestation (baseline), the salmon group had higher percentages EPA, DHA, and total n-3 PUFAs and a lower percentage of ARA compared to the control group. During the intervention period, at 34 and 38 weeks of pregnancy, the salmon group continued to have higher percentages of EPA, DHA, total n-3 PUFAs and also of DPA, and a lower percentage of ARA, and additionally of DGLA and total n-6 PUFAs (Table 4.2).

Figure 4.10 shows the percentages of EPA, DHA, ARA, and DPA in the PC fraction during pregnancy at the 3 timepoints studied (20 weeks, 34 weeks and 38 weeks) and how those changed between the groups at each time point, and within the groups throughout pregnancy. EPA and DHA decreased in the control group but increased in the salmon group during pregnancy. ARA decreased in both groups during pregnancy but less so in the control group. DPA slightly increased in the salmon group whereas it decreased in the control group.

The changes in percentages EPA, DHA and ARA in plasma PC were different between groups ($p<0.001$ for all). The changes in plasma PC DPA % were different between the

two groups only for the calculated 34-20 week change (Table 4.3). Table 4.4 shows the within group comparisons for the calculated changes (34-20 and 38-20 weeks) in the % of EPA, DHA, ARA and DPA in maternal plasma PC. It is clear from these comparisons that within the control group EPA, DPA, DHA, and ARA decreased during pregnancy. In the salmon group EPA and DHA increased, while the decrease in ARA was greater than in the control group and the decrease on DPA was smaller.

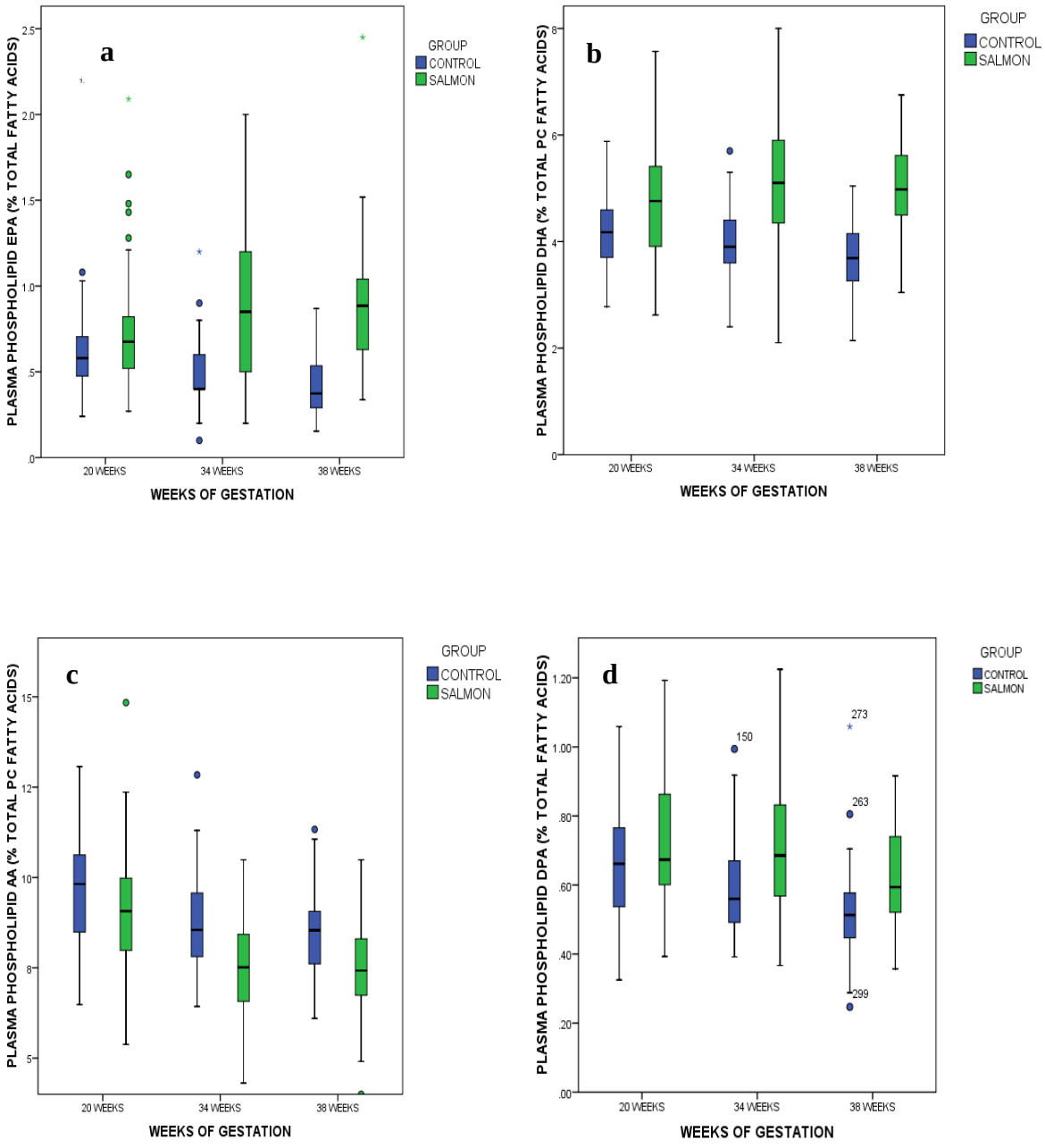


Figure 4.10: Maternal plasma a) EPA, b) DHA, c) ARA, d) DPA expressed as percentage of total fatty acids during pregnancy in both groups; refer to Table 4.2 for exact values

Table 4.2: Maternal plasma phospholipid (PC) fatty acid composition during pregnancy and according to study group

Fatty acid	20 weeks					34 weeks					38 weeks					GLM repeated measures 2-way ANOVA		
	Control group (n=61)		Salmon group (n=62)		p	Control group (n=55)		Salmon group (n=56)		p	Control group (n=41)		Salmon group (n=47)		p	p for time	p for group	p for interaction (grp*time)
	Mean	SD	Mean	SD		Mean	SD	Mean	SD		Mean	SD	Mean	SD				
EPA (20:5n-3)	0.6	0.3	0.7	0.3	**0.028**	0.5	0.2	0.9	0.4	**<0.001**	0.4	0.2	0.9	0.4	**<0.001**	0.758	**<0.001**	**<0.001**
DHA (22:6n-3)	4.2	0.7	4.7	0.9	**0.001**	3.9	0.7	5.1	1.1	**<0.001**	3.7	0.7	5.0	0.9	**<0.001**	0.003	**<0.001**	**<0.001**
14:00	0.4	0.1	0.4	0.1	0.738	0.4	0.1	0.4	0.1	0.669	0.4	0.1	0.4	0.1	0.598	0.590	0.555	**<0.001**
16:00	33.0	1.7	32.8	1.4	0.573	34.1	1.1	34.2	1.7	0.793	34.5	0.9	34.3	0.9	0.390	**0.001**	0.640	**<0.001**
16:1n-7	0.9	0.3	0.8	0.2	0.101	0.9	0.2	0.8	0.2	0.300	0.9	0.2	0.8	0.3	0.555	0.096	0.300	**<0.001**
18:00	10.8	0.9	10.7	1.0	0.379	9.8	0.8	9.7	0.8	0.670	9.8	0.9	9.7	0.8	0.552	**<0.001**	0.728	**<0.001**
18:1n-9	11.2	1.5	11.1	1.4	0.738	11.9	1.2	11.7	1.3	0.317	12.1	1.2	11.9	1.2	0.776	**<0.001**	0.530	**<0.001**
18:1n-9Trans	1.5	0.2	1.5	0.2	0.676	1.4	0.2	1.3	0.2	**0.005**	1.5	0.2	1.4	0.2	**0.008**	**<0.001**	**0.010**	**<0.001**
18:2n-6	21.5	2.5	22.1	2.5	0.176	22.2	2.2	22.7	2.3	0.314	22.7	2.4	22.7	2.2	0.904	**<0.001**	0.666	**<0.001**
18:3n-6	0.1	0.1	0.1	0.1	0.899	0.1	0.1	0.1	0.1	0.789	0.1	0.02	0.1	0.02	0.401	**0.003**	0.790	**<0.001**
18:3n-3	0.3	0.1	0.3	0.1	0.137	0.4	0.1	0.4	0.2	**0.020**	0.4	0.1	0.4	0.2	**0.038**	**<0.001**	0.038	**<0.001**
20:00	0.1	0.1	0.1	0.03	0.702	0.1	0.1	0.1	0.1	0.411	0.05	0.02	0.06	0.03	0.658	**<0.001**	0.934	**<0.001**
20:1n-9	0.2	0.1	0.2	0.2	0.534	0.2	0.04	0.2	0.1	0.343	0.2	0.03	0.2	0.03	0.517	**<0.001**	0.688	**<0.001**
20:2n-6	0.5	0.2	0.5	0.1	0.965	0.5	0.1	0.5	0.1	0.173	0.4	0.1	0.4	0.1	0.358	**<0.001**	0.650	**<0.001**
20:3n-6	4.4	0.7	4.2	0.8	0.148	4.4	0.7	3.7	0.6	**<0.001**	4.2	0.6	3.7	0.6	**<0.001**	**<0.001**	**<0.001**	**<0.001**
ARA (20:4n-6)	9.7	1.5	9.0	1.8	**0.032**	8.7	1.3	7.5	1.4	**<0.001**	8.4	1.2	7.4	1.3	**<0.001**	**<0.001**	**0.002**	**<0.001**
DPA (22:5n-3)	0.7	0.2	0.7	0.2	0.189	0.6	0.1	0.7	0.2	**<0.001**	0.5	0.1	0.6	0.1	**0.003**	**<0.001**	**0.002**	**<0.001**
SFAs	44.3	2.0	44.0	1.7	0.344	44.4	0.7	44.4	1.5	0.893	44.7	0.7	44.5	0.7	0.088	0.078	0.431	**<0.001**
MUFAs	13.8	1.7	13.6	1.6	0.570	14.4	1.3	14.0	1.5	0.167	14.5	1.3	14.3	1.4	0.457	**<0.001**	0.273	**<0.001**
PUFAs	41.9	2.4	42.4	1.9	0.212	41.3	1.6	41.6	1.8	0.285	40.7	1.5	41.2	1.5	0.141	**<0.000**	0.153	**<0.001**
Total n-3 PUFAs	5.8	0.9	6.5	1.2	**<0.001**	5.4	0.8	7.1	1.5	**<0.001**	5.0	0.9	6.9	1.3	**<0.001**	**0.005**	**<0.001**	**<0.001**
Total n-6 PUFAs	36.1	2.3	35.9	2.1	0.576	35.8	1.5	34.5	1.7	**<0.001**	35.7	1.740	34.2	1.9	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Values are mean (SD) percentage (%) of total fatty acids in PC; p-values from ANOVA and independent samples t-test

Table 4.3: Changes in the percentage of the four fatty acids of interest in maternal plasma PC according to study group

Fatty acid	Control group n=55		Salmon group n=56		p
	Mean	SD	Mean	SD	
EPA change 34-20 weeks	-0.16	0.30	0.15	0.45	**<0.001**
DHA change 34-20 weeks	-0.17	0.50	0.38	0.93	**<0.001**
ARA change 34-20 weeks	-1.09	0.94	-1.54	1.36	**0.042**
DPA (n-3) change 34-20 weeks	-0.09	0.17	-0.01	0.18	**0.017**

Fatty acid	Control group n=41		Salmon group n=47		p
	Mean	SD	Mean	SD	
EPA change 38-20 weeks	-0.17	0.16	0.13	0.40	**<0.001**
DHA change 38-20 weeks	-0.41	0.49	0.13	0.69	**<0.001**
ARA change 38-20 weeks	-1.29	0.82	-1.86	1.08	**0.007**
DPA (n-3) change 38-20 weeks	-0.14	0.17	-0.11	0.16	0.318

Values are mean (SD) change in percentage; independent samples t-test p-values

Table 4.4: Changes in the percentage of the four fatty acids of interest in maternal PC according to study group

	Control group					Salmon group				
	34-20 weeks		38-20 weeks		p	34-20 weeks		38-20 weeks		p
Fatty acid	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
EPA	-0.16	0.30	-0.17	0.16	0.324	0.15	0.45	0.13	0.40	0.614
DHA	-0.17	0.50	-0.41	0.49	**<0.001**	0.38	0.93	0.13	0.69	**0.054**
ARA	-1.09	0.94	-1.29	0.82	**0.014**	-1.54	1.36	-1.86	1.08	0.269
DPA (n-3)	-0.09	0.17	-0.14	0.17	**0.004**	-0.01	0.18	-0.11	0.16	**<0.001**

Values are mean (SD) change in percentage; paired t-test p-values

4.3.2 Cord blood fatty acid status

4.3.2.1 Fatty acid composition of lipid classes in cord blood

4.3.2.1.1 Fatty acid composition of plasma phospholipids (PC)

Table 4.5 describes the cord plasma PC fatty acid composition expressed as percentages (%) of each fatty acid and also as absolute concentrations (μg/mL plasma). Independent samples t-tests were conducted so as to compare the PC fatty acid status of the two groups at birth. The salmon group had higher percentages of EPA, DPA, DHA, and total n-3 PUFAs and lower percentages of ARA and total n-6 PUFAs than the control group. For the absolute concentrations of the fatty acids in the cord plasma PC, the same pattern was found. However, only EPA and total n-3 PUFAs were significantly higher in the salmon group compared to the control group i.e. the higher concentrations of DPA and DHA in the salmon group did not reach statistical significance. Fatty acid ratios of interest in the cord plasma PC (ARA/EPA, ARA/DHA, ARA/(EPA+DHA), n-6 PUFA/n-3 PUFA) are also shown. Each of these ratios was higher in the control group. Total PC fatty acids (μg/mL) were not different between the groups.

Table 4.5: Cord plasma phospholipid (PC) fatty acid composition according to study group

Fatty acid percentage (%)	Control group (n=46)		Salmon group (n=47)		p	Fatty acid absolute concentration (μg/mL)	Control group (n=46)		Salmon group (n=47)		p
	Mean	SD	Mean	SD			Mean	SD	Mean	SD	
EPA (20:5n-3)	0.3	0.1	0.6	0.3	**<0.001**	EPA (20:5n-3)	1.79	0.89	3.50	1.77	**<0.001**
DHA (22:6n-3)	6.4	1.3	7.4	1.4	**0.001**	DHA (22:6n-3)	35.71	12.52	40.60	14.64	0.087
14:00	0.3	0.1	0.3	0.1	0.593	14:00	1.58	0.55	1.56	0.59	0.895
16:00	31.1	1.6	31.0	1.5	0.809	16:00	172.77	57.97	172.38	57.86	0.974
16:1n-7	0.9	0.2	1.0	0.2	0.907	16:1n-7	5.37	2.05	5.51	2.45	0.761
18:00	14.6	1.4	14.6	1.1	0.862	18:00	80.23	23.37	80.68	26.20	0.930
18:1n-9	9.0	1.1	8.9	1.1	0.670	18:1n-9	49.59	17.97	49.73	17.93	0.969
18:1n-9 Trans	2.9	0.3	2.8	0.3	0.066	18:1n-9 Trans	15.91	4.81	15.59	5.74	0.773
18:2n-6	8.7	2.3	8.9	1.6	0.500	18:2n-6	49.02	27.61	49.59	18.09	0.906
18:3n-6	0.1	0.03	0.1	0.05	0.251	18:3n-6	0.64	0.27	0.69	0.32	0.467
18:3n-3	0.1	0.05	0.1	0.04	0.966	18:3n-3	0.84	0.25	0.88	0.27	0.525
20:00	0.2	0.05	0.2	0.05	0.377	20:00	0.51	0.37	0.49	0.23	0.885
20:1n-9	0.1	0.05	0.1	0.03	0.291	20:1n-9	0.67	0.32	0.64	0.28	0.616
20:2n-6	0.4	0.1	0.4	0.1	0.089	20:2n-6	2.03	0.86	2.17	0.74	0.391
20:3n-6	6.0	1.0	6.3	1.0	0.138	20:3n-6	32.68	9.48	35.07	11.80	0.283
ARA (20:4n-6)	18.3	2.4	16.6	1.8	**<0.001**	ARA (20:4n-6)	101.27	31.69	91.17	29.97	0.118
DPA (22:5n-3)	0.5	0.2	0.6	0.2	0.019	DPA (22:5n-3)	2.92	1.40	3.47	1.73	0.097
Total SFAs	46.1	1.0	46.1	1.5	0.911	Total SFAs	255.08	79.58	255.12	83.91	0.998
Total MUFAs	13.0	1.4	12.8	1.3	0.399	Total MUFAs	71.54	24.10	71.48	25.83	0.991
Total PUFAs	40.9	1.6	41.2	1.9	0.465	Total PUFAs	226.9	69.73	227.15	71.07	0.987
Total n-3 PUFAs	7.4	1.5	8.8	1.6	**<0.001**	Total n-3 PUFAs	41.27	14.25	48.45	17.26	**0.031**
Total n-6 PUFAs	33.5	1.4	32.4	2.0	**0.002**	Total n-6 PUFAs	185.63	58.38	178.69	56.58	0.562
						Total fatty acids	553.52	171.32	553.74	178.71	0.995
						ARA/DHA	2.92	0.57	2.32	0.51	**<0.001**
						ARA/EPA	65.51	29.40	31.45	16.88	**<0.001**
						ARA/(EPA+DHA)	2.78	0.54	2.13	0.49	**<0.001**
						n-6/n-3	4.64	0.91	3.83	0.92	**<0.001**

Values are mean (SD) percentage (%) of total fatty acids and absolute concentrations (μg/mL) in PC; independent samples t-test p-values

4.3.2.1.2 Fatty acid composition of cord plasma non-esterified fatty acids (NEFAs)

Table 4.6 describes the cord plasma NEFA fatty acid composition expressed as percentages (%) of each fatty acid and as absolute concentrations (μg/mL plasma). Independent samples t-tests were conducted so as to compare the plasma NEFA fatty acid status of the two groups at birth. The salmon group had higher percentages of 18:1n-9 trans, DPA, DHA, and total n-3 PUFAs and a lower percentage of total MUFAs than the control group. Although the absolute concentrations of EPA, DPA, DHA, and total n-3 PUFAs tended to be higher in the salmon group, and the absolute concentrations of ARA and total n-6 PUFAs tended to be lower, the data did not reach statistical significance. The ratios ARA/DHA, ARA/(EPA+DHA), and n-6 PUFA/n-3 PUFA were higher in the control group. Total NEFA concentration (μg/mL) did not differ significantly between the groups.

Table 4.6: Cord plasma NEFA fatty acid composition according to study group

Fatty acid percentage (%)	Control group (n=46)		Salmon group (n=47)		p-value	Fatty acid absolute concentration (μg/ml)	Control group (n=46)		Salmon group (n=47)		p
	Mean	SD	Mean	SD			Mean	SD	Mean	SD	
EPA (20:5n-3)	0.2	0.1	0.2	0.2	0.065	EPA (20:5n-3)	0.26	0.14	0.29	0.24	0.554
DHA (22:6n-3)	2.5	0.7	3.1	1.1	**0.001**	DHA (22:6n-3)	3.86	2.36	4.20	3.35	0.573
14:00	1.5	0.4	1.5	0.6	0.695	14:00	2.41	1.63	2.11	2.15	0.446
16:00	32.1	2.8	31.1	4.1	0.211	16:00	50.49	29.89	42.31	31.80	0.205
16:1n-7	3.3	0.9	3.0	0.9	0.076	16:1n-7	5.48	4.17	4.34	4.53	0.211
18:00	18.8	3.6	19.5	4.8	0.420	18:00	28.39	14.45	25.05	14.3	0.265
18:1n-9	22.4	3.8	20.9	3.8	0.067	18:1n-9	37.26	28.45	29.67	26.63	0.188
18:1n-9 Trans	2.3	0.4	2.1	0.5	**0.026**	18:1n-9 Trans	3.72	2.38	2.72	1.67	**0.022**
18:2n-6	10.8	2.3	10.4	2.4	0.425	18:2n-6	17.64	13.06	14.06	11.39	0.163
18:3n-6	0.5	0.4	0.6	0.5	0.352	18:3n-6	0.61	0.31	0.60	0.42	0.931
20:00	1.2	0.3	1.3	0.3	0.380	20:00	1.85	1.19	1.58	1.05	0.254
20:1n-9	0.4	0.3	0.5	0.5	0.172	20:1n-9	0.56	0.33	0.59	0.48	0.701
20:2n-6	0.2	0.1	0.2	0.1	0.364	20:2n-6	0.30	0.21	0.24	0.21	0.118
20:3n-6	0.5	0.2	0.7	0.6	0.254	20:3n-6	0.89	0.71	0.93	1.42	0.862
ARA (20:4n-6)	3.0	1.1	3.2	2.3	0.584	ARA (20:4n-6)	4.76	3.48	4.52	5.67	0.806
DPA (22:5n-3)	0.3	0.1	0.3	0.1	**0.001**	DPA (22:5n-3)	0.39	0.23	0.42	0.28	0.686
Total SFAs	53.6	4.9	53.4	7.2	0.873	Total SFAs	83.14	46.09	71.05	48.04	0.219
Total MUFAs	28.5	4.3	26.6	4.6	**0.042**	Total MUFAs	47.01	34.52	37.33	32.27	0.166
Total PUFAs	17.9	2.8	18.6	4.4	0.349	Total PUFAs	28.72	18.64	25.26	19.07	0.378
Total n-3 PUFAs	2.9	0.7	3.6	1.2	**0.001**	Total n-3 PUFAs	4.51	2.65	4.90	3.80	0.572
Total n-6 PUFAs	15.0	2.6	15.0	3.6	0.982	Total n-6 PUFAs	24.20	16.3	20.36	15.51	0.247
						Total NEFAs	158.87	97.04	133.63	96.41	0.212
						ARA/DHA	1.23	0.35	1.03	0.33	**0.003**
						ARA/EPA	17.62	8.24	15.59	8.08	0.242
						ARA/ (EPA+DHA)	1.14	0.32	0.95	0.31	**0.003**
						n-6/n-3	5.48	1.61	4.37	1.17	**<0.001**

Values are mean (SD) percentage (%) of total fatty acids and absolute concentrations (μg/mL) in NEFAs; independent samples t-test p-values

4.3.2.1.3 Fatty acid composition of cord plasma cholesteryl esters (CEs)

Table 4.7 describes the cord plasma CE fatty acid composition expressed as percentages (%) of each fatty acid and also as absolute concentrations (μg/mL plasma). Independent samples t-tests were conducted so as to compare the CE fatty acid status of the two groups at birth. The salmon group had a higher percentages of EPA, DHA, ALA, eicosanoic (20:0), and DGLA and lower percentages of tetradecanoic acid (14:0), ARA and DPA than the control group. The absolute concentration of EPA in cord plasma CE was higher in the salmon group compared to the control group. The higher concentrations of DHA and total n-3 PUFAs in the salmon group and the higher concentrations of ARA and total n-6 PUFAs in the control group did not reach statistical significance. Each of the ratios calculated (ARA/EPA, ARA/DHA, ARA/(EPA+DHA), n-6 PUFA/n-3 PUFA) was higher in the control group. Total CE fatty acids (μg/mL) did not differ between the groups.

Table 4.7: Cord plasma cholesteryl ester (CE) fatty acid composition according to study group

Fatty acid percentage (%)	Control group (n=46)		Salmon group (n=47)		p	Fatty acid absolute concentration (μg/ml)	Control group (n=45)		Salmon group (n=46)		p
	Mean	SD	Mean	SD			Mean	SD	Mean	SD	
EPA (20:5n-3)	0.4	0.2	0.6	0.2	**<0.001**	EPA (20:5n-3)	1.92	1.03	2.91	1.53	**<0.001**
DHA (22:6n-3)	2.2	0.9	2.3	0.8	**<0.001**	DHA (22:6n-3)	10.78	6.45	11.49	5.07	0.563
14:00	0.8	0.2	0.8	0.2	**0.050**	14:00	4.17	1.83	4.11	1.82	0.880
16:00	23.2	2.3	22.7	1.8	0.192	16:00	113.08	39.19	114.63	42.48	0.857
16:1n-7	6.8	1.2	6.9	1.3	0.826	16:1n-7	33.60	14.39	36.81	19.94	0.381
18:00	2.7	0.6	2.9	0.9	0.138	18:00	13.10	5.37	14.67	7.53	0.255
18:1n-9	26.2	2.9	26.7	3.3	0.239	18:1n-9	131.12	56.27	138.19	61.84	0.570
18:1n-9 Trans	3.2	0.5	3.2	0.4	0.958	18:1n-9 Trans	15.75	5.68	16.55	8.07	0.588
18:2n-6	18.2	5.2	18.6	2.8	0.181	18:2n-6	97.27	80.44	94.12	40.31	0.815
18:3n-6	0.9	0.2	1.0	0.4	0.279	18:3n-6	4.51	2.09	4.80	2.18	0.514
18:3n-3	0.3	0.2	0.3	0.3	**0.009**	18:3n-3	1.34	1.25	1.31	0.59	0.854
20:00	0.4	0.3	0.5	0.4	**0.047**	20:00	1.92	1.18	2.08	1.21	0.506
20:1n-9	0.2	0.1	0.2	0.3	0.188	20:1n-9	1.04	0.58	1.10	0.79	0.679
20:2n-6	0.2	0.2	0.2	0.2	0.276	20:2n-6	0.84	0.83	0.68	0.78	0.349
20:3n-6	1.4	0.3	1.5	0.3	**0.012**	20:3n-6	6.99	2.47	7.42	3.15	0.474
ARA (20:4n-6)	12.8	2.9	11.4	2.3	**0.021**	ARA (20:4n-6)	62.52	25.27	57.76	26.11	0.379
DPA (22:5n-3)	0.3	0.2	0.3	0.2	**0.009**	DPA (22:5n-3)	1.30	0.98	1.31	0.98	0.967
Total SFAs	27.1	2.8	26.9	2.2	0.732	Total SFAs	132.26	45.89	135.49	50.45	0.750
Total MUFAs	36.4	3.9	37.1	4.4	0.415	Total MUFAs	181.52	74.91	192.65	88.96	0.520
Total PUFAs	36.6	4.9	36.0	4.1	0.577	Total PUFAs	187.47	102.51	181.79	73.22	0.762
Total n-3 PUFAs	3.1	1.2	3.5	1.0	0.138	Total n-3 PUFAs	15.35	8.37	17.02	6.96	0.305
Total n-6 PUFAs	33.5	4.9	32.6	4.0	0.351	Total n-6 PUFAs	172.12	97.56	164.77	68.15	0.679
						Total CE fatty acids	501.26	212.19	509.94	203.13	0.843
						ARA/DHA	6.41	2.11	5.38	1.72	**0.013**
						ARA/EPA	37.17	19.69	22.12	9.96	**<0.001**
						ARA/ (EPA+DHA)	5.32	1.69	4.25	1.37	**0.001**
						n-6/n-3	11.68	3.99	10.23	2.97	**0.054**

Values are mean (SD) percentage (%) of total fatty acids and absolute concentration (μg/mL) in CEs; independent samples t-test p-values

4.3.2.1.4 Fatty acid composition of cord plasma triglycerides (TAGs)

Table 4.8 describes the cord plasma TAG fatty acid composition expressed as percentages (%) of each fatty acid and also as absolute concentrations (μg/mL plasma). Independent samples t-tests were conducted so as to compare the TAG fatty acid status of the two groups at birth. The salmon group had higher percentages of ALA, EPA, DPA, DHA, and total n-3 PUFAs and lower percentages of LA, ARA and total n-6 fatty acids than the control group. For the absolute concentrations of the fatty acids in the cord plasma TAGs, the same pattern was found: EPA, DPA, DHA, and total n-3 PUFA concentrations were higher in the salmon group compared to the control group, while the concentration of. ARA was higher the control group. Each of the ratios (ARA/EPA, ARA/DHA, ARA/(EPA+DHA), n-6 PUFA/n-3 PUFA) was higher in the control group. Total TAG fatty acids (μg/mL) did not differ between the groups.

Table 4.8: Cord plasma triglyceride (TAG) fatty acid composition according to study group

Fatty acid percentage (%)	Control group (n=46)		Salmon group (n=47)		p	Fatty acid absolute concentration (µg/ml)	Control group (n=46)		Salmon group (n=47)		p
	Mean	SD	Mean	SD			Mean	SD	Mean	SD	
EPA (20:5n-3)	0.3	0.2	0.6	0.3	**<0.001**	EPA (20:5n-3)	0.50	0.32	0.92	0.59	**<0.001**
DHA (22:6n-3)	3.2	1.2	4.7	1.7	**<0.001**	DHA (22:6n-3)	4.81	2.74	7.79	4.39	**<0.001**
14:00	1.7	0.3	1.7	0.4	0.433	14:00	2.86	2.31	3.17	2.28	0.509
16:00	28.4	1.8	28.5	1.8	0.887	16:00	48.77	38.85	51.74	34.56	0.697
16:1n-7	6.2	1.1	6.3	1.0	0.751	16:1n-7	10.15	6.42	11.75	8.66	0.316
18:00	4.2	1.0	4.7	1.5	0.086	18:00	6.77	4.18	8.10	5.11	0.173
18:1n-9	30.7	3.6	30.0	2.9	0.308	18:1n-9	53.79	51.18	54.21	35.60	0.964
18:1n-9Trans	3.6	0.9	3.5	0.6	0.439	18:1n-9Trans	5.97	4.07	6.33	4.17	0.680
18:2n-6	12.5	1.9	12.4	2.2	0.875	18:2n-6	21.61	19.02	22.95	15.73	0.711
18:3n-6	0.7	0.3	0.6	0.2	**0.050**	18:3n-6	1.02	0.63	0.97	0.61	0.703
18:3n-3	0.4	0.2	0.5	0.2	**0.015**	18:3n-3	0.81	0.98	1.00	0.83	0.313
20:00	0.1	0.04	0.1	0.1	0.372	20:00	0.13	0.05	0.15	0.08	0.088
20:1n-9	0.2	0.1	0.2	0.1	0.305	20:1n-9	0.36	0.52	0.31	0.21	0.561
20:2n-6	0.2	0.1	0.2	0.1	0.725	20:2n-6	0.39	0.30	0.42	0.26	0.512
20:3n-6	0.9	0.2	0.9	0.2	0.361	20:3n-6	1.39	0.74	1.58	1.09	0.334
ARA (20:4n-6)	6.2	2.4	4.7	1.7	**0.001**	ARA (20:4n-6)	9.05	4.77	7.98	4.71	0.275
DPA (22:5n-3)	0.4	0.2	0.6	0.2	**<0.001**	DPA (22:5n-3)	0.69	0.54	1.10	0.79	0.004
Total SFAs	34.4	2.4	34.9	2.4	0.247	Total SFAs	58.52	44.84	63.17	41.43	0.605
Total MUFAs	40.7	4.2	40.0	3.7	0.360	Total MUFAs	70.28	61.00	72.59	48.25	0.840
Total PUFAs	24.9	4.6	25.1	4.1	0.849	Total PUFAs	40.25	26.19	44.70	27.36	0.425
Total n-3 PUFAs	4.4	1.5	6.4	2.1	**<0.001**	Total n-3 PUFAs	6.80	4.08	10.81	6.35	**0.001**
Total n-6 PUFAs	20.5	3.5	18.7	2.8	**0.009**	Total n-6 PUFAs	33.45	22.97	33.89	21.63	0.924
						Total TAG fatty acids	169.05	129.89	180.46	115.40	0.656
						ARA/DHA	2.06	0.79	1.14	0.70	**<0.001**
						ARA/EPA	20.38	7.94	9.71	4.92	**<0.001**
						ARA/ (EPA+DHA)	1.85	0.69	1.01	0.60	**<0.001**
						n-6/n-3	5.12	1.82	3.28	1.40	**<0.001**

Values are mean (SD) percentage (%) of total fatty acids and absolute concentration (µg/mL) in TAG; independent samples t-test p-values

Total cord plasma EPA, DHA, ARA, and DPA concentrations

Table 4.9 describes the total cord plasma EPA, DHA, DPA, and ARA concentrations calculated by summing up the concentrations of each of these fatty acids in the four lipid fractions (i.e. PC + CE + NEFA + TAG). Independent samples t-test between the groups showed that total plasma EPA, DHA, and DPA concentrations in the cord plasma were higher in the salmon group. Total plasma ARA concentration was not different between the two groups. Irrespective of the group difference, the concentration of DHA was much (approx 10-fold) higher in the cord plasma compared to EPA.

Table 4.9: Total cord plasma concentrations (µg/mL) of the 4 fatty acids of interest

Fatty acid	Control group (n=32)		Salmon group (n=35)		p
	Mean	SD	Mean	SD	
EPA	4.92	1.77	7.09	2.66	**<0.001**
DHA	53.49	17.23	62.24	19.06	**0.05**
ARA	165.81	36.69	163.13	40.22	0.78
DPA (n-3)	5.13	1.83	6.31	2.31	**0.02**

Values are mean (SD) absolute concentration (µg/mL); independent samples t-test p-values

4.3.2.2 Fatty acid composition of cord blood mononuclear cells (CBMCs)

Table 4.10 describes the fatty acid composition of CBMCs, expressed as percentages (%) of each fatty acid, for the two groups. Independent t-tests between the 2 groups were conducted and the results showed that some fatty acids tended to follow the same pattern as in the plasma lipid fractions (Section 4.3.2.1), but no significant differences between groups were observed. EPA, DHA and total n-3 PUFAs tended to be higher in CBMCs in the salmon group, while the ratios ARA/DHA and ARA/(EPA+DHA) tended to be higher in the control group. The ratio of n-6 PUFA/n-3 PUFA was higher in the control group compared to the salmon group.

Table 4.10: Cord blood mononuclear cell (CBMC) fatty acid composition according to study group

Fatty acid	Control group (n=40)		Salmon group (n=40)		p
	Mean	SD	Mean	SD	
EPA (20:5n-3)	0.3	0.3	0.4	0.3	0.339
DHA (22:6n-3)	3.8	1.4	4.3	0.9	0.110
ARA (20:4n-6)	17.5	3.9	17.7	3.4	0.843
14:00	2.2	2.2	2.1	1.7	0.755
16:00	24.9	2.9	24.7	2.3	0.711
16:1n-7	1.3	0.9	1.1	0.8	0.517
18:00	22.1	4.6	21.9	3.7	0.799
18:1n-9	13.9	2.6	13.5	2.2	0.459
18:1n-9Trans	2.7	2.6	2.9	2.4	0.695
18:2n-6	6.0	2.9	5.4	1.6	0.248
20:00	0.7	0.7	0.6	0.4	0.590
20:1n-9	0.7	1.4	0.9	1.4	0.507
20:2n-6	0.4	0.4	0.6	0.4	0.093
20:3n-6	2.5	1.0	2.9	2.2	0.310
DPA (22:5n-3)	0.9	0.5	1.0	0.5	0.190
Total SFAs	49.9	5.5	49.3	4.3	0.550
Total MUFAs	18.6	4.0	18.5	3.3	0.915
Total PUFAs	31.5	4.8	32.2	4.2	0.458
Total n-3 PUFAs	5.1	1.8	5.7	1.3	0.069
Total n-6 PUFAs	26.4	4.5	26.5	4.2	0.910
ARA/DHA	5.1	1.9	4.4	1.5	0.071
ARA/EPA	94.7	78.2	71.3	53.7	0.125
ARA/(EPA+DHA)	4.7	1.8	4.0	1.4	0.080
n-6 PUFA/n-3 PUFA	5.8	2.1	4.9	1.4	0.029

Values are mean (SD) percentage (%) of total fatty acids; independent samples t-test p-value

4.3.3 Relationships between fatty acids in different pools

4.3.3.1 Correlations between LC PUFAs in maternal plasma PC (%), cord plasma PC (%) and CBMCs (%)

Figure 4.11 to Figure 4.13 present correlations between maternal plasma PC fatty acids at 38 weeks gestation, cord plasma PC fatty acids, and CBMC fatty acids. Figure 4.11 shows that the percentages of EPA, DPA, DHA, and ARA in maternal plasma PC at 38 weeks gestation correlate significantly with the percentages of those fatty acids in cord plasma PC. Figure 4.12 shows that the percentages of EPA, DPA, DHA, and ARA in cord plasma PC correlate significantly with the percentages of those fatty acids in CBMCs. Figure 4.13 shows that the percentages of DPA and DHA in maternal plasma PC at 38 weeks gestation correlate significantly with CBMC percentages of those fatty acids; this was not found for EPA or ARA.

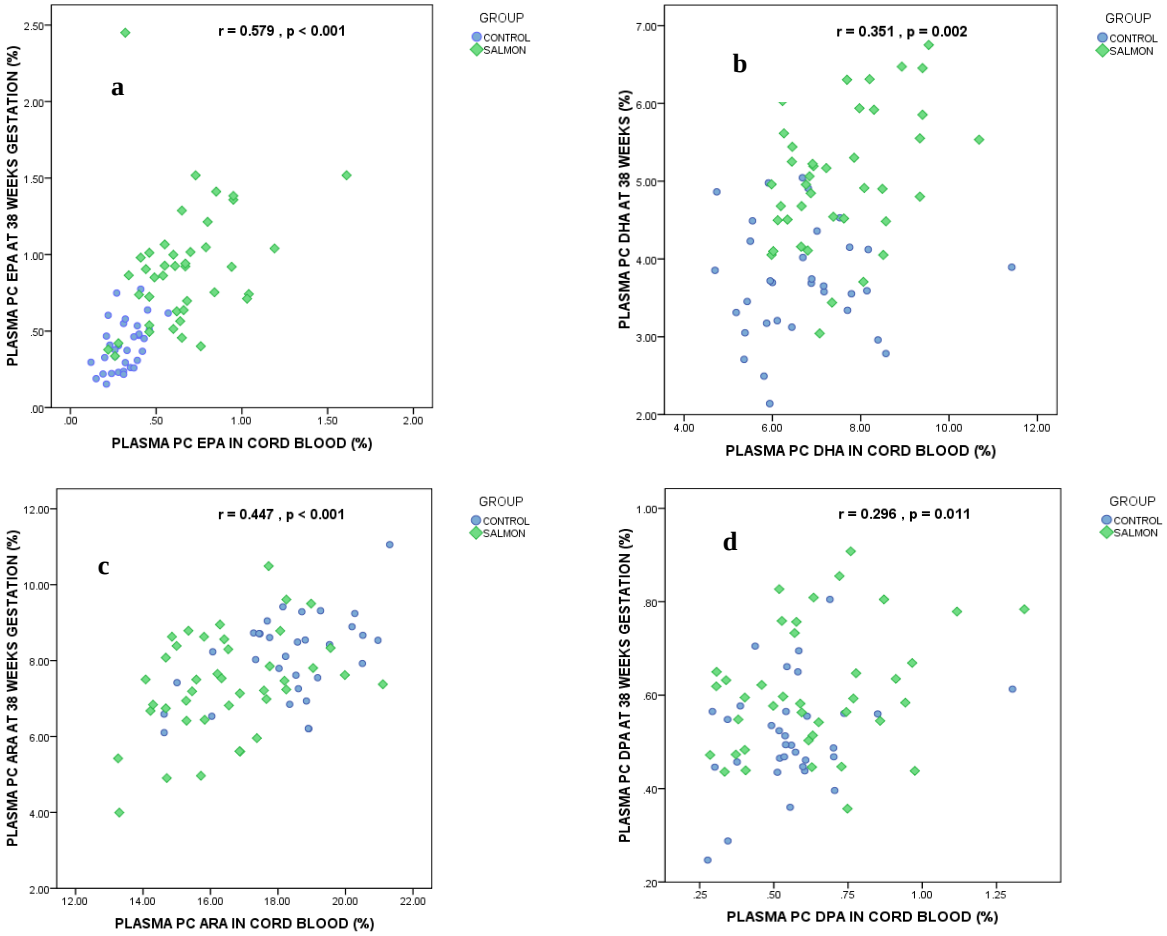


Figure 4.11: Correlations between maternal plasma PC fatty acids (%) at 38 weeks gestation and cord plasma PC fatty acids (%): a) EPA, b) DHA, c) ARA, d) DPA

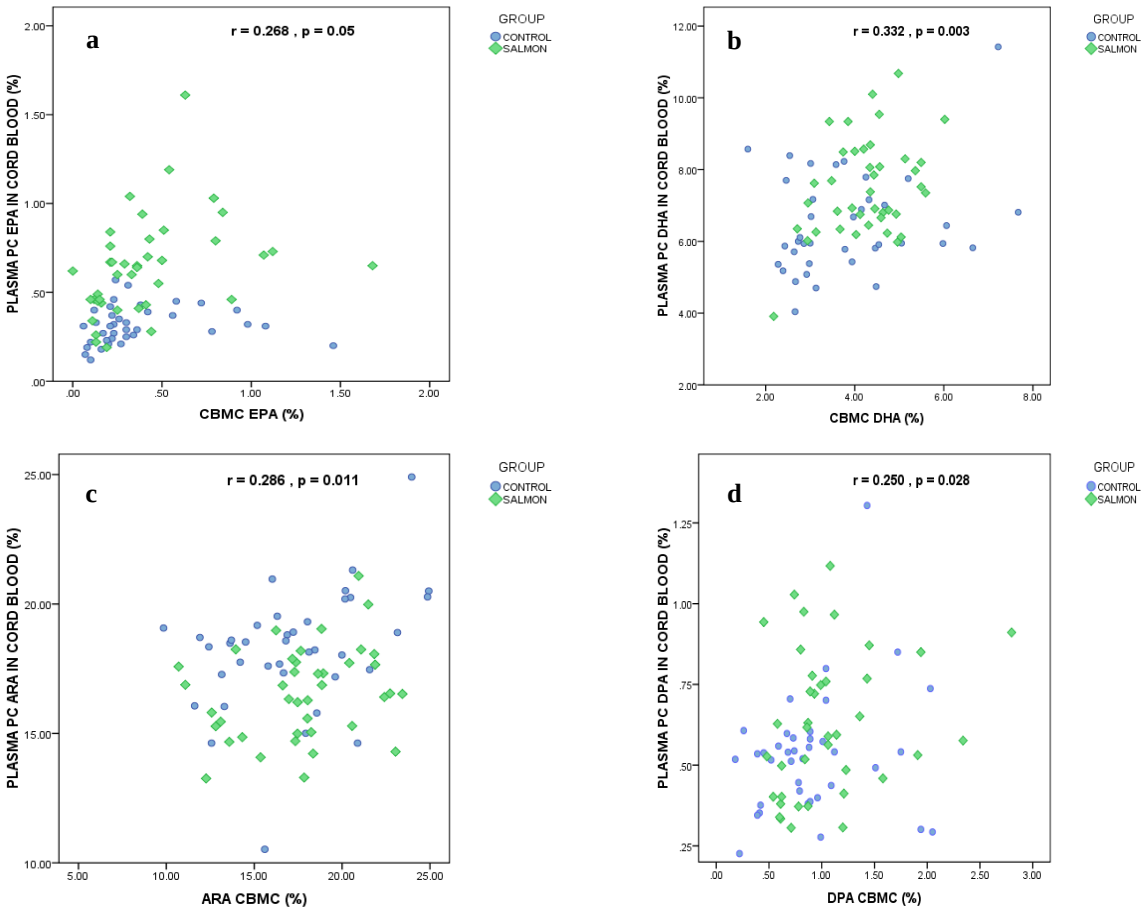


Figure 4.12: Correlations between cord plasma PC fatty acids (%) and CBMC fatty acids (%): a) EPA, b) DHA, c) ARA, d) DPA

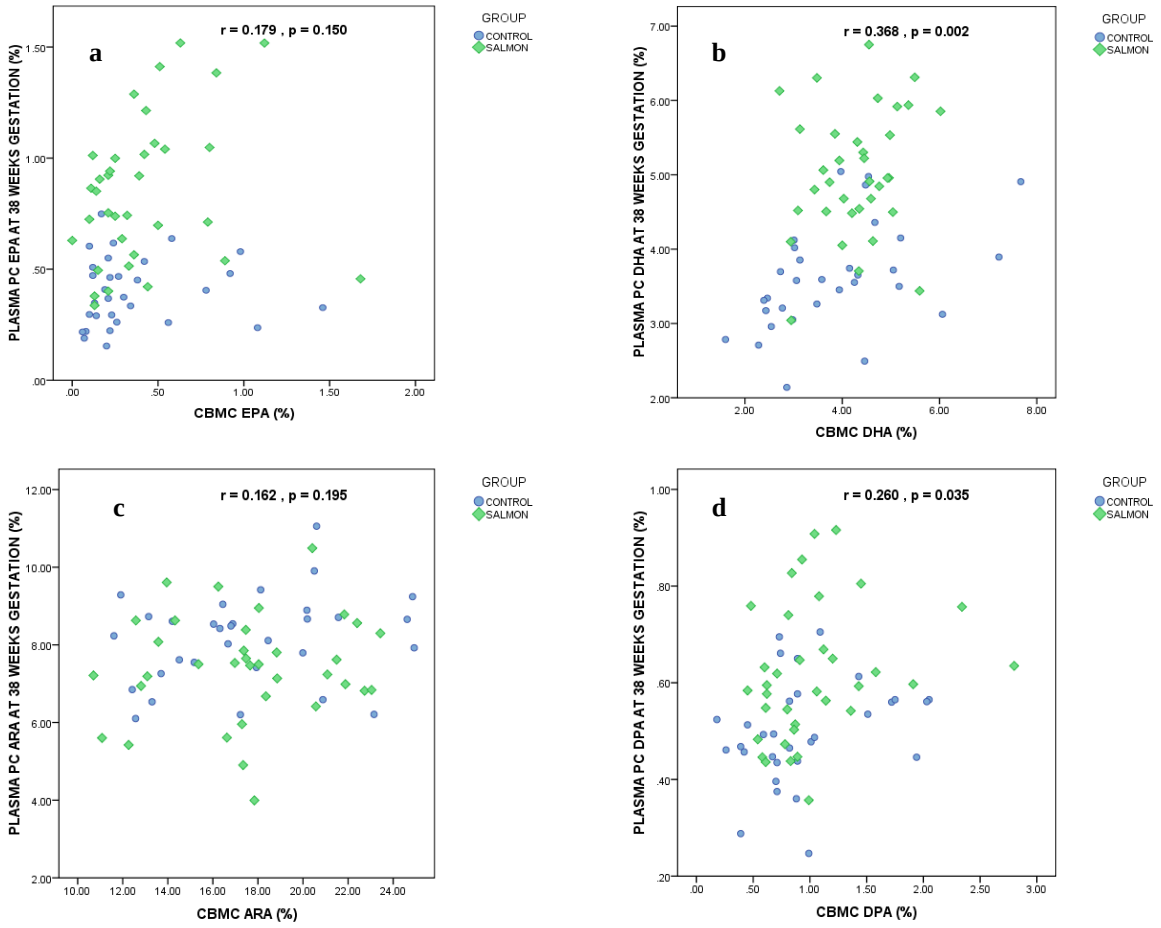


Figure 4.13: Correlation between maternal plasma PC fatty acids (%) at 38 weeks gestation and CBMC fatty acids (%): a) EPA, b) DHA, c) ARA, d) DPA

4.3.3.2 Correlations between cord fatty acids and birth data and pregnancy outcomes

Figure 4.14 shows the highly significant positive correlation between the DHA status in the cord blood both in plasma PC (expressed as percentage and absolute concentration) CBMCs with the duration of gestation. Gestational duration was not correlated with the maternal PC LC PUFAs (% of total fatty acids) at either 34 weeks or 38 weeks of gestation.

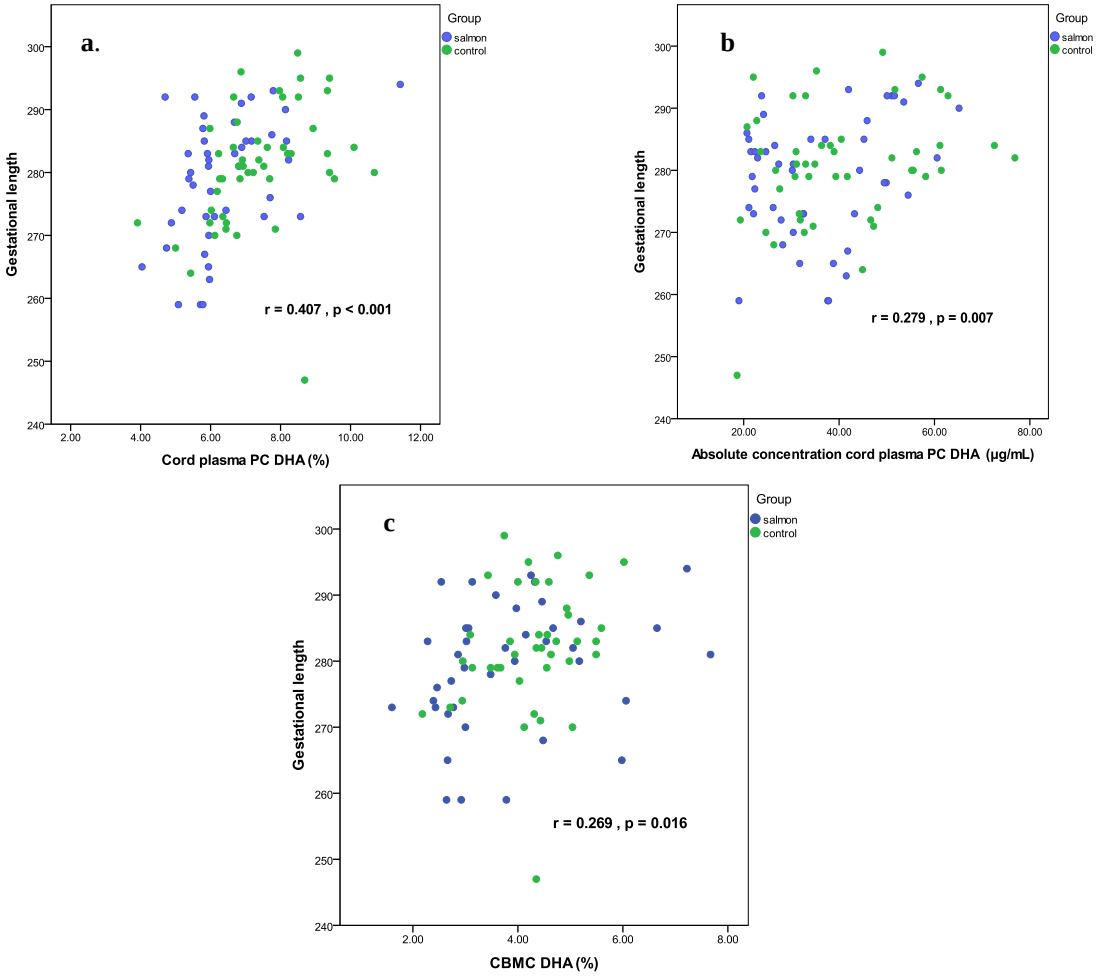


Figure 4.14: Correlation between gestational duration (days) and a) cord plasma PC DHA %, b) cord plasma PC DHa (µg/mL), c) CBMC DHA %

Correlations were performed between the cord blood LC PUFAs (EPA, DHA, ARA) and birth data (birth weight, head circumference, infant length). Of those the only correlation close to significant was the negative correlation between cord blood plasma PC ARA (%) (not in the other lipid fractions or CBMCs) and infant length (r=-0.232, p-value=0.061).

4.3.3.3 Correlations between LC PUFA intake during pregnancy (at 34 weeks gestation) and LC PUFA status in the cord fatty acid status

For both study groups EPA, DHA and ARA intake from the total diet at 34 weeks gestation (calculated from the FFQ) were not correlated with maternal plasma PC EPA, DHA or ARA percentages (%) at 34 weeks gestation. EPA, DHA and ARA intake from the total diet were not correlated with any of the LC PUFAs (%) in cord plasma PC or NEFAs. However, there were a number of significant positive correlations between the dietary intakes of EPA, DHA and ARA from the total diet and the absolute concentrations of various LC PUFAs in various cord plasma lipid fractions. Among these correlations were:

- Dietary EPA with cord plasma EPA in NEFAs (r=0.335, p=0.002)
- Dietary EPA with cord plasma TAG EPA (r=0.322, p=0.003)
- Dietary DHA with cord plasma DHA in NEFAs (r=0.590, p<0.001)
- Dietary DHA with cord plasma TAG DHA (r=0.251, p=0.023)
- Dietary ARA with cord plasma ARA in NEFAs (r=0.247, p=0.026)

- Finally, EPA and DHA from the total diet at 34 weeks gestation correlated positively with CBMC EPA (r=0.260, p=0.029 for EPA vs. EPA; r=0.267, p=0.024 for DHA vs. EPA).

EPA, DHA and ARA intake from the total diet at 34 weeks gestation (calculated from the FFQ) were not correlated with maternal plasma PC EPA, DHA or ARA percentages (%) at 34 weeks gestation. EPA, DHA and ARA intake from the total diet were not correlated with any of the LC PUFAs (%) in cord plasma PC or NEFAs for the control group. For the salmon group DHA intake from total diet at 34 weeks was significantly correlated with % DHA in cord plasma NEFAs (r=0.318, p=0.042). EPA, DHA and ARA intakes from the total diet at 34 weeks gestation correlated with absolute concentrations (AC) of EPA, DHA and ARA in the NEFA and TAG fractions in cord blood for salmon and control groups and with the PC fraction only for the control group. These correlations were:

For the control group:

- Dietary EPA and DHA with cord plasma PC EPA (for EPA: r=0.388,p=0.012; for DHA: r=0.384, p=0.013)
- Dietary EPA, DHA and ARA with cord plasma NEFA EPA (for EPA: r=0.487, p=0.001; for DHA: r=0.488, p=0.001; for ARA: r=0.439, p=0.004), NEFA DHA (for EPA: r=0.649, p<0.001; for DHA: r=0.636, p<0.001; for ARA: r=0.332, p=0.034) and NEFA ARA (for EPA: r=0.712, p<0.001; for DHA: r=0.696, p<0.001; for ARA: r=0.398, p=0.034)
- Dietary EPA and DHA with cord plasma TAG EPA (for EPA: r=0.353, p=0.024; for DHA: r=0.354, p=0.023), TAG DHA (for DHA: r=0.636, p<0.001) and TAG ARA (for EPA: r=0.422, p=0.006, for DHA: r=0.404, p=0.009)

For the salmon group:

- Dietary EPA, DHA and ARA with cord plasma NEFA EPA (for EPA: r=0.339, p=0.003;for DHA: r=0.329, p=0.042), NEFA DHA (for EPA: r=0.621, p<0.001; for DHA: r=0.589, p<0.001; for ARA: r=0.362, p=0.02) and NEFA ARA (for EPA: r=0.563, p<0.001; for DHA: r=0.518, p=0.001)
- Dietary EPA, DHA and ARA with cord plasma TAG EPA (for EPA: r=0.428, p=0.005; for DHA: r=0.402, p=0.009, for ARA:r=0.319, p=0.042), TAG DHA (for EPA: r=0.338, p=0.031, for DHA: r=0.316, p=0.044) and TAG ARA (for EPA: r=0.504, p=0.001, for DHA: r=0.490, p=0.001, for ARA: r=0.411, p=0.008)

4.4 Discussion

4.4.1 Discussion of my findings

SIPS is the first randomised controlled trial with oily fish in pregnant women. In this chapter, the effect of the salmon intervention on the fatty acid composition of maternal plasma PC and of cord plasma lipids and immune cells is reported. The LC n-3 PUFAs of interest (EPA and DHA) have an important role in development and in the health of the fetus and neonate. The hypotheses of the work described in this chapter are that the maternal oily fish intervention will result in higher amounts of EPA and DHA in maternal PC, and in neonatal (cord) plasma lipids, and will result in higher amounts of EPA and DHA in CBMCs. It is very clear that the data obtained support the first of these hypotheses (i.e. in the salmon group EPA and DHA in maternal plasma PC increased and were higher than in the control group and EPA and DHA were higher in cord plasma lipids in the salmon group). However, the data obtained do not support the second hypothesis, in that EPA and DHA were not significantly higher in CBMCs in the salmon group. Nevertheless higher cord plasma PC DHA was associated with higher CBMC DHA, suggesting that as supply of this fatty acid to the fetus increases (e.g. by increased oily fish consumption) then there is enhanced incorporation into immune cells.

The results of the maternal plasma PC fatty acid analysis provide biochemical proof that the intervention (i.e. increased salmon consumption) had been successful and that the subjects had complied to it.

Regarding the maternal plasma PC fatty acid data, at baseline (20 weeks gestation) the salmon group had higher percentages of EPA, DHA and total n-3 PUFAs and a lower percentage of ARA compared to the control group. This baseline difference in LC n-3 PUFA status matches with the baseline difference in fish consumption reported in chapter 3. Although the subjects were randomly assigned to the two groups at study entry, this baseline difference is a limitation of SIPS study.

The measurements of maternal plasma PC fatty acids represent the effect of fairly short term changes in diet; others have reported measurement of fatty acid status in erythrocytes during pregnancy, as these reflect longer term diet effects (they have a lifespan of 120 days in blood

circulation) (174). For SIPS, fatty acids in maternal immune cells and erythrocytes have been analysed, in order to better capture longer term changes in diet, but these findings are not reported in this thesis.

In the control group, maternal plasma PC EPA, DPA, DHA, and ARA declined over the course of pregnancy. These results suggest that the mother is progressively depleted in these fatty acids (especially DHA and ARA), as they are transported to the fetus to meet the demands for growth and development. This decline in maternal LC PUFA status over the course of pregnancy has been demonstrated previously and the extent of the decline in both DHA and ARA seen here is consistent with this earlier work (258;259). This gradual decrease is a normal phenomenon of pregnancy, and might reflect the maternal inability to obtain or to mobilise adequate amounts of these fatty acids for optimal fetal development The decrease in EPA may also be associated with its metabolism to DPA and eventually to DHA which is highly demanded in the fetus (58;253). The pregnancy-associated depletion of maternal DHA and ARA reflects the low dietary intake of these LC PUFAs and the possible maternal inadequacy to provide optimal amounts to the fetus.

In contrast to the control group which had a mean daily intake of about 30 mg of EPA plus DHA from the total diet, the salmon group, which had a mean daily EPA+DHA intake of over 400 mg from the total diet, the maternal status of EPA, DPA and DHA (expessed as % of fatty acids) did not decline in the salmon group and was higher than in the control group. This is the first time that this effect of maternal salmon consumption in pregnancy has been demonstrated. A higher LC n-3 PUFA status in maternal blood may mean that the mother can supply increased amounts of these important fatty acids to the fetus. This may be important for fetal growth and development and also might play a key role in the developmental plasticity of the fetal immune system (202).

The salmon intervention enhanced the pregnancy associated decline in ARA in maternal plasma PC. This may not be a desirable effect as ARA has important roles in fetal growth and develolment too (10;243;263).

The higher LC n-3 PUFA status of the women in the salmon group is also important because it may indicate better capacity to store these fatty acids for later supply to the neonate through breast milk.

The results of the cord plasma fatty acid analysis have been presented in two ways: quantitatively (μg/mL plasma) and qualitatively (% of total fatty acids). Percentages reflect the level of each fatty acid relative to the rest of the fatty acids in the lipid fraction studied. This means that an increase in one fatty acid will be compensated by a decrease in % of at least one other fatty acid. It is worth noting that the use of fatty acid percentages is the most common way of presenting this sort of data in the literature. On the other hand the absolute concentration of a fatty acid is not relative or dependent to the concentration of other fatty acids and therefore a change in the concentration of one fatty acid is not necessarily followed by a change in the concentration of any other. It must also be realised that a change in the absolute concentration of one fatty acid does not mean that there will be a change in the percentage of the same fatty acid and vice versa. The inclusion of both expressions in SIPS is a strength of the study because the combination provides complete information of the differences between the groups. In general greater and more significant effects were observed when the percentage data were used.

This is the first study to show that a dietary intervention with fish during pregnancy increases LC n-3 PUFA status of the fetus/neonate. Higher cord erythrocyte LC n-3 PUFAs have been reported following fish oil supplementation in pregnant women (174).

DHA and ARA in the cord plasma PC were much higher than in the maternal plasma PC (at 38 weeks). DHA was on average 57% higher and ARA 219% higher in cord plasma PC (Figure 4.15). These findings agree with those from previous studies that found that relative amounts of ARA and DHA in umbilical cord blood were significantly higher than in maternal blood (258;264). This might point to a high neonatal need for ARA and DHA which are the most abundant n-6 and n-3 PUFAs in neural and retinal tissue, respectively (and also in immune cells). These high proportions could also be attributed to de novo synthesis by the fetus, although the capacity of the fetal liver to synthesise such LC PUFAs is limited (265). It is more probable that preferential transport through the placenta is favouring the transfer of these fatty acids to the fetal circulation, making them available to developing tissues like brain and retina (253;266). The study of Berghaus *et al*. showed that the fetus obtains fatty acids from a combination of de novo synthesis, a passive gradient dependent transplacental passage of NEFAs and a selective materno-fetal placental transport for certain fatty acids, such as physiologically important LC PUFAs (254).

The calculation of total concentrations of EPA, DPA, DHA, and ARA across all four plasma lipid fractions showed higher concentrations of the three LC n-3 PUFAs in the salmon group but no difference in ARA, although the relative amount of ARA was lower in the salmon group.

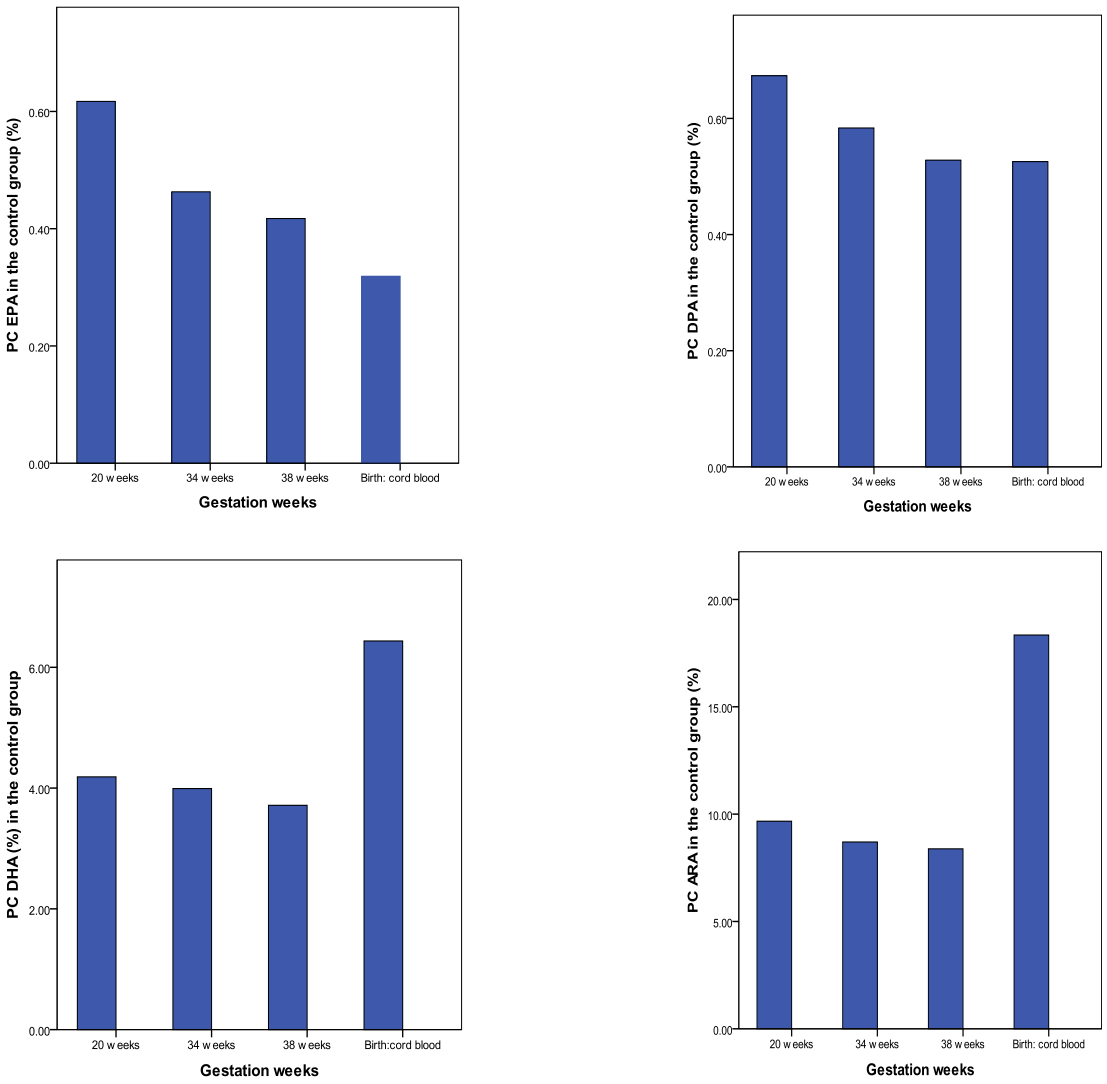


Figure 4.15: Mean relative contributions of EPA, DPA, DHA and ARA (% of total fatty acids) to maternal plasma PC throughout pregnancy (20, 34, 38 weeks of gestation) and to umbilical cord plasma PC in the control group. Data are taken from Table 4.2 and Table 4.5

The positive correlations found between the DHA status in the cord blood [plasma (expressed as percentage and absolute concentration) and mononuclear cells] with gestational length is a very important finding. This result is supported by evidence in the literature that relates

maternal dietary intake of DHA (and ARA) with gestational length and also infant characteristics at birth (birth weight, head circumference) (39;243;248;263).

Increased production of PGE2 by decidual macrophages has been shown to regulate labour initiation at birth (267).There is evidence that a high intake of long chain (n-3)-fatty acids prolongs gestation in humans by reducing uterine prostaglandin formation, possibly by inhibiting formation of prostaglandin F2 alpha and prostaglandin E2 (39;268).

Longer gestational length could be mediated by altered arachidonic acid-derived-prostaglandin production in the mother, since it was observed that matenal PBMC PGE_2 production was lower in pregnant women in the salmon group (unstimulated cells, 34 weeks gestation) (p=0.023) (as shown in the thesis of L-S Kremmyda) and PGE2 production tended to be lower in CBMC after stimulation with LPS (p=0.056) (Chapter 5).

4.4.2 Comparison with the findings of other studies

4.4.2.1 Fatty acid status at birth

Previous studies have shown variations in LC n-3 PUFA status in women during pregnancy and in infant blood and this was supported by a review reporting moderate variations in DHA and ARA status in cord blood lipids in different populations (269).

Mattoras *et al*. assessed in a cross-sectional study in Spain the relationship between intake of LC n-3 PUFAs during pregnancy (162 mother–neonate pairs from normal at-term pregnancies) and the levels of these fatty acids in maternal and neonatal plasma lipids and erythrocytes (270). The n-3 PUFA intake assessed by interview was significantly correlated with n-3 PUFA levels in the plasma of both mothers and neonates. The levels of n-3 PUFAs in mothers and neonates were significantly correlated both in plasma fatty acids (expressed both as a percentage and as absolute values) and in erythrocyte phospholipids (percentages). In conclusion, in a well-nourished population the n-3 PUFA levels of the newborn infants are clearly influenced by those of their mothers and the higher the levels in mothers, the higher those in the neonates (270). Observations made in this chapter are in accordance with this.

De Vriese *et al*. described dietary fat intake during pregnancy (by FFQ) and the relationship between the intake of PUFAs and the fatty acid composition of maternal and umbilical plasma

PC and CEs at delivery. Maternal and umbilical blood samples were collected at delivery from 30 healthy pregnant women. Meat and poultry contributed most to the intake of ARA whereas fish was the major source of EPA and DHA in the diet. EPA and DHA (%) of maternal plasma PC were positively related to the intake of these fatty acids during pregnancy. EPA and the sum of n-6 PUFAs (%) in cord plasma PC were positively correlated with the dietary intake of these fatty acids (256). In SIPS, EPA and DHA dietary intake measured at 34 weeks gestation, was not correlated positively with those fatty acids (%) in cord plasma PC. Dietary EPA intake was correlated positively with DHA (r=0.219, p-value=0.048) in cord plasma PC (absolute concentrations).

Donahue *et al.* recently assessed second trimester maternal diet with FFQs (n =1666), mid-pregnancy maternal erythrocyte PUFA concentrations (n =1550), and umbilical cord plasma PUFA concentrations (n =449) in the US (271). Mean maternal intake of total n-3 PUFAs was 1.17 g/d and of EPA+DHA 0.16 g/d. In SIPS, at 20 weeks gestation mean maternal intake assessed by FFQ of total n-3 PUFAs in the control group was 1.8 g/day and in the salmon group 1.9 g/day, while EPA+DHA was 0.05 g/day for the control group and 0.125 g/day for the salmon group. Compared to the study of Donahue *et al.* conducted in the US, pregnant women in the UK (SIPS control and salmon groups) have similar total n-3 PUFA intake and similar EPA plus DHA intake (similar to the SIPS salmon group rather than the control). Mean maternal erythrocyte and cord plasma PC PUFA concentrations were 7.0% and 5.2% (total n-3) and 5.0% and 4.6% (EPA+DHA). In SIPS, at 20 weeks gestation (second trimester) mean maternal cord plasma PC PUFA concentrations were 5.8 % in the control group and 6.5 % in the salmon group (total n-3 PUFAs) and 4.8% and 5.4% (EPA+DHA) in the control and salmon group respectively. Again these values are similar to those reported by Donahue *et al*. (271). Mid-pregnancy diet–blood and blood–blood correlations were strongest for EPA+DHA (r = 0.38 for diet with maternal blood, r = 0.34 for diet with cord blood, r = 0.36 for maternal blood with cord blood). In SIPS, maternal diet (EPA plus DHA) was not correlated with maternal plasma PC fatty acids (expressed as percentages in PC fraction) or cord plasma PC EPA or DHA (%), but was positively correlated with CBMC EPA (%) (r=0.265, p-value= 0.026). This is a strong indication of the dependence of the fetus on the mother and how important the window of pregnancy and antenatal nutrition on determining fetal growth and child health may be.

Zhang *et al*. recently investigated maternal dietary intake of EPA and DHA (by FFQ) and the levels of these fatty acids in plasma PC of pregnant women and their neonates from river/lake, coastal and inland regions of China. The median maternal plasma PC levels of DHA and EPA were higher in the river/lake and coastal regions compared to the inland region. Neonates in the inland group had higher levels of ARA and lower levels of EPA and DHA in their cord plasma PC compared to those in the lake/river and coastal regions. In conclusion, there was a close association between dietary intake of EPA and DHA and the corresponding levels of these fatty acids in maternal plasma PC and maternal plasma EPA and DHA were also positively associated with the corresponding levels in cord plasma PC (272). In SIPS significant correlations were observed between maternal dietary EPA and DHA status and those fatty acids in cord blood plasma in PC, NEFAs, and TAGs (expressed as absolute concentrations and not percentages) as well as with those fatty acids in CBMCs (expressed as %). The most interesting is the positive correlations between dietary EPA and DHA (at 34 weeks gestation) with CBMC EPA (Section 4.3.3.3).

4.4.2.2 Supplementation studies during pregnancy

Although SIPS is the first study to examine oily fish consumption in pregnancy, there are several studies of maternal supplementation with fish oil as a strategy to increase EPA plus DHA intake.

The European trial by Kraus-Etschmann *et al*. showed that supplementation of pregnant women from gestation week 22 until delivery with a fish oil preparation (0.15 g EPA plus 0.5 g DHA/day) was associated with increased maternal plasma PC EPA (0.18% at 20 weeks to 0.53% at 30 weeks gestation) and DHA (5.75% at 20 weeks to 7.26% at 30 weeks) and subsequently greater cord plasma PC DHA compared to the placebo group (9.90% in the salmon group vs. 8.74% in the control group) (177). SIPS identified the same pattern of increase of EPA and DHA in maternal and cord plasma PC, although the extent of the increase seen was smaller in SIPS. This could be attributed to the fact that the EPA plus DHA weekly dose in the supplementation study (0.65 g x 7 = 4.55 g EPA+DHA) was higher compared to the dose of EPA plus DHA provided from fish in SIPS in a week (3.46 g EPA+DHA). Also, the size of the effect could be due to the fact that supplementation studies provide a constant

amount of selected nutrients on a daily basis in contrast to a food intervention that provides specific foods (in this occasion salmon) for certain times in the week.

A RCT, conducted in Australia by Dunstan *et al*. with LC n-3 PUFA supplementation via fish oil (4 g/day that provided 1.1 g/day EPA and 2.2 g/day DHA) in healthy pregnant women from 20 weeks gestation to delivery, showed increased maternal erythrocyte PC EPA and DHA percentages and significantly decreased ARA percentage compared to baseline. At birth, erythrocyte PC EPA and DHA percentages were significantly higher in the salmon group and ARA % was significantly lower in the fish oil group compared to the control (174). The size of the effect of this supplementation was large due to the high dose of EPA plus DHA provided: Dunstan *et al*. provided 21.7 g (3.1 g/day x 7 days) EPA+DHA per week from fish oil while SIPS provided 3.46 g (1.73 g/portion x 2 portions) EPA+DHA per week from oily fish (salmon).

Supplementation of healthy Norwegian women with 1.2 g/d DHA and 0.8 g/d EPA from gestation week 18 till delivery, led to higher DHA proportion in cord plasma than in the control group which received corn oil (247) in accordance to SIPS results. Although this study did not assess the maternal plasma DHA status, DHA was increased in breastmilk, showing the activation of maternal stores during lactation.

Consistent with the previous study, Velzing-Aarts *et al*., examining the effect of three low-dose fish oil supplements, observed an effect on higher neonatal EPA and DHA % of total cord plasma fatty acids after supplementation of pregnant women from the second trimester to delivery with 0.5-1 g of LC n-3 PUFAs per day (273).

A lower amount of DHA (200 mg/day) was administered to 50 healthy women in the UK from gestation week 15 until term. This increased DHA proportions in maternal plasma and erythrocytes but had no effect on cord plasma or erythrocyte fatty acid composition (274). In the study of Sanjurno *et al*., 20 pregnant women received 200 mg/day of DHA during the last trimester of pregnancy. DHA was significantly higher and ARA was significantly lower in plasma in the study group compared to the unsupplemented control group. The neonates showed no differences of LC n-3 and n-6 PUFAs between the two groups. The results showed that an oral supplementation with 200 mg/day of DHA during the final stages of pregnancy is

reflected in an increase in the plasma level of this fatty acid in the mother, but not the offspring (275).

In these latter two supplementation studies the dose of LC n-3 PUFAs used was closer to what SIPS delivered as 2 portions of salmon per week (equivalent of 165 mg/day DHA and 82 mg/day EPA (247 mg/day EPA plus DHA)). Although the "low dose" fish oil supplements did not find changes in fetal fatty acid status at birth, SIPS found such an effect of salmon. This may mean that the oily fish as a whole food (twice per week) is more effective than a low dose supplement on a daily basis.

In another RCT, by van Houwelingen *et al*., 23 Norwegian women were supplemented with 2.3 g/day of DHA from week 30 of pregnancy and this high dose supplementation resulted in increase both in maternal and subsequent neonatal DHA status (276).

From the results of the studies above and of SIPS it seems that dosage, and duration of supplementation/dietary intervention as well as habitual dietary fish consumption of the subjects could explain the variations in fetal DHA levels. When DHA stores are depleted, especially in low consumers of oily fish or less well nourished women, low dose DHA supplementation may primarily affect the fetal DHA status rather than the maternal, as DHA is selectively transported to the fetus by the placenta (266;277). However, a higher dose supplementation might be needed so as to satisfy the maternal stores as well. The European trial used intermediate amounts of DHA that succeeded in repletion of maternal and fetal DHA stores. This was in agreement with the study conducted by Velzing-Aarts *et al*. (273). On the other hand a very high dose supplementation may additionally help to sustain maternal n-3 PUFA stores during breastfeeding (175) but it is still unclear whether the improved fatty acid status of the offspring will have any functional effect on the developing infant such as growth, cognitive development or immunomodulatory effects.

Epidemiological studies have shown that neonates with higher levels of plasma phospholipid DHA had longer gestational length and birth weight (263) and this is suggested by some intervention trials (247;278) but not others (279). In agreement with those epidemiological studies, in SIPS there was a strong positive correlation between DHA status in cord blood (in plasma and CBMCs) and gestational duration. The salmon group had about 4 days longer

gestation than the control group, although this difference was not statistically significant (Chapter 2, Table 2.9; p=0.094).

Also a number of studies have demonstrated improved visual and other neurological outcomes particularly in preterm infants supplemented with DHA (10). However, in the latter study postnatal rather than antenatal supplementation with DHA was been associated with subtle differences in neurodevelopment outcomes. This suggests that longer term and larger intervention studies during the antenatal period are required (with fish oils and oily fish) so as to address the potential effects of fetal DHA enrichment on a variety of health issues and especially on immune outcomes (202;203).

4.5 Conclusion

Pregnancy is associated with changes in the blood lipid profile and in the fatty acid components of these lipids. The literature has shown that percentages of EPA and DHA in maternal plasma lipids decline during pregnancy. This was also observed in the control group in SIPS, probably related with preferential transfer of DHA thought the placenta to the growing fetus. SIPS, being the first randomised controlled trial with fish during pregnancy, showed that consumption of two portions of salmon per week (between week 20 until delivery) prevented the pregnancy associated decrease in percentages of EPA and DHA in the phospholipid (PC) fraction, and also resulted in increased levels of these LC n-3 PUFAs in maternal PC compared to the control group. Thus the consumption of 2 portions of salmon per week that was confirmed by the dietary assessments (FFQs and fish diaries) and provided an intake of over 400 mg/day EPA+DHA was proven also biochemically, by the increased status of those LC n-3 PUFAs in the mothers.

In the cord blood, significant changes in the status of LC n-3 (EPA and DHA) and n-6 (ARA) PUFAs were mostly observed in all lipid fractions when data was expressed as percentages rather than absolute amounts. The pattern observed in SIPS was that EPA and DHA status was increased significantly whereas the ARA status was decreased significantly in the cord blood plasma in the salmon group infants compared to those of the control group. A similar trend was found for those same LC PUFAs measured in the immune cells (CBMCs) but here the effect of group was not statistically significant. However in the next chapter (immune chapter) it is shown that these changes had some impact on the functionality of these cells (eicosanoid and cytokine production).

To conclude, the salmon intervention increased EPA and DHA concentrations in maternal plasma, and these fatty acids were successfully passed to the fetus in the cord plasma but not in the immune cells. The following chapter explores the immunomodulatory effect of this improved fatty acid composition in cord blood.

5 Salmon in pregnancy study (SIPS): The effect of salmon intervention on infant immune outcomes in cord blood

5.1 Introduction

The maturation of the fetal immune system depends on several maternal influences including the maternal immune response (1). Therefore, the focus of early life origins of immunity is on factors influencing maternal immune status and the subsequent effect on the immune response of the fetus (280). During pregnancy, immunological changes occur in the mother in order to suppress cell-mediated responses to fetal and paternal antigens which thus allows the normal progression of pregnancy (84). The maternal immune response is skewed during pregnancy towards type 2 helper T-cell (Th2) responses so as not to reject the fetus, and this Th2-dominant response is "transferred" to the fetus. Th1 responses normally up-regulate in the infant immune system during the first two years of life as a result of antigenic exposure. However, different temporal changes have been observed in the infants of atopic mothers, which show immature Th1 immunity and altered Th2 responses (82). Although Prescott *et al*. reported that newborns (irrespective of their atopy heritability) have skewed cord blood mononuclear cell (CBMC) Th2 cytokine responses (interleukin (IL)-4, IL-5, IL-6, IL-9, IL-3) to common environmental allergens and a high level of IL-10 production (regulatory cytokine) (82;112), other studies have failed to demonstrate this Th2 bias at birth and further have shown no association between maternal allergen exposure during pregnancy and CBMC responses (281;282). Smillie *et al.* reported no association between levels of Der p1 in the maternal mattress during pregnancy and CBMC proliferative responses after stimulation with Der p1 in 225 neonates participating in an allergen avoidance study in Manchester, UK (281). Similarly Miller *et al.* reported no association between measured levels of house dust mite, mouse and cockroach allergens during pregnancy and CBMC responses, while CBMC responses were observed in the absence of allergen induced maternal mononuclear cell responses (282).

Substantial immune development happens both *in utero* and post-natally (106), and it is possible that such development can be influenced by nutritional factors (58). However, relatively little attention has been devoted to the potential for early life programming of the immune system by dietary factors. The food consumed by the mother during pregnancy can contribute to the immunologic profile of herself and the offspring (70). During pregnancy, a diet enriched in n-6 polyunsaturated fatty acids (PUFAs), which are contained, for example, in margarine and vegetable oils, seems to be more likely to be associated with higher risk of

eczema in the child during the first 2 years than n-3 PUFAs, which are contained in fish, at least when consumed during the last 4 weeks of pregnancy (77;152). Possible mechanisms might be that higher levels of n-3 PUFAs are associated with less neonatal oxidative stress, lower production of inflammatory leukotriene (LT) B_4 and inflammatory cytokines, and enhanced activity of protein kinase c-z (inverse association with allergic diseases) in T cells, as shown in human (131) and murine (143) studies. On the other hand, not only the absolute content but also the ratio of n-6 to n-3 PUFAs might influence the development of either tolerance or sensitization to food allergens: in a rat model a ratio of 9:1 in the mothers' diet prevented tolerance induction in neonatal rats, which otherwise could be achieved when the diet was n-3 PUFA enriched (283). Fish oil supplementation during pregnancy has been shown to increase the levels of the long-chain (LC) n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in breast milk up to 6 weeks after delivery in a randomized controlled human trial (175). Other human studies showed that fish or fish oil consumption decreases atopic dermatitis risk (153) or at least the severity of atopic dermatitis in high risk children at 1 year of age (77). A body of mainly epidemiological literature has developed, which associates temporal changes in the patterns of intake of n-6 and n-3 PUFAs with temporal changes in the incidence and prevalence of atopic sensitisation or its clinical manifestations (allergies, atopic dermatitis, hayfever and allergic asthma), and there is a proposed molecular and cellular mechanism to explain the observed association (112).

5.1.1 PUFA hypothesis and atopic disease

There have been many reports associating an "abnormal" n-6 and n-3 PUFA exposure or composition to the occurrence of atopic diseases. The elongated and desaturated products of the essential fatty acids linoleic acid (LA) and α-linolenic acid (ALA) regulate inflammatory processes. Arachidonic acid (ARA) is a precursor of inflammatoery eicosanoids, and mediates chemotactic signals and smooth muscle cell contraction, while EPA, dihomo-γ-linolenic acid (DGLA) and DHA are known to be involved in suppressing inflammatory pathways. An abnormal PUFA composition has been demonstrated in cord blood (CB) serum, plasma and erythrocytes of infants at high risk for atopic diseases compared with CB of infants at no risk for atopy (129;130;148). One study reported an altered CB serum PUFA composition to be associated with the onset of atopic diseases after a follow-up of 1 year in a cohort of 57 newborns at risk (148). Another study reported abnormal proportions of n-3 and n-6 PUFAs in the serum of allergic mothers; in this study, a higher content of n-3 PUFAs was found in

women whose infants did not develop atopic disease during the first 6 years of life (130;149). The predictive value of PUFA exposure for atopy was investigated in a large UK cohort (Avon longitudinal study of parents and children) of over 1000 newborns (150). By analyzing ratios of different n-6 to n-3 PUFAs and their products in erythrocytes the authors could detect a significant association of alteration in ratios to wheezing and eczema although these associations were no longer significant after adjustment for multiple comparisons. The authors concluded that exposure to n-6 and n-3 PUFAs is not a likely predictive factor for an early onset of atopic diseases (150).

A recent review by Sala-Vila *et al*. summarized studies of fatty acid composition in relation to atopy (287). Some studies report elevated LA in atopy, while some studies report lower amounts of the n-6 PUFAs, including ARA, and of LC n-3 PUFAs in atopy, although observations on this were not consistent. These data did not support the hypothesis that atopy is associated with a high exposure to, and status of, n-6 PUFAs. Intervention studies with n-3 PUFAs in pregnant women, infants and children suggest some clinical benefits, although how long lasting these are remains to be determined. The authors proposed that the observation of low ARA (LC n-6 PUFA) status in atopy suggests that fish oil intervention (LC n-3 PUFAs), which targets ARA status and metabolism, may not be ideal and that a combination of fish oil with some LC n-6 PUFAs could be more efficient (284). Nevertheless, intervention trials with larger sample sizes and longer follow up period of the infants are needed so as to draw more definite conclusions.

Additionally, in the general introduction (Chapter 1) are explored the other two hypotheses associated with atopic disease: 'hygiene' hypothesis and 'antioxidant' hypothesis.

5.1.2 Mediators of atopy in cord blood and in childhood

Miles *et al*. observed significantly lower numbers of $CD25^+$ (activated) T cells in the cord blood of high risk babies who had developed both allergic symptoms and positive skin prick tests by one year of age when compared with the low risk group (285). $CD45RO^+$ (memory) T cells were detected in both high and low risk babies with a trend for lower numbers of memory cells to be detected in high risk infants who later developed allergic symptoms and/or positive skin prick tests. The significantly lower numbers of memory T cells in the high risk babies suggested a suppression of T cell activation or lack of antigenic priming in this group (285). Devereux *et al.* determined the CD45 isoform of neonatal Th cells that respond to

allergen stimulation (286). They demonstrated that 50% of CBMC proliferative responses after stimulation with timothy grass allergen are mediated by T cells expressing $CD45RA^{high}$ and 50% are mediated by T cells expressing the $CD45RO^{high}$ isoforms. It was concluded that timothy grass allergen specific fetal Th cells can be sensitised *in utero,* but that this priming does not occur in all individuals even if their CBMCs are able to respond to the allergen (286). This evidence questions the reliability of CBMC responses to allergen sensitisation *in utero* and the complexity of the relation between antenatal allergen exposure and *in utero* sensitisation.

Allam *et al*. have reviewed the role of soluble mediators of atopy in the serum or plasma of the patients in the pathophysiology of atopic diseases (287). A characteristic feature of atopy is a Th2-dominated immune response; Th2 related cytokines like IL-4, IL-5 and IL-13 are involved in the induction of IgE synthesis (84;288). Th2 related cytokines have also been analysed in CB. A recent publication reported that detectable levels of IL-4 and IFN-γ in CB of newborns were associated with a lower risk of developing allergic asthma and sensitization to some inhalant allergens after the follow-up period to 6 years (289).

Hinz *et al*. within the LINA (Lifestyle and environmental factors and their Influence on Newborns Allergy risk) mother-child study determined the frequency and function of Treg cells, cytokine production and the total IgE concentration in pregnant women in the 34^{th} week of gestation and in corresponding CB at birth. Because CB IgE has been shown to be predictive for allergic diseases in early childhood, these results indicated that reduced maternal Treg cell numbers and increased Th2 cytokine production during pregnancy might influence the allergy risk of the child (290). Balossini *et al.* found that in newborns of allergic fathers IL-10 levels tended to be lower and transforming growth factor (TGF)-β1 levels were significantly lower than in newborns of non-allergic parents (291). Schaub *et al*. investigated Treg cells in CB in the context of maternal atopy. In offspring of atopic mothers, Treg cell numbers, expression, and function were impaired at birth. Th17 cells correlated with Th2 cells, independently of maternal atopy (292). Smith *et al*. found that children with egg allergy (at 12 months) had evidence of reduced neonatal $CD4^{+}CD25^{+}CD127^{low}$ Treg cell function. This study confirmed the presence and activity of Treg cells in CB and provided preliminary evidence of differences in neonates who progress to allergic disease in the first year of life (293).

5.1.2.1 Cytokine production in cord blood mononuclear cells

Lappalainen *et al*. found that there is maturation of cytokine production from birth to the first year of age. Stimulated cytokine responses were higher in mothers' than in children's blood samples; children of food sensitised mothers had elevated cord blood IL-10 responses and a decreased IFN-γ/IL-5 ratio. The results suggested that both adaptive and innate immune responses increased from birth to 1 year of age, although they are weak when compared to the adult response (294). Warner *et al.* found reduced IFN-γ secretion in neonates that had a high risk of atopy (295), and this reduced IFN-γ secretion was significantly associated with future atopic dematitis (81). Liao *et al.* observed decreased production of IFN-γ and increased production of IL-6 by CBMCs from newborns with a high risk of allergy. $CD4^+$ T cells in the presence of monocytes and isolated monocytes from the high-risk group produced a much greater amount of IL-6, either spontaneously or after stimulation, than did those of the low-risk group; $CD4^+$ T cells from the low-risk group produced a significantly greater amount of IFN-γ than did those from the high-risk group; cord blood basophils stimulated with PHA could produce a significant amount of IL-4; there was an inverse correlation between the production of IFN-γ and cord blood IgE level and the number of natural killer (NK) cells was significantly lower in high-risk group than the low-risk group. In conclusion this data suggested increased production of IL-6 and decreased production of IFN-γ by CBMCs appear to be the characteristic of newborns with high risk of allergy (296).

Many studies have focused on the relevance of Th2 cytokine production in neonates for the development of atopic disorders in early infancy with sometimes conflicting results. In this context, the predictive value of the Th2 cytokine IL-13 as an early marker for atopy has been investigated. Williams *et al.* showed that babies at risk of atopic disease in infancy demonstrate defective IL-13 production at birth (297). This might represent an inherent immaturity in the development of T cell-cytokine responses in babies at genetic risk for atopy or could be a consequence of downregulation of responses by other factors. Normal pregnancy, irrespective of atopic status, is associated with the production of appreciable quantities of IL-13 initially by the placenta and subsequently by the fetus (297). Another study reported an association between newborns producing elevated IL-13 levels and increased risk of developing atopic symptoms (298). Boyle *et al.* found that infants at high risk of allergic disease had a reduced percentage of $CD14^+$ monocytes and reduced CD14 mean fluorescence intensity on mononuclear cells. They also had a decreased dendritic cell (DC) percentage in

mononuclear cells cultured with ovalbumin (OVA) or lipopolysacharide (LPS) as compared with infants at intermediate or low risk of allergic disease. No relationship was seen between risk of allergic disease and toll-like receptor (TLR) 2 or TLR4 expression, or FoxP3 expression in cultured cells. In conclusion, infants with a biparental history of allergic disease had altered markers of innate immunity at birth, with reduced expression of membrane bound CD14 and consequently reduced in vitro development of DCs. Further work is needed to understand the role that these alterations play in the pathogenesis of allergic disease, and whether interventions to up-regulate fetal CD14 expression can prevent allergic disease (299).

IL-10 and IL-5 responses at birth predict risk for respiratory infections in children with atopic family history. Zhang *et al.* measured cord blood T-cell capacity to produce IL-10 and IL-5, and related these to subsequent infection history (300). IL-10 and IL-5 were associated, respectively, with resistance or susceptibility to infections. The greatest contrasting effects of these two cytokines were seen when they were considered in combination by generating IL-10/IL-5 response ratios for each subject. The low IL-10/high IL-5 T-cell response phenotype was strongly associated with susceptibility to all grades of acute respiratory infection, relative to the more resistant high IL-10/low IL-5 phenotype. In conclusion, excessive production of IL-5 by T cells at birth was associated with increased risk for subsequent severe respiratory infections, and this risk was attenuated by concomitant IL-10 production (300).

Innate immune responses early in life are critical for the development of allergic illnesses. The underlying mechanisms remain unknown, but include T-cell responses either along Th1/Th2 pathways or via Treg and Th17 cells. Schaub *et al.* investigated the impairment of T helper and Treg cell responses at birth (292). The aim of this study was to investigate T-cell responses to innate and adaptive stimuli at birth and to compare these findings with adult immune responses. Proliferation and cytokine responses to innate stimuli were less mature at birth than in adulthood. Treg and Th17 cells were less expressed in cord than in adult blood. Mitogen-induced suppression of Treg cells on T-effector cell function was less efficient in cord than in adult blood. At both ages, Th17 cells were correlated with Th1/Th2 cells, but not with IL-10 secretion following innate-stimulation. So, innate immune responses were immature at birth. Furthermore, the functions of Treg and Th17 cells were impaired. Th17 cells in association with Th1/Th2 cells may be involved in early immunomodulation. Potent innate immune stimulation early in life can potentially contribute to protection from allergic diseases (292).

5.1.2.2 Cord blood IgE production

Atopic disorders are closely linked to elevated serum IgE levels in early life. Taking positive atopy family history into consideration, the percentage of elevated CB IgE concentrations increased with the number of close family members suffering from atopic diseases (287). Karmaus *et al.* found that IgE is reduced with increasing birth order. The findings suggested that cord IgE is reduced in pregnancies with higher order, indicating that the sibling effect upon atopy may have its origin *in utero* (301). Liu *et al*. (302) suggested that maternal, but not paternal, total IgE level correlated with elevated infant IgE levels and infant atopy. This provided a high specificity (83%) and a sensitivity of 34% for prediction of infant atopy. Thus, maternal factors, placental factors, or both have an impact on perinatal allergic sensitization. Kaan *et al.* suggested that cord blood IgE is a significant risk factor for the development of urticaria due to food allergy at 12 months of age. As urticaria due to food allergy is a prodrome for anaphylaxis, measurement of IgE in cord blood may be indicated in infants at high risk for developing allergic diseases so that preventive measures can be applied (303).

Ferguson *et al*. recently found that maternal atopy and birth in winter months were risk factors associated with detectable CB IgE. CB IgE was found to be significantly associated with allergic sensitization and recurrent wheeze at 7 yrs but not with other outcomes. CB IgE may be a useful measure for identifying children at high risk of atopic diseases for the purpose of primary prevention (304). Pesonen *et al*. found after 20 years follow up of 200 newborns that the combination of elevated cord serum IgE and positive family history of allergy was strongly associated with subsequent atopic manifestations. Nevertheless, it showed a reduced sensitivity as compared to cord serum IgE or family history of allergy. They concluded that an elevated cord serum IgE level predicts subsequent atopy up to age 20 years (305).

5.1.3 Summary and hypotheses

In summary, there is evidence (as discussed in the previous sections) that atopic disease is initiated *in utero* and that risk can be identified in cord blood by T-cell phenotyping, by measuring cytokine production (from Th1, Th2, T-reg cells), and IgE production by B cells or IgE concentrations. Early exposure to LC n-3 PUFAs may influence early immune responses, in a manner that decreases the likelihood of atopic sensitization. There is evidence to support this from epidemiologic observations (section 1.5.1) and from a small number of fish oil supplementation studies (section 1.5.2). Oily fish is a good source of LC n-3 PUFAs, but there

are no published intervention trials with fish during pregnancy. Since eating fish is advised, but fish are also a source of contaminants, aquaculture of fish to have low contaminant levels, while maintaining their LC n-3 PUFA content, could be an ideal solution to enable oily fish consumption by pregnant women and possibly promote the intake of the recommended amounts of LC n-3 PUFAs. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish in pregnant women. SIPS focuses on pregnant women whose offspring have increased risk of developing atopic disease, aiming to identify whether there is an effect on atopy outcomes in the offspring. A total of 123 women were randomly assigned to consume either 2 portions of farmed salmon per week (from week 20 until delivery) or to continue their habitual diet low in oily fish (less or equal to two portions per month).

The aim of the work described in this chapter is to investigate the effect of the increased maternal oily fish consumption on markers of infant immunity at birth (cord plasma and CBMC responses) which may potentially influence the development of atopy risk at 6 months of age (Chapter 6).

The hypotheses of the work described in this chapter are that the maternal oily fish intervention will:

1. alter neonatal immune cell phenotypes;
2. decrease PGE_2 production by cultured CBMCs;
3. alter neonatal innate and adaptive immune T-cell responses (increased Th1, decreased Th2, T reg) measured as cytokine expression in plasma and by cultured CBMCs;
4. decrease cord plasma IgE concentration.

5.2 Materials and methods

5.2.1 Study design and subjects

Chapter 2 describes the study design, the subjects' characteristics, and the intervention. In summary, the population studied were pregnant women with high risk of having an atopic offspring. The study groups were two, of which the control group (n=61) was asked to keep their habitual diet unchanged during the study period (from 20 weeks until delivery) and the salmon group (n=62) which was asked to consume 2 portions of farmed salmon per week (from 20 weeks until delivery). Every portion of salmon provided 1.73 g of EPA plus DHA; the weekly intake of LC n-3 PUFAs was 3.46 g and the daily intake was 494 mg/day. The nutrient analysis of salmon is described in Chapter 2; Table 2.3 The consumption of the study salmon provided more EPA plus DHA than the recommended minimum intake in the UK for pregnant women (450 mg/day; (26)). Subjects in the salmon and the control groups came into the maternity hospital fasted at three time points during pregnancy (20, 34, 38 weeks gestation), and fasting blood was taken. At birth, umbilical cord blood was collected to enable fatty acid analysis of plasma and CBMCs (see Chapter 4) and measurement of infant immune markers related to possible manifestations of atopic disease. This chapter describes the cord blood immune outcomes.

5.2.2 Laboratory materials and methods

5.2.2.1 Cord blood collection

5.2.2.1.1 Blood Tubes

Plastic 6 mL lithium heparin (LH) vacutainer blood bottles, plastic 2 mL ethylenediaminetetraacetic acid (EDTA) vacutainer blood bottles, alcohol wipes – sterets, 19 gauge needles, 50 ml tubes – sterile (Elkay Laboratories) and 50 ml syringes were obtained from NHS stores, Southampton General Hospital.

5.2.2.1.2 Procedure

Midwifery staff at Princess Anne Hospital assisted with all cord blood collections. Umbilical cord blood was only collected from normal full-term (> 37 weeks) deliveries with informed consent

from the mothers. Cord blood was preferentially collected during the third phase of delivery while the placenta is still in the uterus, in order to ensure a good volume of collection. If this was not possible, cord blood was collected after delivery and the placenta had been expelled. In either instance, the umbilical cord was clamped immediately and cleaned with 70% alcohol and/or iodine swab, this minimised maternal blood contamination. Whole cord blood was collected using a 16-19G needle on a 50 mL syringe. After umbilical cord vein puncture, the cord blood was collected into the 50 mL syringe. The volume collected ranged from 10 to 30 ml depending on the condition of the placenta. Cord blood samples were collected into 2 LH tubes and 2 EDTA tubes, one of each was kept at room temperature and the other in the fridge (4°C) for less than 8 hours for further analysis. The EDTA bottle which was stored at room temperature was sent for haematology testing. The cord blood samples were then either stored at room temperature or in fridge and within the next 8-12 hours blood was separated into plasma, CBMCs and erythrocytes by gradient centrifugation.

5.2.2.2 Analysis of total cord blood leukocytes

The cord blood kept in the EDTA tube at room temperature was sent to Chemical Pathology, SUHT for the assessment of total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils. The analysis was performed using a Sysmex XE-2100 flow cytometer for haematology analysis.

5.2.2.3 Analysis of cord blood immune cell subsets

5.2.2.3.1 Materials

Mouse anti-human fluorochrome-conjugated antibodies were purchased from Serotec. Mouse RPE-labelled anti-human CD127 was purchased from IOTest-Beckman Coulter. FACS lysing solution, cell wash, and cell fix were purchased from BD Biosciences.

5.2.2.3.2 Procedure

Flow cytometry was used to measure the presence of markers on the surface of leukocytes. Heparinised whole blood (100 μL) was incubated with fluorochrome-labelled monoclonal antibodies (Table 5.1) for 30 min at 4˚C in the dark to identify different cell subsets. After this incubation, erythrocytes were lysed for 10 min using 2 mL 10% FACS lysing solution in

deionised distilled water. After centrifugation (7 min, 200 × *g*), lysed erythrocytes were discarded and leukocytes were washed with 2 mL cell wash (7 min, 200 × *g*), and then fixed with 200 µL 10% cell fix solution in deionised distilled water. Fixed leukocytes were analysed by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) equipped with a 488 nm argon blue laser. Lymphocytes were identified by their forward scatter (FSC) and side scatter (SSC) properties. FSC and SSC were set to a linear scale and collection of 10,000 events was required for all analyses, apart from the determination of Treg cells where approximately 100,000 events were collected. CaliBRITE 3 beads (Becton, Dickinson) were employed for instrument calibration and the same voltage settings were used for each sample. In all experiments, gates were set so that isotype control staining consistently yielded < 1% positive cells. Data were analysed using CELLQuest software (Becton, Dickinson). Figure 5.1 to Figure 5.10 how typical profiles for each stain set. These profiles were used to identify the proportion of a smaller cell population within a major cell group of the leukocytes (i.e. lymphocytes and monocytes). These profiles can also give information on mean fluorescence intensity (MFI), a parameter that indicates the level of expression of the marker protein on the cell surface (how dense a marker is expressed on the surface of the cells). MFI was determined for the expression of CD127 on T-regulatory cells (Figure 5.11).

Table 5.1: Labelled monoclonal antibodies ("stains") used to identify different cord blood cell subsets

Stain	Cell type identified	Phenotype of cell type identified	Volume of stain used (µl)
None	Control (No stain)	-	
Anti-CD3-FITC/anti-CD4-RPE	T helper/inducer cells	$CD3^+CD4^+$	10
Anti-CD3-FITC/anti-CD8-RPE	T cytotoxic/suppressor cells	$CD3^+CD8^+$	10
Anti-CD3-FITC/anti-CD16-RPE	NK cells	$CD3^-CD16^+$	10
Anti-CD3-FITC/anti-CD19-RPE	B-cells	$CD3^-CD19^+$	10
Anti-CD14-FITC + anti-CD282-RPE	TLR2 bearing monocytes	$CD14^+TLR2^+$	10+10
Anti-CD4-FITC + anti-CD25-RPE-Cy5 + anti-CD127-RPE	T regulatory cells	$CD4^+CD25^+CD127^{low}$	10+5+20
Anti-CD4-FITC + anti-CD25-RPE-Cy5 + IgG1-RPE	T regulatory cells control	-	10+5+10
IgG1-FITC + IgG1-RPE + IgG1-RPE-Cy5	Triple isotype control	-	10+10+10

FITC, Fluorescein isothiocyanate isomer 1 (liquid); RPE, R. Phycoerythrin (lyophilised)

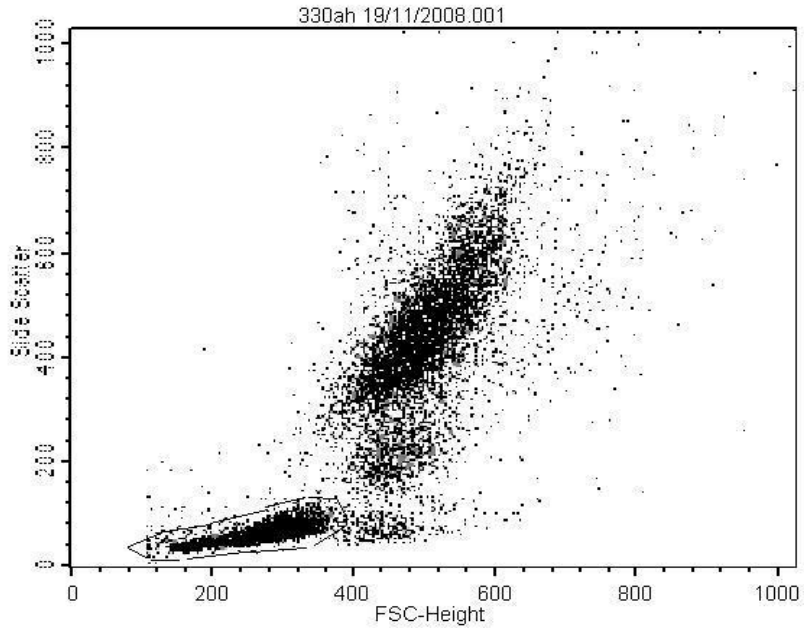
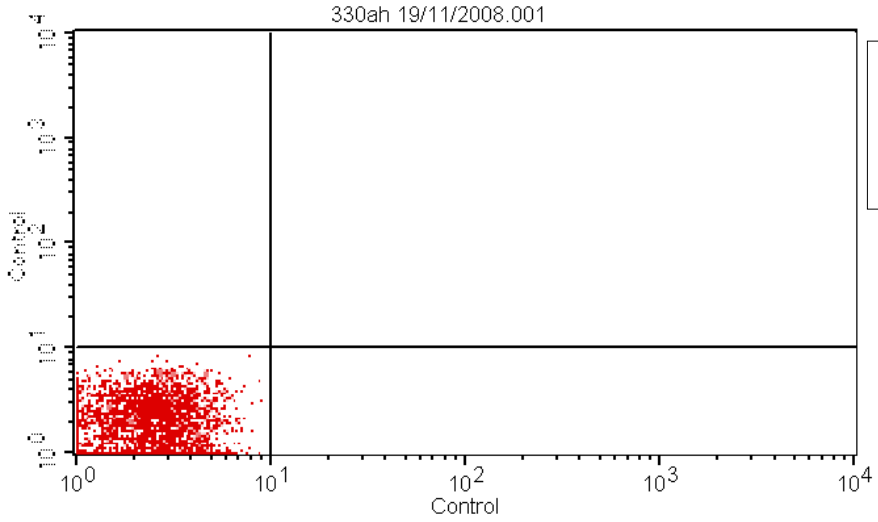
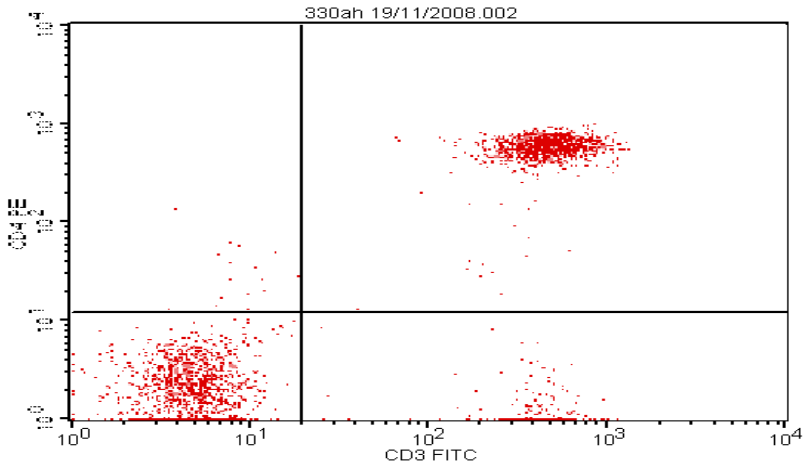


Figure 5.1: Typical dot plot of leukocyte populations from human blood. A gate has been drawn around the lymphocyte population



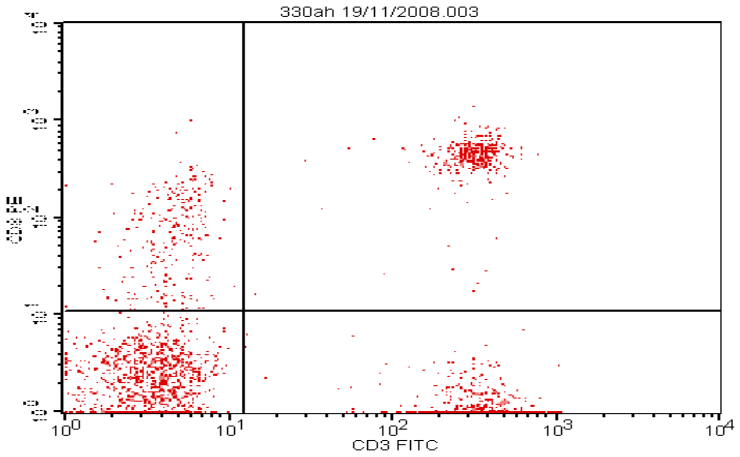
Control, (lower left quadrant): 100% of the gated population, 27.33% of the total leukocyte population.

Figure 5.2: Typical profile of the control (no stain) for the gated lymphocytes from Figure 5.1



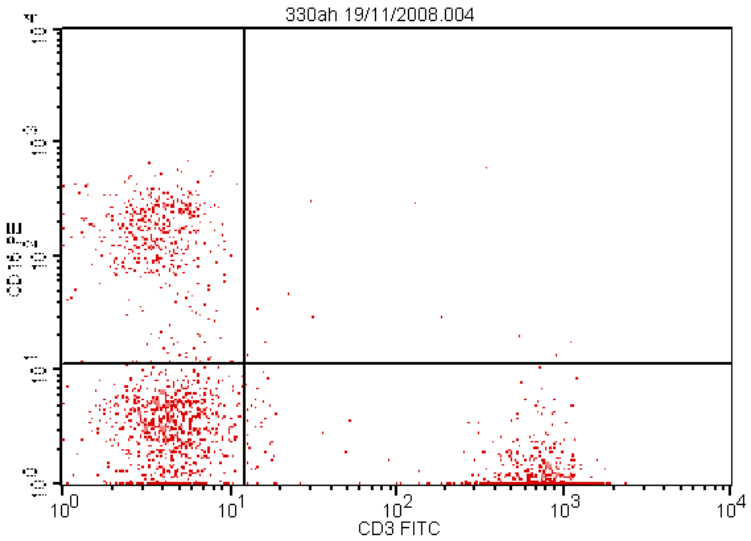
T helper lymphocytes (upper right quadrant): 41.16% of the gated population, 10.92% of the total leukocyte population.

Figure 5.3: Typical profile of T helper lymphocytes (CD3+/CD4+) for the gated lymphocytes from Figure 5.1



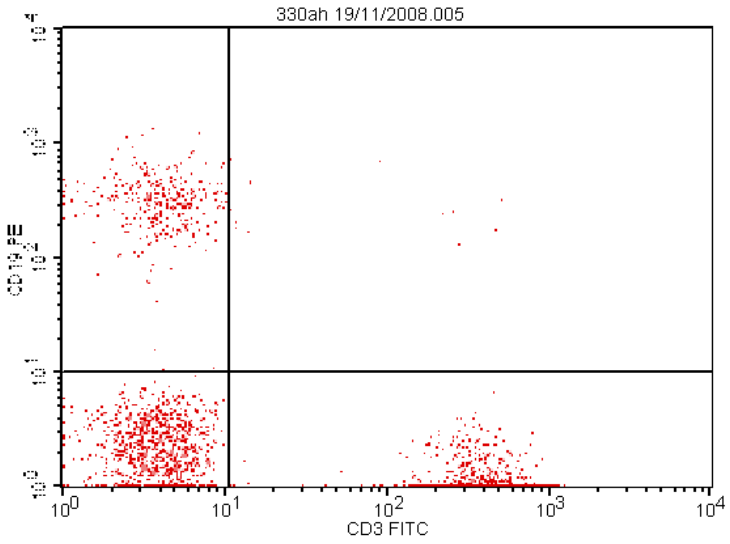
Cytotoxic T lymphocytes (upper right quadrant): 12.77% of the gated population, 3.61% of the total leukocyte population.

Figure 5.4: Typical profile of cytotoxic T lymphocytes (CD3+/CD8+) for the gated lymphocytes from Figure 5.1



NK cells (upper left quadrant): 15.12% of the gated population, 4.46% of the total leukocyte population.

Figure 5.5: Typical profile of natural killer (NK) cells (CD3-/CD16+) for the gated lymphocytes from Figure 5.1



B lymphocytes (upper left quadrant): 11.45 % of the gated population, 3.22% of the total leukocyte

Figure 5.6: Typical profile of B lymphocytes (CD3-/CD19+) for the gated lymphocytes from Figure 5.1

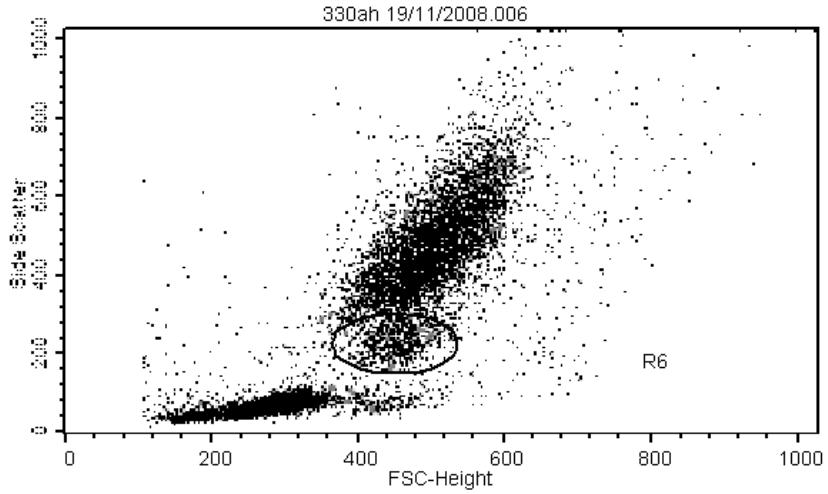
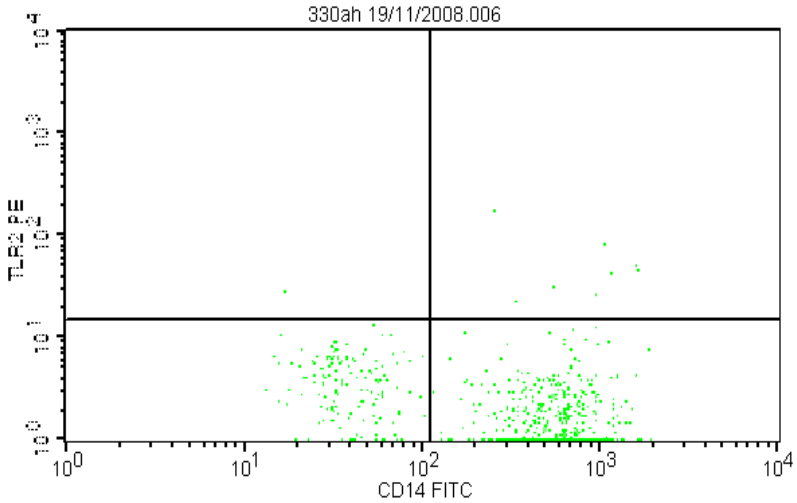


Figure 5.7: Typical dot plot of leukocyte populations from human blood. A gate (R6) has been drawn around the monocyte population



TLR2 bearing monocytes (upper left quadrant): 1.06% of the gated population, 0.08% of the total leukocyte population.

Figure 5.8: Typical profile of TLR2 bearing monocytes (CD14+/TLR2+) for the gated monocytes (R4) from Figure 5.7

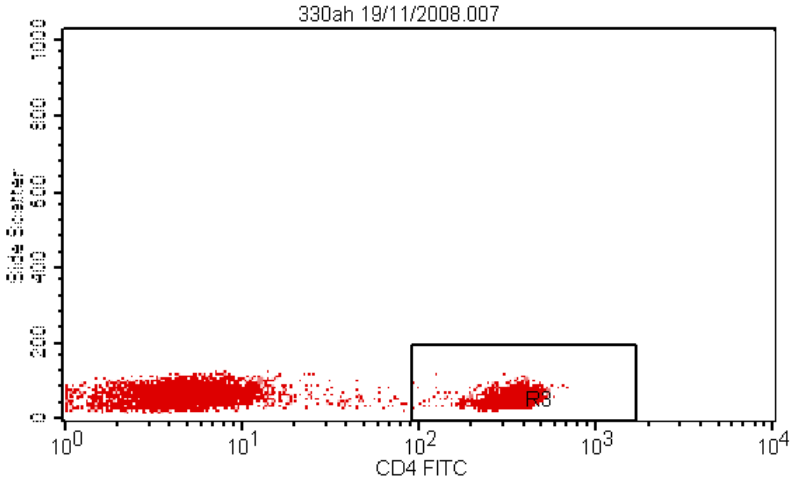
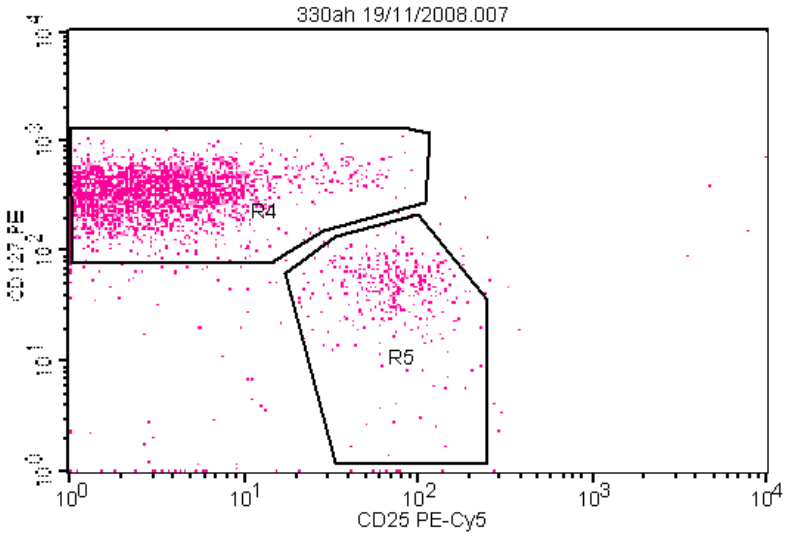


Figure 5.9: Gate R3 drawn around the CD4+ lymphocytes included in the gate of Figure 5.1.



T regulatory cells at gate R5 (lower right quadrant)

Figure 5.10: Typical profile of T regulatory cells (CD4+/CD25+/CD127 ^{low}) included in gate R5, from the gated lymphocytes (R3) from Figure 5.9.

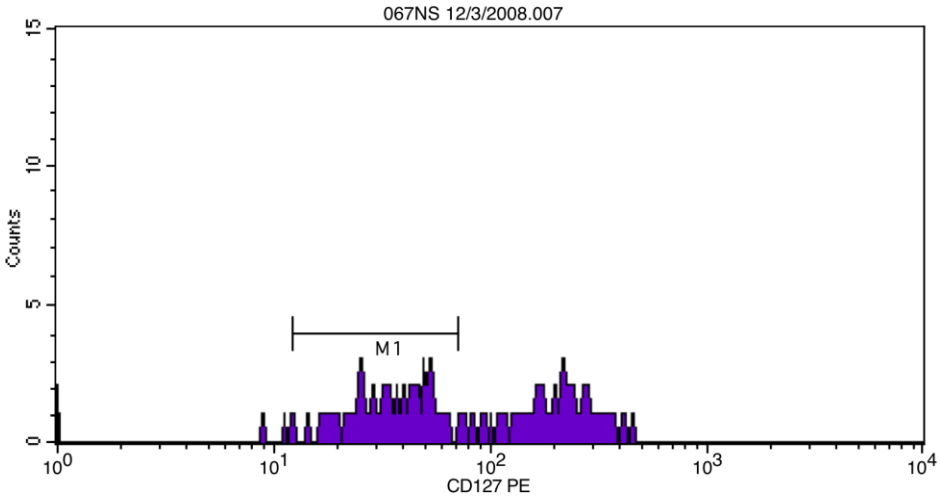


Figure 5.11: Typical mean fluorescence intensity (MFI) gated (M1) on the T-regulatory cells CD4+/CD25+/CD127 ^{low}

5.2.2.4 Preparation of CBMCs and plasma

5.2.2.4.1 Materials

Histopaque (density 1.077 g/L, containing polysucrose 5.7 g/dL, and sodium diatrizoate 9.0 g/dL), L-glutamine (200 mM), antibiotics (penicillin, streptomycin), EDTA, potassium bicarbonate, ammonium chloride, and crystal violet were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid. RPMI medium (without L-glutamine and antibiotics) and heat-inactivated fetal bovine serum (HI-FBS) were purchased from PAA Laboratories. Dimethyl sulphoxide (DMSO) and acetic acid were purchased from Fisher Scientific. Saline (0.9% sodium chloride) was purchased from SUHT Pharmacy, Southampton General Hospital.

White cell counting fluid was made as follows: 100 mL PBS, 2 mL acetic acid, approximately 5 g crystal violet. The solution was millipore filtered before use.

5.2.2.4.2 Procedures

Heparinised whole blood was layered onto Histopaque (ratio of blood to Histopaque: 2:1) and centrifuged for 15 min at 800 × *g* (2000 rpm, R = 18 cm) at room temperature. Plasma was collected from the top layer and frozen at -80˚C for future culturing of CBMCs and for determination of total IgE.

The CBMCs were collected from the interphase and washed once for 10 min at 450 × *g* (1500 rpm, R = 18 cm) with RPMI medium containing 0.75 mmol/L glutamine and antibiotics (penicillin and streptomycin). After being resuspended in 1 mL RPMI medium, the cells were counted with a haemocytometer using white cell counting fluid (crystal violet). After counting total CBMCs, 2×10^6 cells were removed and frozen at -80˚C for fatty acid analysis (see chapter 4).

The remaining CBMCs were washed once (10 min, 450 × *g*) with RPMI medium containing 2% HI-FBS. The CBMCs were resuspended in RPMI medium containing 2% HI-FBS and were cryopreserved with 15% DMSO in HI-FBS (freezing medium). The freezing medium was added to the resuspended CBMCs drop-wise on ice (ratio of resuspended cells in RPMI medium containing 2% HI-FBS to freezing medium: 1:1). CBMC concentrations were adjusted to 12-20 × 10^6 cells/ml/cryovial and were frozen at -80˚C in a Nalgene Cryo 1˚C

freezing container (filled with 250 mL isopropanol). After at least 4 hours of freezing, cryovials containing the CBMCs were stored in liquid nitrogen for later thawing and culturing.

5.2.2.5 Measurement of cord plasma IgE concentration

Cord blood plasma was sent to the Chemical Pathology, SUHT for analysis of total IgE. The analysis was done on Phadia ImmunoCAP 250 and the lowest detection of total IgE was 0.00 kU/L. The test is designed as a sandwich immunoassay. ImmunoCAP provides high sensitivity and very low concentrations of IgE antibodies can be detected.

5.2.2.5.1 Principle

Anti-IgE, covalently coupled to ImmunoCAP, reacts with the total IgE in the infants' cord plasma sample. After washing, enzyme-labelled antibodies against IgE are added to form a complex. After incubation, unbound enzyme-labelled anti-IgE is washed away and the bound complex is then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate is measured. The response is then transformed into a concentration through a calibration curve. The fluorescence is directly proportional to the concentration of IgE in the plasma sample.

5.2.2.6 Measurement of cord plasma interleukin 13 (IL-13) concentration

5.2.2.6.1 Principle

The human IL-13 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human IL-13. An anti-human IL-13 coating antibody is adsorbed onto microwells. Human IL-13 present in the plasma sample or standard binds to antibodies adsorbed to the microwells. A conjugate mixture (biotin-conjugated anti-human IL-13 antibody and Streptavidin-HRP) is added and binds to human IL-13 captured by the first antibody. Following incubation, unbound biotin-conjugated anti-human IL-13 antibody and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human IL-13 present in the plasma sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human IL-13 standard dilutions and human IL-13 plasma sample concentrations are determined.

5.2.2.6.2 Materials

The human IL-13 platinum ELISA kit purchased from by eBiosciece included: a microwell plate (96 wells) coated with monoclonal antibody to human IL-13, biotin-conjugate anti-human IL-13 monoclonal antibody, streptavidin-HRP, human IL-13 standard lyophilized, assay buffer concentrate, wash buffer concentrate, substrate solution, stop solution, blue-dye and green-dye.

5.2.2.6.3 Procedure

Serial dilutions of a human IL-13 standard were prepared and then pipetted (100 µl) into wells of the ELISA plate. Sample (50 µl) was added to the other wells and 50 µl of assay buffer added to these wells. Conjugated mixture (50 µl) was added to all wells. The plate was covered with an adhesive film and incubated at room temperature for 2 hours, on a microplate shaker set at 100 rpm. Then the microwell strips were washed 3 times. Then 100 µl of substrate solution was added to all wells. The microwell strips were then incubated at room temperature (18° to 25°C) for about 10 min avoiding direct exposure to intense light. After a period of time (judged by the colour change that occurred in the well containing the highest concentration of standard) stop solution (100 µl) was added into each well. Plates were read within one hour on a microplate reader (Thermo Labsystems) set at 450 nm. IL-13 concentration of each sample was calculated from the standard curve. The limit of detection was 0.7 pg/ml.

5.2.2.7 Resuscitation and culturing of cryopreserved cord blood mononuclear cells

5.2.2.7.1 Materials

L-glutamine (200mL), antibiotics (penicillin, streptomycin), trypan blue and phytohaemaglutinin (PHA; HA16) were purchased from Sigma-Aldrich. RPMI medium without L-glutamine and antibiotics was purchased from PAA laboratories. Ultra pure LPS (*Escherichia coli* K12 strain), bacterial peptidoglycan (*Staphylococcus aureus*) and polyinosine-polycytidylic acid (poly (I:C)) were purchased from InvivoGen. LoTox *Dermatophagoides pteronyssinus* allergen 1 (Der p 1) was purchased from Indoor

Biotechnologies. Low endotoxin ovalbumin (Ova) was purchased from Profos AG (Germany). Salmon paralbumin (Sal s1) was kindly provided by John Doe (University of Bergem Norway).

5.2.2.7.2 Procedures

Cryopreserved CBMCs were thawed rapidly in the waterbath at 37°C and immediately resuspended dropwise with 15 mL ice cold RPMI (containing 0.75 mmol/L glutamine and antibiotics). After centrifugation, cells were resuspended in 1 mL RPMI, 10 μL of which were stained with trypan blue and counted using a haematocytometer. Cell viability was assessed (approximately 80%) and live cells were counted. After counting, cells were resuspended in PRMI plus 10% (v/v) autologous plasma (used as culture medium) to correct the concentration for culturing.

All cultures were plated in duplicate in 96-well, round bottom plates (2 x 10^6 cells/ml) in 200 μL of RPMI plus autologous plasma and incubated at 37°C in a 5% CO_2 atmosphere for the times indicated below. The stimuli used were: Der p1, Ova, Sal s1, PHA, TLR-2 ligand (peptidoglycan (PGN)), TLR 3 ligand (synthetic double stranded RNA Poly I:C), TLR-4 ligand (LPS). Control wells were left unstimulated. Table 5.2 describes the exact concentration, amount and volume of each stimulus used in the cultures. Stock concentrations of stimulants were stored at -20°C and the same stimulant stocks were used throughout the study. The incubation period for cultures with TLR ligands was 24 h, whereas for the rest (allergens and mitogen) it was 48 h. After incubation, the plates were centrifuged and the supernatants collected and frozen at -20°C for later analysis of cytokines and PGE_2.

Table 5.2: Stimuli used for culturing of cord peripheral blood mononuclear cells

Stimulant	Type	Final concentration	Volume/well	Amount/well	Culture time
Der p 1	Allergen	10 μg/mL	10 μL	200 ng	48 h
Sal s 1	Allergen	200 μg/mL	10 μL	2000 ng	48 h
Ova	Allergen	15 μg/mL	10 μL	150 ng	48 h
PHA	Mitogen	7.5 μg/mL	10 μL	75 ng	48 h
PGN	TLR-2 ligand	10 μg/mL	10 μL	100 ng	24 h
Poly I:C	TLR-3 ligand	100 μg/mL	10 μL	1000 ng	24 h
LPS	TLR-4 ligand	10 ng/mL	10 μL	0.1 ng	24 h

5.2.2.8 Measurement of cytokine concentrations in cord blood mononuclear cell culture supernatants by cytometric bead array (CBA) and flow cytometry

5.2.2.8.1 Principle

The cytometric bead array (CBA) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. The CBA is combined with flow cytometry to create a multiplexed assay. Six bead populations with distinct fluorescence intensities are coated with capture antibodies specific for Th1 and Th2 cytokines. The six bead populations are mixed together to form the CBA that is resolved in the FL3 channel of a fluorescence-activated cell sorter (flow cytometer). The CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume of sample. The cytokine capture beads are mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Acquisition of data is done using the flow cytometer.

5.2.2.8.2 Materials

The human Th1/Th2 CBA kit purchased from BDBiosciences included human IL-2, IL-4, IL-5 and IL-10, tumour necrosis factor-α (TNF-α) and IFN-γ capture beads, human Th1/Th2 PE detection reagent, lyophilised human Th1/Th2 cytokine standards (all contained in one vial), cytometer set up beads, PE positive control detector, FITC positive control detector, wash buffer, and assay diluent.

5.2.2.8.3 Procedure

The BD CBA protocol was followed. The Th1/Th2 cytokine standard containing all 6 human Th1/Th2 cytokines was diluted in assay diluent and was allowed to equilibrate for 15 minutes. A serial dilution of the top standard was performed using assay diluent to achieve final concentrations of 5000 (top standard), 2500 (1:2), 1250 (1:4), 625 (1:8), 312.5 (1:16), 156, (1:32), 80 (1:64), 40 (1:128), and 20 (1:256) pg/ml for each cytokine in the standard.

All culture beads were pooled together into a 'mixed capture beads' tube before analysis prior to very good vortexing of each of the beads. For each test conducted (standard, control and samples) 4.5 μL of each capture bead was used. Each assay tube was added with: 25 μL 'mixed capture beads', 25 μL human PE detection reagent, 25 μL standard dilutions to the standard assay tubes, 25 μL assay diluent to the negative control assay tube, and 25 μL CBMC culture supernatant to the test assay tubes. The human Th1/Th2 cytokine detection reagent that was used included PE-conjugated anti-human IL-2, IL-4, IL-5, IL-10, TNF-α and IFN-γ antibodies.

The tubes were incubated for 3 h at room temperature. After incubation, 1 mL of wash buffer was added to each tube and the tubes centrifuged (200 × *g*, 5 min). After discarding the supernatant, the bead pellet was resuspended with 300 μL of wash buffer. The samples were analysed by flow cytometry using a FACSCalibur (Becton Dickinson, Oxford, UK). The number of events counted was set at 1800 of gated events (i.e 300 events per capture bead). The cytometer and instrument settings setup were performed according to the instructions of the BD CBA protocol. Compensation for special fluorochrome overlap was performed. The BD CellQuest software was used for data acquisition and formatting. The results were generated in graphical and tabular format using the Becton Dickinson CBA analysis software.

The limits of detection for IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ were 2.6, 2.6, 2.4, 2.8, 2.8, 7.1 pg/ml respectively. Values below level of detection (LOD) were set at ½ LOD for all cytokines. Figure 5.12 shows a typical acquisition template example with the six distinct bead populations in the negative control standard and Figure 5.13 that of a culture supernatant from CBMCs stimulated with PHA.

The CBMC cytokine responses reported in the results (Section 5.3.3) are presented in two ways, firstly, as the unstimulated control responses and the responses to various stimuli. There were some differences in the background (i.e. unstimulated) cytokine responses between the salmon and control groups that make interpretation of the raw results difficult. For this reason, the second way for the results to be presented is by difference in cytokine production over the corresponding unstimulated secretion (e.g. CBMC IL-10 production after PHA stimulation minus CBMC IL-10 production without stimulant (control)) so as to give the stimulus-specific response.

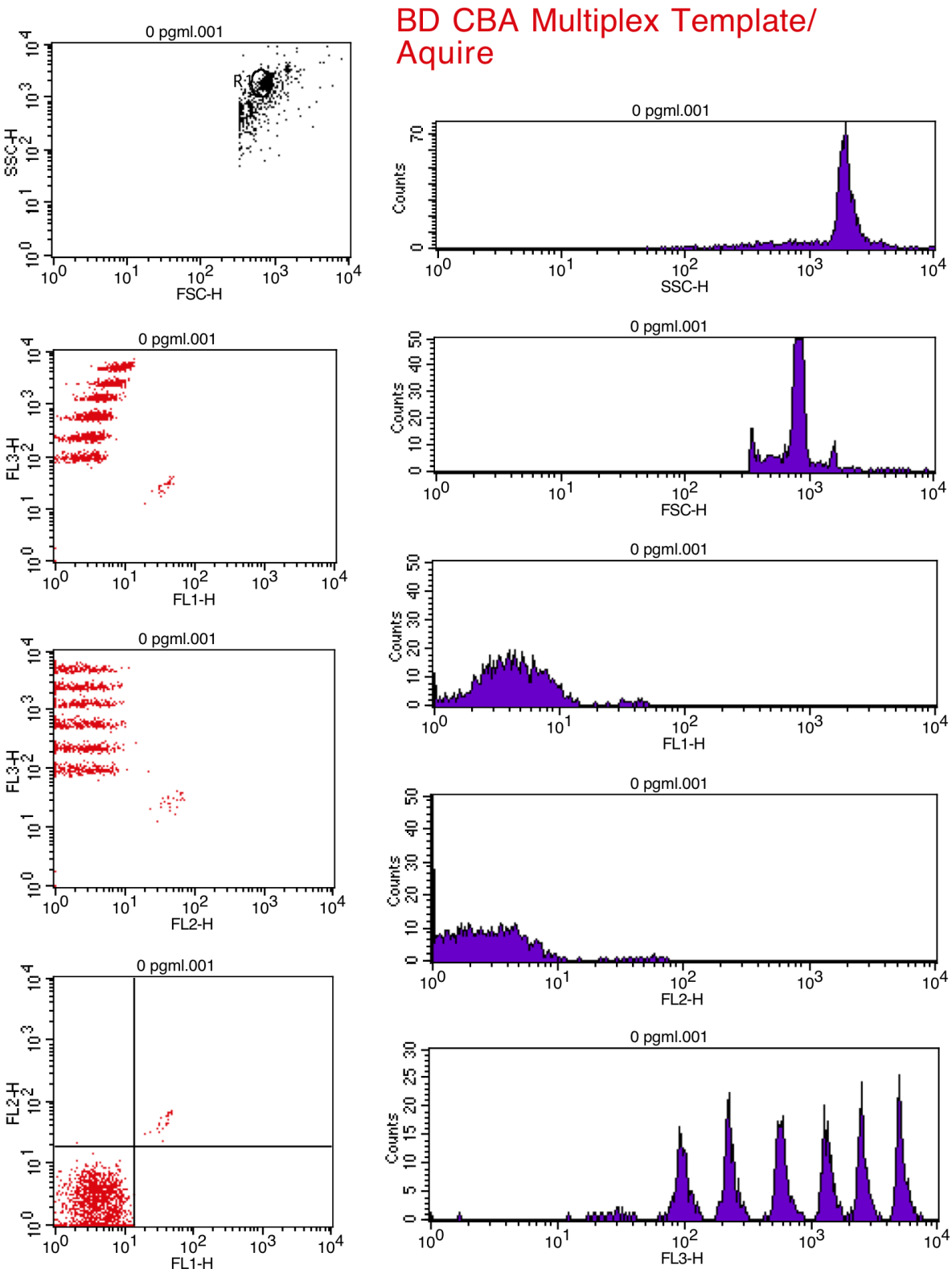


Figure 5.12: Cytometric bead array acquisition template for negative control standard

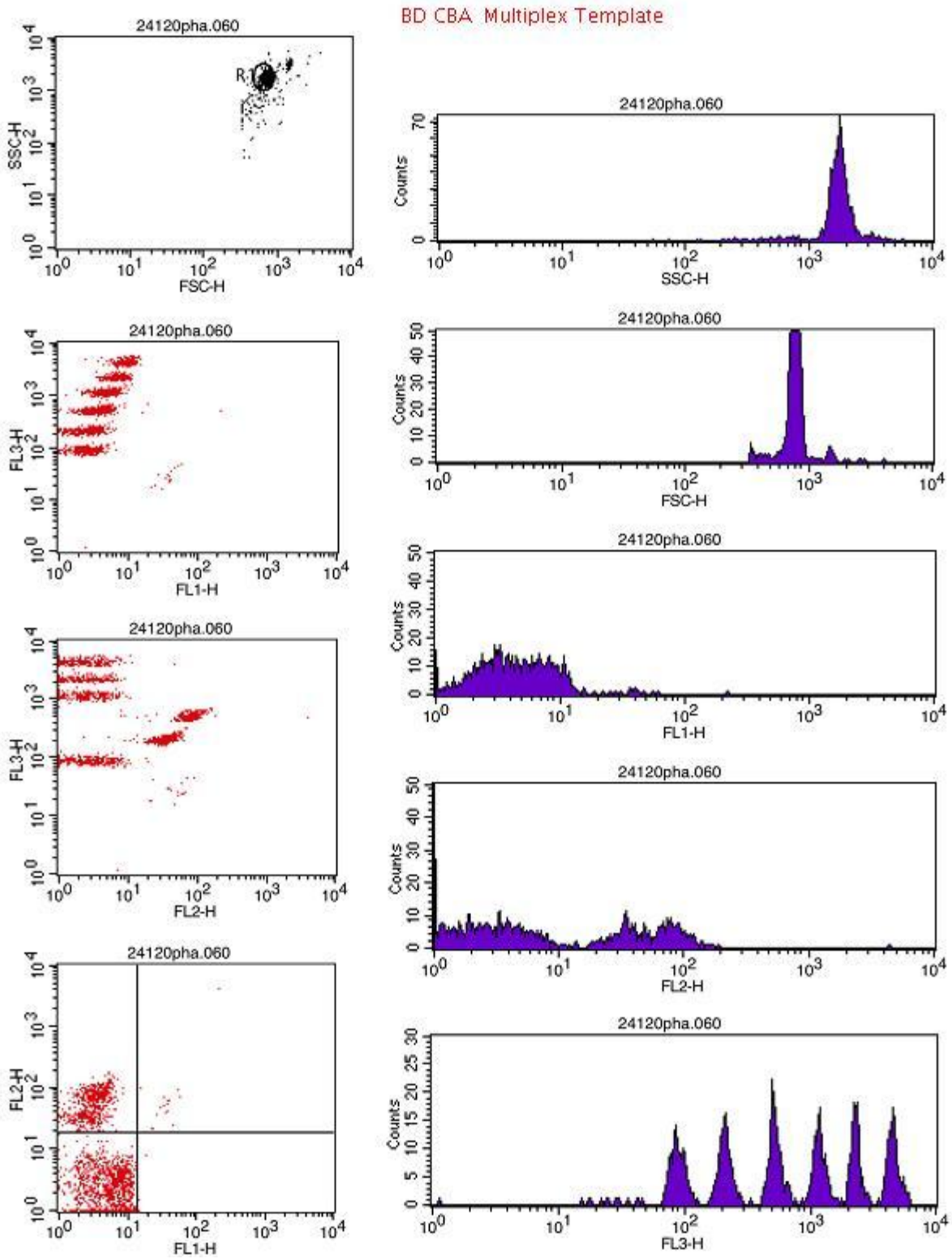


Figure 5.13: Cytometric bead array acquisition template for culture supernatant from PHA stimulated CBMC (48 hours)

5.2.2.9 Measurement of prostaglandin E_2 concentration in mononuclear cell culture supernatant by enzyme-linked immunosorbent assay

5.2.2.9.1 Principle

The enzyme-linked immunosorbent assay (ELISA) is based on the forward sequential competitive binding in which prostaglandin E_2 (PGE_2) present in a sample competes with horseradish peroxidase (HRP)-labelled PGE_2 for a limited number of binding sites on a mouse monoclonal antibody which is bound to goat anti-mouse polyclonal antibodies covering the bottom of the ELISA plate. PGE_2 in the sample is allowed to bind with the mouse antibody in the first incubation. During the second incubation, HRP-labelled PGE_2 binds to the remaining antibodies sites. Following a wash to remove unbound materials, a substrate solution is added to the wells to determine the bound enzyme activity. The colour is inversely proportional to the concentration of PGE_2 in the sample.

5.2.2.9.2 Materials

PGE_2 ELISA kit purchased from R&D Systems included: goat anti-mouse 96-well microplate, HRP-PGE_2 conjugate, PGE_2 standard, primary antibody (monoclonal antibody to PGE_2), a calibrator diluent, wash buffer, colour reagent A and B, stop solution.

5.2.2.9.3 Procedure

The procedure followed was according to the manufacturer's protocol. Culture supernatants were diluted (1:3, 1:10 or 1:100) prior to the ELISA so that PGE_2 could be detected within the limits of the assay. All reagents, working standards, and buffers were prepared according to the protocol. The lyophilized PGE_2 standard was reconstituted with 1 mL distilled water to produce a stock of 25,000 pg/ml. This was left for 15 min to stabilize. A serial dilution of the stock standard was performed using calibrator diluent to achieve final concentrations of: 2500, 1250, 625, 313, 156, 78, 39 pg/ml. Calibrator diluent was used as negative control (0 pg/mL). 200 μL of calibration diluent was added to the non specific binding (NSB) wells. 150 μL of calibration diluent was added to the negative control well. 150 μL of standard, control or sample was added to the remaining wells and 50 μL of the primary antibody solution was added to each well, turning their colour to blue apart from the NSB wells. The plate was then covered and incubated for 1 hour at room temperature on horizontal orbital microplate shaker. Without washing the plate, after incubation 50 μL of HRP-PGE_2 conjugate was added to each

well. All wells turned a violet colour apart from the NSB. The plate was incubated for another 2 hours at room temperature on the shaker. The plates were then aspired, washed, and blot dried 4 times with wash buffer 200 μL of substrate solution (colour reagents A and B mixed 15 minutes prior to use) was added to each well. The plate was incubated for 30 minutes at room temperature in the dark. Finally, 100 μL stop solution was added to all wells. The optical density was measured immediately with a microplate reader (Thermo Labsystems) set at 450 nm. Wavelength correction was set at 450 nm. The NSB optical density was subtracted from that of the standards, controls and samples. A standard curve was created for the optical density and PGE_2 standard concentration. PGE_2 concentration of each sample was calculated from the standard curve. The concentration was multiplied by the dilution factor of the samples.

5.2.3 Statistical analysis

For the cord blood immune cell phenotypes independent samples t-tests were used for comparisons between groups since the data were normally distributed. IgE data and the cytokine and PGE_2 data from cultured CBMCs were not normally distrubuted. These data were not log transformed prior to analysis, but instead 2 independent samples Mann-Whitney tests were used for comparisons between groups. The level of statistical significance was for all tests at p-value ≤ 0.005.

5.3 Results

5.3.1 Cord blood leukocytes

Table 5.3 describes the umbilical cord blood leukocyte counts (cells x 10^3/μL whole blood) at birth in the salmon and the control groups. The salmon group had higher counts of neutrophils, lymphocytes and total leukocytes compared to the control group.

5.3.2 Cord mononuclear cell phenotypes

Table 5.4 describes the CBMC phenotypes at birth in the salmon and control groups; data are expressed as percentages of total lymphocytes or or $CD4^+$ lymphocytes (for Treg cells) except for TLR-2^+ monocytes which are expressed as percentage of total monocytes. There were no differences in the percentages of any of the cell subsets between the two groups.

Table 5.5 describes the CBMC phenotypes at birth in the two groups expressed as percentages of total leukocytes. Again, there were no differences between the two groups.

Table 5.6 describes the CBMC subset numbers (cells x 10^3/mL whole blood) in both groups. These were calculated using the information from the leukocyte percentages of the cell subsets (Table 5.5) and total leukocyte counts (Table 5.3). The numbers of helper T-cells, cytotoxic T-cells and Treg cells were higher in the salmon group compared to the control group.

Figure 5.14 shows the mean fluorescent intensity (MFI) of CD127 on Treg cells in cord blood. MFI tended be higher in the salmon group compared to the control group, but the difference was not significant.

Table 5.3: Cord blood leucocyte counts (cells x 10^3/µL whole blood) at birth according to study group

Cell type	Control group (n=30)		Salmon group (n=35)		p
	Mean	SD	Mean	SD	
Neutrophils	**6.940**	2.072	**8.277**	2.640	**0.026**
Lymphocytes	**4.430**	1.503	**5.860**	2.770	**0.011**
Monocytes	1.167	0.511	1.454	0.762	0.075
Eosinophils	0.393	0.224	0.449	0.262	0.363
Basophils	0.103	0.100	0.229	0.511	0.165
Total Leukocytes	**13.060**	3.691	**16.263**	5.051	**0.005**

Values are mean (SD), independent sample t-test p-values

Table 5.4: Cord blood mononuclear cell phenotypes at birth (percentage of the gated cell population) according to study group

Cell subsets	Control group (n=26)		Salmon group (n=29)		p
	Mean	SD	Mean	SD	
Helper T-cells ($CD3^+CD4^+$) (% Lymphocytes)	39.7	9.5	40.8	12.1	0.701
Cytotoxic T-cells ($CD3^+CD8^+$) (% Lymphocytes)	13.1	5.9	14.9	7.4	0.302
NK cells ($CD3^-CD16^+$) (% Lymphocytes)	11.8	6.7	12.4	7.4	0.740
B-cells ($CD3^-CD19^+$) (% Lymphocytes)	11.7	5.2	10.7	5.4	0.484
TLR-2^+ monocytes($CD14^+TLR2^+$) (% Monocytes)	49.6	18.1	43.8	21.7	0.279
T-regulatory ($CD4^+CD25^+CD127^{low}$) (% $CD4^+$ lymphocytes)	54.2	15.1	56.9	16.4	0.516

Values are mean (SD) percentage of lymphocytes or monocytes (for TLR-2+ monocytes), independent sample t-test p-values

Table 5.5: Cord blood cell phenotypes (percentage of the total leukocytes) according to study group

Cell subsets	Control group (n=26)		Salmon group (n=29)		p
	Mean	SD	Mean	SD	
Helper T-cells ($CD3^+CD4^+$) (% Leukocytes)	11.0	3.5	11.4	4.2	0.670
Cytotoxic T-cells ($CD3^+CD8^+$) (% Leukocytes)	3.5	1.5	4.3	2.5	0.171
NK cells ($CD3^-CD16^+$) (% Leukocytes)	3.5	2.6	3.7	2.8	0.758
B-cells ($CD3^-CD19^+$) (% Leukocytes)	3.4	2.2	2.9	1.6	0.275
TLR-2^+ monocytes ($CD14^+TLR2^+$) (% Leukocytes)	2.8	1.7	2.4	1.2	0.305
T-regulatory ($CD4^+CD25^+CD127^{low}$) (% Leukocytes)	1.1	0.5	1.2	0.7	0.642

Values are mean (SD) percentage of the total leukocyte population, independent sample t-test p-values

Table 5.6: Cord blood mononuclear cell subset numbers (cells x10^3/µL whole blood) at birth according to study group

Cell type	Control group (n=20)		Salmon group (n=25)		p
	Mean	SD	Mean	SD	
Helper T-cells ($CD3^+CD4^+$)	1.847	0.752	2.369	0.956	**0.042**
Cytotoxic T-cells ($CD3^+CD8^+$)	0.586	0.248	0.820	0.448	**0.028**
NK cells ($CD3^-CD16^+$)	0.496	0.385	0.734	0.510	0.075
B-cells ($CD3^-CD19^+$)	0.551	0.329	0.688	0.410	0.210
TLR-2^+ monocytes ($CD14^+TLR2^+$)	0.660	0.345	0.654	0.400	0.963
T-regulatory ($CD4^+CD25^+CD127^{low}$)	0.134	0.064	0.218	0.168	**0.030**

Values are mean (SD) number of cells x 103/µL whole blood, independent sample t-test p-values

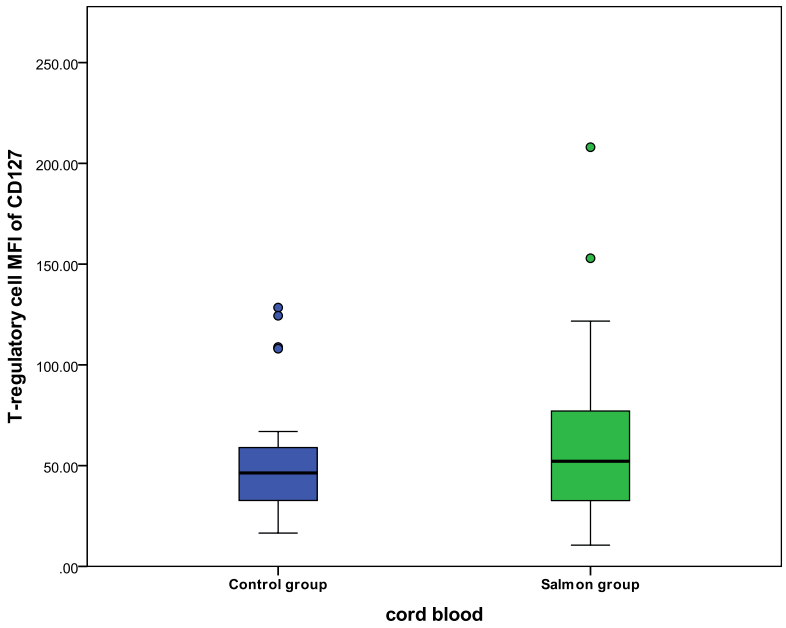


Figure 5.14: Geometric mean fluorescence intensity (MFI) of CD127 receptor on T regulatory cells at birth according to study group
independent samples t-test, p= 0.310.

5.3.3 Production of cytokines by cord blood mononuclear cells cultured with various stimulants

Table 5.7 to Table 5.9 describe the concentrations of five cytokines (Treg (IL-10), Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5)) in the culture medium of CBMCs stimulated with allergens (Der p 1, ovalbumin, salmon paralbumin), a mitogen (PHA), and TLR (-2, -3, -4) ligands.

Table 5.7 presents the concentrations of IL-2, -4, -5 after stimulation of cultures of CBMCs with PHA. The concentrations of these cytokines were also measured after stimulation with the TLR ligands and with the allergens but the concentrations were below the limits of detection. In this table are also included the ratios of IFN-γ/IL-4 and IFN-γ/IL-10 representing the ratios of Th1/Th2 cytokine responses and Th1/Treg cell responses. CBMCs from the salmon group produced lower concentrations of all four cytokines and this was significant for IL-2, -4 and -5 compared to those from the control group. IFN-γ concentration and the ratios IFN-γ/IL-4 and IFN-γ/IL-10 did not differ between the two groups.

Table 5.8 presents the concentration of IL-10 after stimulation of cultures of CBMCs with various stimulants for 24 and 48 hours. The concentrations produced in response to all stimulants were above detection level. CBMCs from the salmon group produced a lower concentration of IL-10 in all conditions tested; this was significant for unstimulated cells at 48 hr, in response to LPS at 24 hr and in response to PHA at 48 hr.

Table 5.9 presents the concentration of TNF-α after stimulation of cultures of CBMCs with various stimulants for 24 and 48 hours. The concentrations produced in response to all stimulants were above detection level. CBMCs from the salmon group produced a lower concentration of TNF-α in all conditions tested; this was significant for unstimulated cells at 48 hr and in response to PHA at 48 hr.

Table 5.10 to Table 5.12 describe the concentrations of five cytokines (Treg (IL-10), Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5)) in the culture medium of CBMCs stimulated with allergens (Der p 1, ovalbumin, salmon paralbumin), a mitogen (PHA), and TLR (-2, -3, -4) ligands after subtracting the corresponding background (i.e. unstimulated) secretion.

Table 5.10 presents the concentrations of IL-2, -4, -5 after stimulation of CBMCs with PHA minus the concentrations seen for unstimulated cultures incubated for 48 hours. In this table are also included the ratios of IFN-γ/IL-4 and IFN-γ/IL-10 representing the ratios of Th1/Th2 cytokine responses and Th1/Treg cell responses. CBMCs from the salmon group produced significantly lower concentrations of IL-2, -4, -5 after correcting for background secretion.

Table 5.11 presents the concentration of IL-10 after stimulation of cultures of CBMCs with various stimulants for 24 or 48 hours minus the concentrations seen for unstimulated cultures. Some of these values are negative as the control responses were higher than the specific stimulus responses. CBMCs from the salmon group produced a lower concentration of IL-10 in response to LPS at 24 hr after correcting for background secretion.

Table 5.12 presents the concentration of TNF-α after stimulation of cultures of CBMCs with various stimulants for 24 or 48 hours minus the concentrations seen for unstimulated cultures. Again, some of these values are negative as the control responses were higher than the specific stimulus responses. There were no differences between the two groups.

Table 5.7: Interleukin -2,-4,-5, and Interferon-γ concentrations (pg/mL) and ratios of IFN-γ/IL-4 and IFN-γ/IL-10 in the medium of cultured CBMCs collected at birth and stimulated with a mitogen (PHA) for 48 hours

Cytokine	Control group (n=30)			Salmon group (n=27)			p
	Median	IR		Median	IR		
		25th perc	75th perc		25th perc	75th perc	
IL-2	**196.4**	86.9	509.9	**69.5**	28.8	185.3	**0.012**
IL-4	**6.9**	2.4	14.1	**1.3**	1.3	7.2	**0.019**
IL-5	**12.3**	3.9	34.1	**4.9**	1.2	20.5	**0.032**
IFN-γ	121.2	34.4	286.4	86.6	24.5	178.2	0.218
IFN-γ/IL-4	17.6	8.7	42.9	18.8	7.6	63.6	0.743
IFN-γ/IL-10	1.2	0.6	2.5	2.0	0.7	2.5	0.492

Values are median (interquartile range (IR)), Mann-Whitney test p-values

Table 5.8: Interleukin-10 concentration (pg/mL) in the medium of cultured CBMCs collected at birth and stimulated with various TLR ligands, allergens and mitogens for 24 or 48 hours

Stimulant	Control group (n=29)			Salmon group (n=26)			p
	Median	IR		Median	IR		
		25th perc	75th perc		25th perc	75th perc	
None [1]	41.9	10.5	67.2	13.5	4.7	67.9	0.143
PGN [1]	166.7	36.1	309.6	68.0	32.9	177.5	0.081
Poly 1:C [1]	26.1	8.2	52.8	11.6	6.9	51.4	0.284
LPS [1]	**143.5**	53.7	281.2	**71.1**	33.4	159.5	**0.043**
None [2]	**49.1**	13.1	83.5	**14.9**	6.1	75.0	**0.051**
Der p 1 [2]	41.8	12.1	63.6	17.2	5.4	60.9	0.192
OVA [2]	46.5	14.8	84.2	16.7	7.9	75.6	0.086
Sal s1 [2]	62.2	30.2	87.3	26.9	13.8	77.4	0.070
PHA [2]	**106.9**	56.3	166.3	**43.3**	18.4	132.1	**0.022**

Values are median (interquartile range (IR)), Mann-Whitney test p-values
The limit of detection was 2.8pg/ml. When the value was below the LOD was set at LOD/2=1.4 pg/ml
[1]24 hour culture, [2]48 hour culture

Table 5.9: Tumor necrosis factor-α concentration (pg/mL) in the medium of cultured CBMCs collected at birth and stimulated with various TLR ligands, allergens and mitogens for 24 or 48 hours

Stimulant	Control group (n=29)			Salmon group (n=26)			p
		IR			IR		
	Median	25th perc	75th perc	Median	25th perc	75th perc	
None [1]	78.6	22.9	150.7	47.1	15.4	140.7	0.338
PGN [1]	591.3	255.8	1850.2	486.4	185.5	931.1	0.484
Poly 1:C [1]	64.3	21.1	138.1	38.6	9.3	87.2	0.338
LPS [1]	247.2	124.6	448.5	190.8	126.9	500.1	0.331
None [2]	**47.5**	28.9	93.4	**22.2**	4.3	61.6	**0.032**
Der p 1 [2]	39.4	11.4	79.2	21.3	8.7	44.9	0.152
OVA [2]	35.8	18.8	92.1	22.5	9.5	54.3	0.186
Sal s1 [2]	54.6	26.4	97.3	33.1	19.7	53.4	0.066
PHA [2]	**224.9**	62.5	429.5	**67.0**	26.6	129.4	**0.01**

Values are median (interquartile range (IR)), Mann-Whitney test p-values
The limit of detection was 2.8 pg/ml. When the value was below the LOD was set at LOD/2=1.4 pg/mL
[1]24 hour culture, [2]48 hour culture

Table 5.10: Interleukin -2,-4,-5, and Interferon-γ concentrations (pg/mL) and ratios of IFN-γ/IL-4 and IFN-γ/IL-10 in the medium of cultured CBMCs collected at birth and stimulated with a mitogen (PHA) for 48 hours after subtraction of the concentrations in the unstimulated controls

Cytokine	Control group (n=30)			Salmon group (n=27)			p
	Median	IR		Median	IR		
		25^{th} perc	75^{th} perc		25^{th} perc	75^{th} perc	
IL-2	**195.1**	85.6	505.3	**68.2**	27.5	184.0	**0.012**
IL-4	**5.6**	1.1	12.3	**0.0**	0.0	5.9	**0.019**
IL-5	**11.1**	2.5	32.3	**3.7**	0.0	19.3	**0.031**
IFN-γ	109.2	30.8	232.4	83.0	20.7	174.6	0.243
IFN-γ/IL-4	19.2	3.7	55.8	14.6	8.0	40.8	0.754
IFN-γ/IL-10	2.4	1.1	8.3	2.7	0.7	5.6	0.767

Values are median (interquartile range (IR)), Mann-Whitney test p-values

Table 5.11: Interleukin-10 concentration (pg/mL) in the medium of cultured CBMCs collected at birth and stimulated with various TLR ligands, allergens and mitogens for 24 or 48 hours after subtraction of the concentrations in the unstimulated controls

Stimulant	Control group (n=29)			Salmon group (n=26)			p
	Median	IR		Median	IR		
		25th perc	75th perc		25th perc	75th perc	
PGN [1]	110.5	20.6	257.4	54.7	22.3	93.4	0.071
Poly 1:C [1]	-2.1	-18.3	6.8	0.5	-10.5	4.3	0.578
LPS [1]	**99.5**	32.1	211.7	**56.4**	14.5	84.4	**0.036**
Der p 1 [2]	-7.4	-17.0	0.0	-0.5	-13.5	1.2	0.247
OVA [2]	0.0	-7.5	8.5	0.0	-1.8	6.0	0.872
Sal s1 [2]	5.3	-5.0	20.0	3.8	0.1	11.4	0.879
PHA [2]	37.3	8.7	105.9	22.5	3.5	50.7	0.150

Values are median (interquartile range (IR)), Mann-Whitney test p-values
The limit of detection was 2.8pg/ml. When the value was below the LOD was set at LOD/2=1.4 pg/ml
[1]24 hour culture, [2]48 hour culture

Table 5.12: Tumor necrosis factor-α concentration (pg/mL) in the medium of cultured CBMCs collected at birth and stimulated with various TLR ligands, allergens and mitogens for 24 or 48 hours after subtraction of the concentrations in the unstimulated controls

Stimulant	Control group (n=29)			Salmon group (n=26)			p
		IR			IR		
	Median	25th perc	75th perc	Median	25th perc	75th perc	
PGN [1]	460.2	180.1	1505.7	432.1	154.1	896.9	0.553
Poly 1:C [1]	-10.9	-43.3	9.1	-3.6	-41.6	3.7	0.735
LPS [1]	136.2	33.5	323.1	125.7	45.1	315.4	0.877
Der p 1 [2]	-16.6	-26.5	2.8	-4.2	-19.2	0.0	0.404
OVA [2]	-12.3	-30.4	1.4	-1.5	-12.9	1.7	0.174
Sal s1 [2]	-9.4	-33.3	16.2	0.1	-119.6	9.5	0.543
PHA [2]	49.8	8.1	327.2	25.2	9.7	103.8	0.338

Values are median (interquartile range (IR)), Mann-Whitney test p-values
The limit of detection was 2.8 pg/ml. When the value was below the LOD was set at LOD/2=1.4 pg/mL
[1]24 hour culture, [2]48 hour culture

5.3.4 Production of prostaglandin E_2 by cord blood mononuclear cells cultured with various stimulants

Table 5.13 presents the concentration of PGE_2 after stimulation of cultures of CBMCs with LPS for 24 hr or PHA for 48 hr. Although CBMCs from the salmon group produced a lower concentration of PGE_2 in all conditions tested, this did not reach significance, although the trend towards a difference was strong in response to LPS (p=0.056). Figure 5.15 depicts the PGE_2 production in response to LPS. When PGE_2 (unstimulated and stimulated CBMCs with LPS and PHA) was correlated against EPA, ARA and ARA/EPA content in CBMCs, no significant associations were identified.

Table 5.13: Prostaglandin E_2 concentration (pg/mL) in the medium of cultured CBMCs collected at birth and stimulated with TLR-4 ligand (LPS) and a mitogen (PHA)

Stimulant	Control group (n=32)			Salmon group (n=29)			
		IR			IR		
	Median	25th perc	75th perc	Median	25th perc	75th perc	p
None [1]	2539	799	4191	1971	559	4456	0.563
LPS [1]	**4463**	1577	6773	**2518**	419	5951	**0.056**
PHA [2]	3874	1829	8456	2365	322	6068	0.129

Values are median (interquartile range (IR)), Mann-Whitney test p-values
[1] 24 hour culture, [2] 48 hour culture

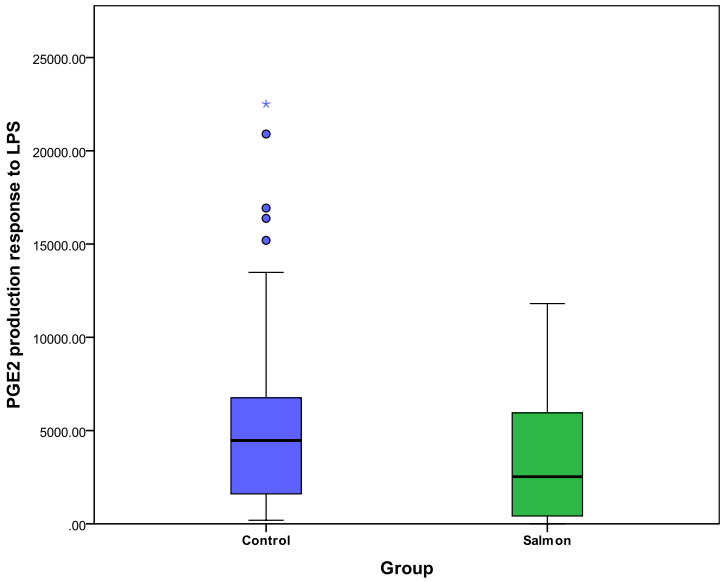


Figure 5.15: PGE_2 production (pg/mL) from CBMCs after stimulation with lipopolysaccharide (LPS; TLR-4 ligand) according to study group
Mann-Whitney test p-value 0.056

5.3.5 Cord blood plasma IgE concentration

Figure 5.16 shows the concentration of IgE in umbilical cord blood plasma at birth. There was no difference between groups.

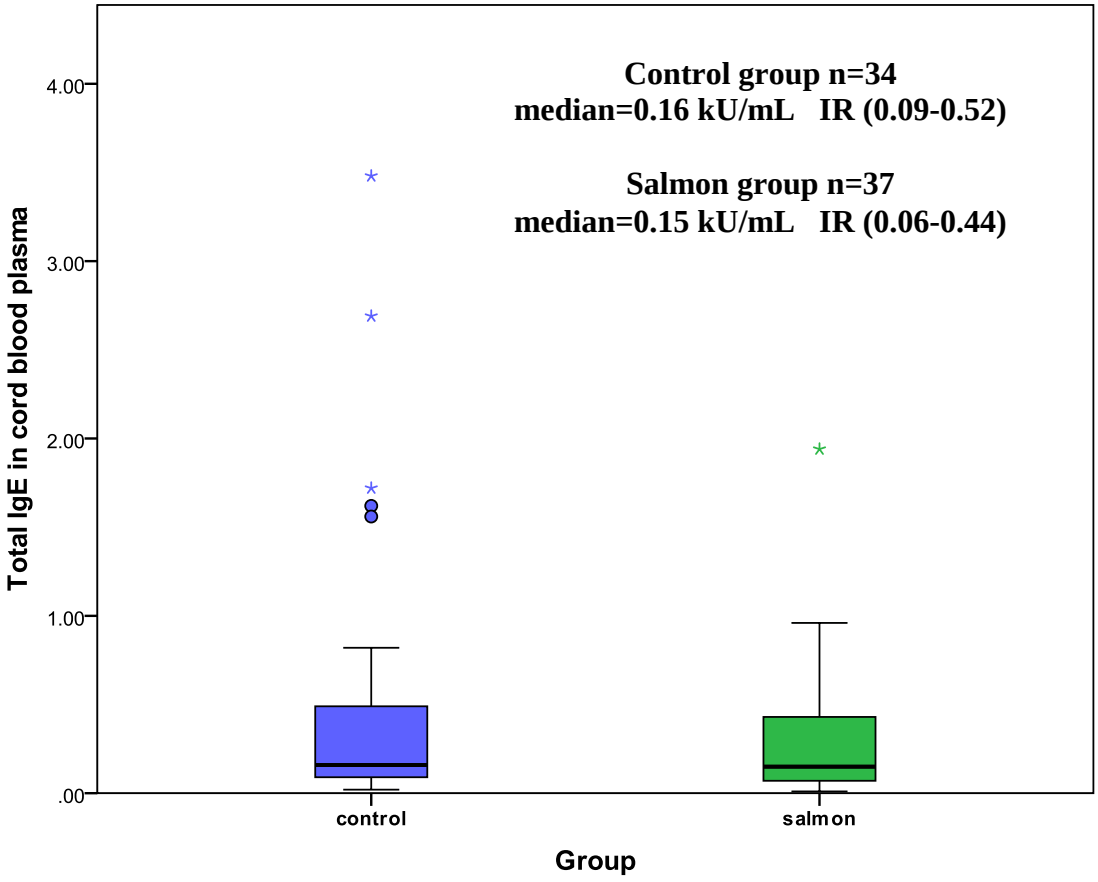


Figure 5.16: Concentration (IU/mL) of total IgE measured in cord blood plasma according to study group
Values are medians and interquartile range (IR), p-value=0.616 (Mann-Whitney test)

5.3.6 Cord blood plasma IL-13 concentration

IL-13 was not detected in most samples; IL-13 was detectable in three samples in the control group and two in the salmon group (7% of samples in total), the concentration in these five samples ranged from 0.2 to 156.7 pg/mL.

5.4 Discussion

In Chapter 4 it was shown that increased intake of salmon during pregnancy results in higher total n-3 PUFA status in cord plasma and in a tendency to higher total n-3 PUFA in CBMCs. Because of the importance of LC n-3 PUFAs in regulating aspects of the immune and inflammatory responses (48), salmon intake could influence the development of infant immune system and predisposition of the infant to atopy. Consequently, the hypotheses of the work described in this chapter are that the maternal oily fish intervention will:

1. alter neonatal immune cell phenotypes;
2. decrease PGE_2 production by cultured CBMCs;
3. alter neonatal innate and adaptive immune T-cell responses (increased Th1, decreased Th2, Treg) measured as cytokine expression in plasma and by cultured CBMCs;
4. decrease cord plasma IgE concentration.

The results obtained indicate that maternal salmon intake during pregnancy results in some differences in cord blood immune cell phenotypes at birth (more leukocytes, neutrophils, lymphocytes, helper T-cells, cytotoxic T-cells and Treg cells), a generalised decrease in production of cytokines in response to a T cell mitogen, TLR agonists and allergens, that became significant for several cytokines in response to the mitogen PHA and the TLR-4 ligand LPS, and a tendency to lower PGE_2 production in response to LPS. The cord plasma concentration of IgE was not affected by the salmon intervention. IL-13 was not detected in most cord plasmas and no other cytokines were measured in those samples. Thus, there is evidence to support two of the hypotheses (altered immune cell phenotypes and altered T cell responses) and to support in part a third (decreased PGE_2 production) while there is no evidence in support of the fourth (decreased IgE concentration).

These findings will be discussed in relation to the findings of studies using LC n-3 PUFAs from fish oils as an exposure in pregnancy (observational or intervention studies) and measuring immunological parameters in the cord blood of the infants compared to the controls. There are two such studies: the first was conducted in Australia by Dunstan *et al.* (35;131;132;136;173;176) and the second in Germany by Krauss-Etschmann *et al.* (178). Dunstan *et al.* examined the effect of maternal fish oil supplementation providing 3.7 g LC n-3

PUFAs/day on immune outcomes in cord blood (IgE concentration, IL-13 concentration, cytokine production by CBMCs stimulated with various agents including a mitogen and several allergens) of neonates at risk of developing atopic disease. They also examined the effect of the supplementation on clinical development of atopic disease at 12 months of infant age (35;132;173;176). Krauss-Etschmann *et al.* examined the effect of supplementing pregnant women with 0.5 g/day DHA plus 0.15 g/day EPA, or 400 μg/day of methyl-tetra-hydrofolic acid, or both or placebo on modulation of allergy-related parameters in the cord blood (178).

5.4.1 Whole blood immune cell subsets at birth (cord blood) and the effect of increased salmon consumption

The immune cell subset analysis in combination with the immune cell numbers obtained from the SUHT Pathology Laboratory analysis showed that the numbers of T helper cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$) and Treg cells ($CD4^+CD25^+CD127^{low}$) were higher in the salmon group compared to the control group. The percentages of these cells in the lymphocyte and total leukocyte populations did not differ between the two groups, but the numbers of lymphocytes and total leukocytes (and also neutrophils) were higher in the salmon group. Dunstan *et al.* (35) observed no significant difference in the frequency of various lymphocyte subsets (% and absolute concentration) between the fish oil supplemented and the control group; they reported total T cells, T helper cells, cytotoxic T cells, NK cells and B cells. The current results for NK cells and B cells are in agreement with these earlier findings, as are the current findings for percentages of the T cell subsets. Kraus-Etschmann *et al.* observed that the number of NK cells ($CD16^+$) was significantly decreased in the fish oil group compared with the placebo group, as were $CCR3^+CD8^+$ T cells (178).

It should be clarified that the method used to identify the Treg cells in SIPS was not optimal; one antibody stain (to the FOXP3 transcription factor), which according to the literature (306) would definitely determine $CD4^+CD25^+CD127^{low}$ cells as Treg cells was not used. The reason for this omission is that the flow cytometer used for the SIPS analysis could only detect three antibody stains at the same time, and the use of a fourth stain was not possible.

One limitation of SIPS is that the cord blood immune cell subsets were characterised by extracellular surface markers and not intracellular markers which describe the functional properties of these cells. However, the production of Th1 and Th2 cytokines by unstimulated

and stimulated CBMCs was investigated as a measure to obtain information about the functional phenotype of these cells (discussed in the next section).

5.4.2 Infant innate and adaptive immune responses at birth from CBMCs cytokine production and cord plasma IL-13 concentration

SIPS used different stimulants in order to explore different aspects of the immune response including both innate and adaptive immunity. The allergens and one mitogen (PHA) were used to investigate adaptive immune responses, whereas innate immune responses were tested with stimulation of the CBMCs by TLR ligands (to TLR-2, TLR-3 and TLR-4). The adaptive immune responses are so-called because these are specific immune responses of the T cells to antigens, which for the purpose of SIPS were allergens. The T cells that respond are already sensitised to these allergens and have immunologic memory of this event, so that when are cultured ex vivo they will respond to the allergen. However, because the number of the T cells responding to specific allergens is relatively small, the culture time required for the expression of this response is long (48 hours). Nevertheless the cytokine-inducing capacity of all allergens tested was limited, especially in comparison with that of PHA or LPS, which do not act in an antigen-specific way. Thus it seems likely that the number of allergen sensitised cells in the CBMC samples studied here was low. Another stimulus which activates adaptive immune responses is the T-cell mitogen PHA. PHA activates T-cells non-specifically via the CD3 receptor present on their surface, so that all T-cells respond to the mitogen in a non-specific way and thus can be used as a positive control. There were strong cytokine responses to PHA.

Innate immune responses were investigated stimulating via TLR-2, -3, and -4 with specific ligands. TLR ligands stimulate mainly monocytes which express the different TLRs on their surface. TLR stimulation is non-antigen specific and TLRs being responsible to recognise generalised features of ‘invaders’ termed pathogen associated molecular patterns (PAMPs). The response to TLR ligands is quite rapid compared with T cell responses, because of the non-specific nature of the interaction with the responder cells.

In general cytokine responses were lower from CBMCs in the salmon group, although not always significantly so and after correcting for the unstimulated concentrations, the statistical significance for CBMC IL-10 and TNF-α responses after mitogen stimulation (PHA) was lost. Controlling for maternal smoking did not affect the findings (data not shown).

The only cytokine measured in plasma, IL-13, was detectable in less than 10% of the samples.

Dunstan *et al.* measured cytokines in the cord plasma (IL-4, -5, -6, -10, -12, -13, TNF-α, IFN-γ) and also production by CBMCs (IL-5, -10, -13 and IFN-γ) after stimulating with 3 allergens (HDM, OVA and cat hair extract (CAT)) or PHA. All cytokines were measured with ELISAs. In the cord plasma IL-13, but not the other cytokines measured, was lower in the fish oil group compared to the placebo reflecting the decrease in Th2 cytokines after the fish oil supplementation (35;132). Dunstan *et al.* could detect IL-13 in 64% of plasma samples in the control group and in 45% of plasma samples in the fish oil group with an assay with a limit of detection of 3 pg/ml. In the current study IL-13 was not detected in the majority (93%) of the samples, yet the limit of detection was 0.7 pg/ml.

In the study of Dunstan *et al*., IL-10 responses of CBMCs stimulated with HDM or CAT were significantly lower in the fish oil group. In fact, responses to OVA and CAT for all cytokines tended to be lower in the fish oil group but, apart from IL-10, the difference was not significant. Responses to PHA did not differ between the two groups for any cytokine (35;132). However, it is worthy noting that the cytokine responses of CBMCs to allergens (HDM, OVA and CAT) and mitogen (PHA) observed by Dunstan *et al*. were not entirely consistent with the clinical features of the infants assessed at one year of age (atopic dermatitis, recurrent wheeze, asthma, food allergy) (132). However, the observation of generally lower cytokine responses seen in SIPS agrees with the findings of Dunstan *et al.*, although these are some differences in the detail. For example, in SIPS all cytokine responses to PHA (apart from IFN-γ) were lower in the fish oil group. Dunstan *et al.* measured innate immunity in the cord blood mononuclear cells and found no differences in the proportions of lymphocytes or monocytes expressing HLA-DR either with or without stimulation with rhIFN-γ or in the level of expression (136).

Kraus-Etschmann *et al.* measured cytokine expression patterns in the cord blood by real time RT-PCR looking at the mRNA levels of Th2 associated molecules CCR4, IL-13 and IL-4, Th1 related molecules IL-1 and IFN-γ, and Treg related molecule TGF-β. Fish oil supplementation decreased mRNA levels of the Th2 related molecules compared with placebo, whereas levels of mRNA for Th1 cytokines remained unaffected. TGF-β mRNA expression, which has shown to be involved in tolerance towards allergens, was significantly higher in the fish oil

group (178). So this European study agrees with the Australian study in that the Th2 related molecules (chemokines and cytokines) are reduced with fish oil. In general the current results from SIPS are in agreement with this: production of the Th2 cytokines (IL-4 and IL-5) was lower in the fish oil group while production of the prototypical Th1 cytokine IFN-γ although lower in the fish oil group was not significantly different between groups. Thus it appears that fish oil decreases the Th2 type response with less of an impact on the Th1 type response. Indeed, although there was no significant effect on the IFN-γ to IL-4 ratio (Th1/Th2) this tended to be higher in the salmon group.

5.4.3 IgE concentration in cord plasma

Total IgE concentration was low in the cord blood plasma in both groups with no difference between them. The IgE assay used was of high sensitivity (0 kU/L) and that is why IgE was detectable in all samples examined. In contrast, Dunstan *et al.* could detect IgE in only in 5% of cord plasma; the lowest level of detection of the assay they used was 0.35 kU/L.

5.4.4 PGE_2 production from cord mononuclear cells

Eicosanoid mediators are involved in modulating the intensity and duration of inflammatory responses. Through actions on dendritic cells (DCs), T cell differentiation and Ig class switching in B cells, some eicosanoids such as PGE_2 are believed to play a role in promoting sensitization to allergens (48;182). Indeed, allergic inflammation in animal models is associated with increased PG and LT production. PGE_2 is a vasodilator, increases vascular permeability, inhibits the production of Th1 cytokines and primes naıve T cells to produce IL-4 and IL-5 (Th2 cytokines). PGE_2 also promotes Ig class switching in uncommitted B cells towards the production of IgE (284).

PGE_2 production from unstimulated and PHA-stimulated CBMCs did not differ between the two study groups, but PGE_2 production from LPS-stimulated CBMCs tended to be lower in the salmon group compared to the control group (p-value=0.056). Krauss-Etschmann *et al.* did not report PGE_2 production in infant immune cells at birth. Neither did Dunstan *et al.* although Prescott and Dunstan (176) showed that fish oil supplementation during pregnancy resulted in lower LTB_4 production and higher LTB_5 production by cord blood neutrophils. Cord neutrophil LTB_4 production was inversely related to LC n-3 PUFA status. It could be

speculated that increased provision of EPA and DHA from the mother to the fetus and subsequent decreased incorporation of ARA into CB immune cells and lower production of ARA lipid mediators (PGE_2, LTB_4) as well as increased production of the less potent EPA and DHA lipid mediators (PGE_3, LTB_5) from the neonatal immune cells might occur. This could possibly affect the offsprings' T-cell and B-cell function and predisposition to a less inflammatory environment in early life so that there is less risk of developing atopic disease in infancy and later in life. Atopy outcomes at age 6 months in the infants studied here will be reported in the next chapter (Chapter 6).

5.5 Conclusion

The neonatal immune data shows that eating two portions of salmon during pregnancy results in:

- some differences in cord blood immune cell phenotypes at birth (more leukocytes, neutrophils, lymphocytes, helper T-cells, cytotoxic T-cells and T regulatory cells);
- a generalised decrease in production of cytokines in response to a T cell mitogen (PHA), TLR agonists and allergens, that became significant for several cytokines in response to the mitogen PHA (IL-2, IL-4, IL-5, IL-10, and TNF-α) and the TLR-4 ligand LPS (IL-10, TNF-α);
- a tendency to lower PGE_2 production in response to LPS;

but does not affect IgE concentration in cord plasma. IL-13 was not detected in most cord plasmas and no other cytokines were measured in those samples. Thus, there is evidence to support two of the hypotheses (altered immune cell phenotypes and altered T cell responses) and to support in part a third (decreased PGE_2 production) while there is no evidence in support of the fourth (decreased IgE concentration). This is the first study demonstrating that increased salmon intake in pregnancy can affect fetal/neonatal immune responses. Some of the effects seen are similar to those reported from fish oil supplementation studies in pregnant women. Some of the immune outcomes affected relate to future risk of infant atopy and so it appears that salmon intake in pregnancy has the potential to reduce atopy risk. The next chapter will examine this possibility.

6 Salmon in pregnancy study (SIPS): Atopy outcomes at 6 months of infant age, infant feeding patterns, anthropometry and health

6.1 Introduction

Breast milk is normally the single source of nutrients for the infant during the period of rapid growth and development in the first moths of life. Besides providing the principal source of energy, breast milk fatty acids possess functional properties. Long chain (LC) n-3 and n-6 polyunsaturated fatty acids (PUFAs) (i.e. docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (ARA; 20:4n-6)) are essential for the development of infants' cognitive and visual functions (10;242) and have immunomodulatory properties (97;307;308).

Fish, especially oily fish, is a rich source of LC n-3 PUFAs. An inverse association between fish consumption and childhood symptoms of atopy has been observed in cross-sectional studies (163;309). Frequent maternal consumption of fish during pregnancy has been associated with reduced risk of infant eczema, asthma and rhinitis (151;153;155). Such observations are supported by observations that dietary LC n-3 PUFAs reduce the production of pro-inflammatory and pro-allergic eicosanoid mediators, including prostaglandins and leukotrienes, and thus may reduce the risk of atopic disease (48). Early infancy is believed to be the key time for the programming of the immune system (202). Thus, the fatty acid composition of the diet in early infancy (or of the mother's diet during pregnancy) may have a causal influence on risk of developing atopy. This may relate to fatty acid composition of the infants immune cells and their capacity to produce fatty-acid derived mediators that influence sensitization and allergic responses. A number of studies have examined the fatty acid (FA) profile in breast milk as a risk factor for subsequent atopic disease (172;310-313). These studies have shown mixed results, but have generally found that a higher content of LC n-3 PUFAs in breast milk is associated with lowered risk of atopic diseases. In contrast, Stoney *et al.* reported from the Melbourne Atopy Cohort Study, that LC n-3 PUFAs in colostrum were unexpectedly associated with increased risk of allergic sensitization at 6 and 24 months (314). Using further data from the same prospective birth cohort, Lowe *et al.* found that high levels of docosapentaenoic acid (DPA; 22:5n-3) in both colostrum and expressed breast milk were associated with an increased risk of infantile atopic eczema, and higher concentrations of n-6 PUFAs in colostrum were associated with increased risk of childhood rhinitis at 7 years of age. There were no associations observed between the FA profile and risk of childhood asthma (315). A review by Sala-Vila *et al.* (284) looked at the PUFA concentrations in breast milk from atopic women. They found that the studies reporting PUFA concentration in breast milk

of atopic women are contradictory and with inconsistent effects when examining α-linolenic acid (18:3n-3; ALA) and DHA (284). In SIPS the breast milk fatty acid composition was analysed but the results are not included in this thesis. Breast milk samples were collected at days 1, 5, 14 and 28 after birth and the fatty acid analysis has shown that LC n-3 PUFA status (both EPA and DHA) at all timepoints was higher in the salmon group compared to the control group (unpublished data: Heidi Urwin & Parveen Yaqoob). Thus, SIPS infants in the salmon group who were breast fed would have received a better supply of LC n-3 PUFAs than those in the control gtroup.

The LC PUFA composition of term formula milks has been regulated by EU law for many years. At present, about half of term infant formulas on the UK market are fortified with LC PUFAs and numbers are increasing (316). However, FA intake and FA biomarkers of infants under these conditions need to be investigated. Schwartz *et al.* investigated plasma LC PUFAs in fully milk fed infants that continued consuming breast milk beyond 4 months and those that consumed formula (either fortified with or without LC n-3 PUFAs) beyond 4 months. The main findings were that infants consuming LC PUFA-supplemented formula achieved similar relative plasma levels of LC n-3 PUFAs to breastfed infants, and that those infants consuming non-supplemented formula had lower levels of these important fatty acids (317).

The issue of introduction of solids in relation to allergy development has been widely explored but the conclusions that can be drawn at present are not definite. Kneepkens *et al.* support that breastfeeding for 4-6 months and the postponement of supplementary feeding (solids) until 4 months of age are the main measures considered effective for allergy prevention. Although meta-analyses suggest that hypoallergenic formula after weaning from breastfeeding is protective against the development of allergic disease, the evidence is limited and weak (308). Anderson *et al.* reviewed from currently-available research that introducing solids at 4-6 months may result in the lowest allergy risk (318). However, Fiocchi *et al.* concluded that with assessed risk of allergy, the optimal age for the introduction of selected supplemental foods should be 6 months, dairy products 12 months, hens' egg 24 months, and peanut, tree nuts, fish, and seafood at least 36 months. For all infants, complementary feeding could be introduced from the sixth month, and egg, peanut, tree nuts, fish, and seafood introduction require caution (319). A 2008 report from American Academy of Pediatrics concluded that although solid foods should not be introduced before 4 to 6 months of age, there was no current convincing evidence that delaying their introduction beyond this period had a

significant protective effect on the development of atopic disease regardless of whether infants were fed cows' milk protein formula or human milk. This report did not consider that there is stron evidence for delaying the introduction of foods that are considered to be highly allergic, such as fish, eggs, and foods containing peanut protein (320).

6.1.1 Clinical outcomes of atopic disease

The prevalence of allergic diseases in childhood (atopic dermatitis, asthma, and allergic rhinitis and conjunctivitis) has increased considerably in developed countries in the last three or four decades (70), and accordingly there is increasing need for allergy testing. Population-based studies have reported a cumulative prevalence of allergic diseases in childhood around 25-30%, of atopic dermatitis (AD) around 15-20%, and of asthma between 7-10% (321). In infancy the main symptoms of possible allergic nature are AD, gastrointestinal symptoms, and recurrent wheezing, whereas bronchial asthma and allergic rhinitis and conjunctivitis are the main problems later in childhood. Adverse reactions to foods, mainly cows' milk protein and hens' egg are most common in the first years of life, whereas allergy to inhalant allergens mostly occurs later. Correspondingly, specific immunoglobulin (Ig) E antibodies against milk and egg are most frequent during the first 2–3 years of life, whereas IgE against inhalant allergens is predominant later in childhood. Interestingly, IgE antibodies to allergens (hens' egg white, cows' milk) in infants predict sensitization to inhalant allergens and allergy before 7–10 years of life. Low levels of specific IgE to several allergens - food allergens as well as inhalant allergens - are a normal phenomenon, especially in early childhood and may have no clinical significance (321). Infants with early development of IgE sensitization to cows' milk or hens' egg proteins or early sensitization to inhalant allergens have an increased risk for later development of allergic diseases. Early identification of children with food allergy and AD may provide opportunities to prevent the development of asthma (322). In early infancy food allergy with manifestations from skin, the gastrointestinal or the respiratory tract is more common than inhalant allergy. Children with food allergy almost always show symptoms from two or more organ systems concomitantly, but in some cases, e.g. AD, the children may only show one severe persisting symptom. Cows' milk allergy is the most common food allergy in young children followed by allergy to hens' egg, cereals, and nuts. A few children are sensitized to indoor inhalant allergens already during the first 1-2 year of life, and in case of persisting asthma symptoms, allergy testing for relevant indoor allergens such as houst dust mite and cat hair may be useful.

The potential role of n-6 and n-3 PUFAs in modulating risk of atopic disease has been described previously (202;203). There is quite good evidence from observational and intervention trials that early exposure to LC n-3 PUFAs induces immune effects that may be associated with reduced atopic sensitisation and with a reduction in allergic manifestations. However, data available from existing studies, which are of many different types, are not entirely consistent and so it is not possible to draw a firm conclusion at this stage.

There are no published intervention trials with fish during pregnancy. Since eating fish is advised, but fish area also a source of contaminants, aquaculture of fish to have low contaminant levels could be an ideal solution to enable oily fish consumption by pregnant women and in order to achieve the recommended intake of LC n-3 PUFAs. The Salmon in Pregnancy Study (SIPS) is the first randomised controlled trial with oily fish in pregnant women. SIPS focuses on pregnant women whose offspring have increased risk of developing atopic disease, aiming to identify whether there is an effect on atopy outcomes in the offspring. A total of 123 women were randomly assigned to consume either 2 portions of farmed salmon per week (from week 20 until delivery) or to continue their habitual diet low in oily fish (less or equal to two portions per month).

The aims of the work described in this chapter are to describe the feeding patterns of the infants born to the SIPS mothers at 6 months of age, to identify whether oily fish consumption in pregnancy affects the introduction of particular foods like fish to the infants (up to age 6 months), and to investigate the effect of the increased maternal oily fish consumption on atopy risk at 6 months of age.

The hypotheses of the work described in this chapter are that the maternal oily fish intervention will:

1. reduce sensitization to common allergens in infants at 6 months of age (determined by SPT)
2. reduce AD score in infants at 6 months of age (determined by clinical assessment-scoring for atopic dermatitis (SCORAD))
3. decrease plasma IgE concentration in infants at 6 months of age.

6.2 Materials and methods

6.2.1 Study design and subjects

Chapter 2 describes the study design, the subjects' characteristics, and the intervention. In summary, the population studied were pregnant women with high risk of having an atopic offspring. The study groups were two, of which the control group (n=61) was asked to keep their habitual diet unchanged during the study period (from 20 weeks until delivery) and the salmon group (n=62) which was asked to consume 2 portions of farmed salmon per week (from 20 weeks until delivery). Every portion of salmon provided 1.73 g of EPA plus DHA; the weekly intake of LC n-3 PUFAs was 3.46 g and the daily intake was 494 mg/day. The nutrient analysis of salmon is described in Chapter 2; Table 2.3. The consumption of the study salmon provided more EPA plus DHA than the recommended minimum intake in the UK for pregnant women (450 mg/day; (26)). The total number of infants at birth was 107; 53 in salmon group and 54 in the control group and 101 cord blood samples were collected (n=51 salmon group, n=50 control group). At 6 months of infant age 86 infants were followed up at a family visit (mother, father and sibling) at the Welcome Trust Clinical Research Facility (WTCRF) of Southampton General Hospital (SGH). An infant health and food frequency questionnaire (FFQ) was completed that also included clinical assessment of atopic disease by scoring AD (SCORAD) and skin prick testing (SPT) was performed. The FFQ referred to the 7 days prior to the 6 month visit. An infant blood sample was collected for IgE measurement (total and specific).The parents (mother and father) were also assessed for atopy by SPT and these results are included in the description of the subjects at baseline (Chapter 2).

6.2.2 Infant follow-up (6 month) questionnaire

The aim of the follow-up questionnaire was to collect personal details and information on general health and feeding of the baby at 6 months of age. The questionnaire included a visit checklist for the appointment, the baby's personal details, questions on general health, history of immunisation and atopy, feeding history over the 6 months prior to the visit, FFQ referring to infant food consumption over the 7 days preceding the visit, and results of the infant clinical examination (SCORAD) and SPT (Appendix 20).

6.2.3 Infant dietary assessment

6.2.3.1 Infant feeding and infant health diary

At 38 weeks of pregnancy SIPS mothers were provided with an infant feeding and health diary to keep from birth until 6 months of infant age (Appendix 12). They were asked to record on a daily basis in three separate columns: 1. the type of feeding they used (breast or formula) as well as the brands and types of the formula milks or other milks used; 2. the introduction of solids and relevant details of weaning (timing/specific food item/quantity); 3. any health issues the baby had including sicknesses such as colds, coughs, vomiting, diarrhoea, wheezes, rashes, any medication taken/prescribed as well as a record of the completed vaccinations. The purpose of keeping this diary was to help the mothers for reference and to remember details of the feeding and health of the infant during the administration of the 6 month infant health and FFQ (follow-up questionnaire) at the 6 month visit. These diaries were not analysed as a separate piece of data. The use of these diaries is an advantage of SIPS since a 6 month period is a long time to remember accurately and precisely details for example of all the formula milks that the baby had, and the duration of consumption of each of them or how many times the baby had a cold/cough or runny nose in the past 6 months. Of course the completeness and accuracy of the diary depended on individuality - a factor that could not be controlled for - personal motivation and interest.

6.2.3.2 6 month questionnaire: Infant feeding history and FFQ

The aim of the FFQ at 6 months of age (section 1-3 of the infant FFQ; Appendix 20) was to collect detailed information about the baby's breast milk and formula feeding, as well as the introduction of other food items and supplement usage. The infant feeding questionnaire (feeding history) reflected questions about feeding patterns (breastfeeding/formula feeding) over the first 6 months of life including types of formulas used during this period. The infant FFQ was administered by an appropriately trained nutritionist (myself, L-S Kremmyda) during a structured interview lasting around 30 minutes with the infant's carer supplying the relevant information. The FFQ was developed to assess the diets of infants aged 6 months, and was the FFQ was used for dietary assessment in the infant population as part of the SWS (323). It includes meat, fish, vegetables, fruit, cereals and snack foods divided into 16 food groups and 6 types of non-milk drinks. Human milk, baby formulas and other milk intakes, and introduction of solids (timing/kind of solid), were recorded in separate sections.

The daily non-human milk intake in the previous week was estimated from the average total volume of bottle feeds consumed per day; for human milk, the usual feed length and number of feeds per day were recorded. The frequency of consumption and the amount of each food in the 34 item-FFQ consumed over the last week preceding the 6 month visit were recorded. Foods not listed in the FFQ but consumed in the last week, were recorded in an open section. Also in case there was consumption of fish recorded in the FFQ, a further question was asked about type/types of fish consumed in the past week, and the portion size on each occasion was recorded.

Subjects were asked to recall their infants' intake during one week prior to the time point of the interview. To help them with remembering, subjects were prompted to keep a detailed record of that last week of their infants' food intake (types of food and quantities) in the infant feeding diary. Another help to recall quantities consumed and portion sizes was the presentation and reference to household measures (spoons/cups in different sizes) during the administration of the questionnaire as well as the use of prompt cards with the food items. The main purpose of the portion size measures was to ensure consistent responses. The question 'number of times in the past week' referred to the frequency of consumption and 'average amount on each occasion' was the question asked about the portion size.

This questionnaire has been validated for use on 6 month old infants in Southampton (324). The energy and nutrient intakes obtained from the FFQ were compared with those obtained from a 4-day weighted diary. The authors concluded that although there were differences in absolute energy and nutrient intakes between methods, the correlation coefficients indicated reasonable agreement in the ranking of intakes. Thus the 6 month FFQ is a useful tool for estimating energy and nutrient intakes in infants about 6 months old especially in the Southampton area (324). So the use of this FFQ could be considered as an advantage of the SIPS study. Previous studies have mainly used estimated or weighed records to assess diet at around 6 months of age (325;326). However such methods are expensive to process in larger groups of subjects. FFQs have been used for older age groups to assess diet in large populations but few researchers have examined this tool in infants. One Finnish study (327) reported food and milk patterns by using semi-quantitative FFQs.

The nutrient of focus in SIPS was LC n-3 PUFAs. Gale *et al.* estimated the DHA exposure of infants up to 6 months of age from the feeding history, using average milk volumes for each month of feeding together with the DHA content of the formula milk fed (obtained from the manufacturers). For breast milk they used an estimated value for the UK (9.56 mg per 100 g) (328). In SIPS DHA intake could be estimated in a similar way but it was decided not to do this because it would be a rough estimate and this was not the primary outcome of the study. Nevertheless, DHA was assessed by the FFQ reflecting the intake from the total diet plus supplements. Moreover, from the feeding history information on consumption of formula milks fortified or not with LC n-3 PUFAs (EPA and DHA) and n-6 PUFAs (ARA) was obtained. Also information on the nutritional content of all the formula milks consumed was obtained from the manufacturers so as the calculate PUFA intakes from the total diet to be as accurate as possible.

The SWS nutrition team declared the following nutrients to be complete to calculate daily intakes: protein, fat, carbohydrates, energy values (KJ/Kcal), fibre, sodium, potassium, magnesium, calcium, phosphorus, iron, copper, zinc, retinol, vitamins D, E, thiamin, riboflavin, niacin, vitamins B6, B12, C, and folate.

6.2.4 Infant anthropometry at 6 months of age

Infant length (cm) and weight (kg) were measured (Appendix 20).

6.2.5 Infant clinical assessment at 6 months of age

6.2.5.1 Skin prick testing (SPT)

SPT was performed by introducing a small amount of allergen (droplet), approximately 0.1µl of extract, into the epidermis using a lancet without drawing blood. The immediate wheal and flare reaction is a consequence of allergen-specific IgE molecules being cross-linked by allergen. This results in signalling via Fcε receptors on mast cells which leads to degranulation and release of histamine, proteases, proteoglycans, prostaglandin D_2 (PGD_2), leukotriene C_4 (LTC_4) and platelet-activating factor. Histamine is considered to be the major mediator in the allergic wheal reaction, which is caused by extravasation of serum from capillaries in the skin. Pruritis and a larger erythematous flare accompany the wheal. SPT is a reliable test to

determine sensitivity and subsequent confirmation of allergy in susceptible individuals, even in infants at 6 months of age. All SPTs were conducted by a trained research nurse (Norma Diaper). The SPT was performed using allergen extracts from house dust mite, tree, grass pollen, cows' milk, hens' egg, cat, dog, salmon as well as histamine (positive control) and glycerin (negative control) (Appendices 16 & 17). Table 6.1 presents the allergens and controls used. The potency is expressed in 'Histamine Equivalent in skin Prick testing' (HEP) which is related to the allergic activity of the allergen. All allergens apart from salmon (purchased from Merck) including the positive control and negative control were purchased from Soluprick SQ, ALK Abello. Figure 6.1 shows the application of the SPT, while Figure 6.2 shows the skin area approximately 15-20 minutes after application, demonstrating positive responses to some allergens. A wheal diameter of ≥ 2 mm greater than the negative control was considered to be positive in the current study.

Table 6.1: Allergens used in the infant SPT

Type	Strength
Negative control (glycerine)	Glycerine diluent
Dermatophagoides pteronyssinus (House Dust Mite (HDM))	10 HEP
Felix domesticus (cat)	10 HEP
Canis familiaris (dog)	10 HEP
6 Grass pollen mix (Avena/Dactylis/Poa/Festuca/Lolium/Phleum)	10 HEP
3 Tree pollen mix (Alnus/Betula/Corylus)	10 HEP
Cows' milk raw (Bos spp)	1:20 w/v
Whole hens' egg (Gallus spp)	1:100 w/v
Salmon	5000 NPU/mL
Positive control (histamine)	10 mg/ml

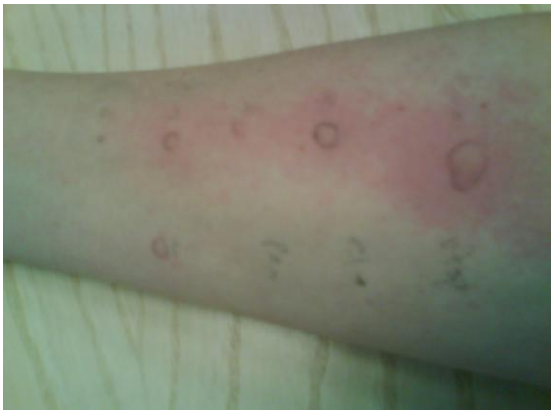


Figure 6.1: SPT application at forearm; Figure 6.2: SPT responses to some allergens

6.2.5.2 Score of atopic dermatitis (SCORAD)

The presence and severity of AD can be quantified by using an international clinical measuring tool, SCORAD. SCORAD has been previously validated in other studies (329;330). SCORAD was assessed with evaluation of the extent, intensity and subjective criteria. Total SCORAD is calculated from the proportion of involved surface area segment by segment. Calculations were made using the body map form (Appendix 18 and 20 (section 9)). SCORAD was assessed by a trained research nurse (Norma Diaper).

On completion of clinical examination any significant clinical findings were discussed with the parents as well as with the clinical supervisor. If further action was required after the assessment, this was advised.

6.2.6 Infant immunological assessment at 6 months

6.2.6.1 Plasma total and specific IgE concentrations

A specialised paediatric nurse collected a small amount (1-2 mL) of infant blood from a hand or foot vein into a plain tube (clot and gel). This blood was analysed by the Chemical Pathology Laboratory of SUHT for total IgE and for specific IgE to cows' milk, hens' egg and salmon. The analysis was done on Phadia ImmunoCAP 250 and the lowest detection of the

total IgE was 5.00 kU/L and of the specific IgE 0.35 kU/L. The test is designed as a sandwich immunoassay which provides high sensitivity and very low concentrations of IgE antibodies can be detected.

6.2.6.1.1 Total IgE

Anti-IgE, covalently coupled to ImmunoCAP, reacts with the total IgE in the infant's cord plasma sample. After washing, enzyme-labelled antibodies against IgE were added to form a complex. After incubation, unbound enzyme-labelled anti-IgE was washed away and the bound complex was then incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate was measured. The response was then transformed into a concentration through the calibration curve.

6.2.6.1.2 Specific IgE

The allergen of interest, covalently coupled to the ImmunoCAP, reacts with the specific IgE in the infant's plasma sample. The rest of the assay procedure is as described for total IgE.

6.2.7 Statistical analysis

Between group comparisons for data that were not normally distributed data were performed using the Mann-Whitney test, as a non-parametric 2 independent samples test, and the data are described using medians and interquartile ranges (infant feeding at 6 months: breastfeeding/formula feeding/exclusive breastfeeding duration). Between group comparisons for normally distributed data were performed using the independent samples t-test and the data are described using the means and standard deviations (SD) (IgE concentration, SPT and SCORAD data, feeding with formula milks, food and nutrient intakes, infant anthropometry). Categorical data were compared between groups by Pearson χ^2 (Chi-square) test (dichotomised clinical data, food categories and reported allergic associated infections at 6 months). A p-value of < 0.05 was considered statistically significant for all tests. The statistical package for Social Sciences (SPSS version 18) was used for all statistical tests.

6.3 Results

6.3.1 Infant diet at 6 months

6.3.1.1 Feeding history

Table 6.2 presents the feeding practises of the infants up to 6 months of age. The duration of breastfeeding did not differ between the 2 groups and was (median) 146 days for the control group and 145 days for the salmon group. Infants in the control group were exclusively breastfed for more days than the salmon group (median 44 vs. 27), but the difference was not significant. Infants in the salmon group received formula milks for (median) 152 days and the control for 124 days. This difference was not significant.

Table 6.2: Feeding practices of the infants up to 6 months of age according to study group

Infant feeding at 6 months	Control group N=39	Salmon group N=47	p	Total (n=86)
Breastfeeding duration (days)	146 (15, 180)	145 (14, 180)	0.981	146 (15, 180)
Formula feeding duration (days)	124 (14, 169)	152 (54, 170)	0.152	136 (46, 169)
Exclusive breastfeeding (days)	44 (10, 163)	27 (9, 125)	0.233	43 (10, 133)

Values are median (interquartile range) days in the last 6months, Mann-Whitney p-values

Table 6.3 shows the numbers and percentages of infants that were breastfed, fed with formula milks or still breastfeeding at 6 months. It is impressive that 43% of the infants in both groups (n=86) were still breastfeeding at 6 months of age (Table 6.3).

Table 6.3: Feeding practices of infants up to 6 months of age according to study group

	Control group (n=39)		Salmon group (n=47)		p
	Yes	No	Yes	No	
Ever breastfed	35 (89.7%)	4 (10.3%)	42 (89.4%)	5 (10.6%)	0.954
Still breastfeeding at 6 months	16 (41%)	23 (59%)	21 (44.7%)	26 (55.3%)	0.828
Formula fed	30 (77%)	9 (23%)	42 (89.4%)	5 (10.6%)	0.149

Values are number of infants (within group percentage); Chi-square test p-values

Table 6.4 shows the mean duration of consumption of formula milks containing LC n-3 PUFAs (EPA and DHA) and milks without such fortification in each group. There was no significant difference observed between the two groups in the duration of consumption of either milk category. Infants had consumed more days of formula milks with added EPA and DHA (85.5 days) than non-fortified formulas (26.5 days).

Table 6.4: Consumption frequency of formula milks either enriched or not with LC n-3 PUFAs according to study group

Formula milks	Control group (n=39)	Salmon group (n=47)	p	Total (n=86)
Feeding with LC n-3 PUFA fortified formula milks (days in the last 6 months)	71 (67)	97 (69)	0.082	86 (69)
Feeding with non fortified formula milks (days in the last 6 months)	33 (63)	21 (43)	0.311	27 (53)

Values are mean (SD) days in last 6 months, independent t-test p-values

6.3.1.2 Food intake at 6 months of age

Table 6.5 describes the mean weekly consumption frequency of breastmilk and the food groups as recorded in the FFQ. There were no differences between the two groups apart from consumption of vegetable jars that were more frequently consumed by the salmon group compared to the control (2.9 vs. 0.9 times per week).

Table 6.5: Consumption of breast milk or various foods according to the FFQ (referring to the past 7 days) at age 6 months according to study group

Food groups	Control group (n=39)			Salmon group (n=47)			p
	n	Times/week		n	Times/week		
		Mean	SD		Mean	SD	
Breast milk	39	3.05	3.52	47	3.04	3.47	0.991
Baby rice	38	1.74	2.62	47	2.47	3.76	0.295
Cereals	38	3.53	3.56	47	2.94	3.25	0.432
Rusks	38	1.21	2.17	47	0.81	1.62	0.347
Porridge jar	24	0.08	0.41	28	0.14	0.59	0.671
Meat jar	24	1.17	1.58	28	1.68	2.09	0.320
Vegetable jar	24	0.92	1.10	28	2.89	2.57	**0.001**
Milk dessert jar	24	1.00	2.19	28	0.71	1.36	0.585
Fruit dessert jar	24	0.58	1.02	28	1.21	1.66	0.101
Pure fruit jar	24	2.08	2.64	29	2.03	2.90	0.949
Potato	35	2.17	2.57	44	1.68	2.65	0.410
Rice	35	0.31	1.23	44	0.07	0.25	0.252
Pasta	35	0.54	1.20	44	0.30	0.88	0.310
Meat	35	1.43	3.01	44	0.86	2.43	0.371
Fish	35	0.09	0.28	43	0.26	1.09	0.332
Beans	35	0.26	0.82	44	0.09	0.29	0.258
Other vegetables	35	4.80	4.30	44	5.55	5.41	0.497
Yogurt	35	2.26	3.22	44	2.14	3.08	0.866
Cooked fruit frequency	35	2.57	2.92	44	2.80	4.15	0.780
Bananas	35	1.54	2.06	44	1.32	2.02	0.629
Other fruit	35	0.74	1.34	44	1.50	3.71	0.215
Bread	35	0.09	0.51	44	0.25	0.75	0.251
Biscuits	35	0.49	1.44	44	0.14	0.63	0.189
Juice	26	0.92	2.10	35	0.66	2.61	0.661
Water	28	5.82	4.51	36	6.25	5.24	0.726

Values are mean (SD) consumption (times/week), independent t-test p-values

Table 6.6 shows the number and proportion of infants in each treatment group that had any breast milk, expressed breast milk, formula or cows' milk, any jars, home made food and any other drink apart from breast milk in the last 7 days as recorded in the FFQ at 6 months. There were no statistical significant differences in the number of infants that consumed any of those food categories between the two groups.

Table 6.6: Number of infants that consumed breast milk, expressed breast milk, formula milk, jars, home made food, or other drinks as recorded in the 6 month FFQ referring to the past 7 days

Categories of food	Control group (n=39)		Salmon group (n=47)		p
	Yes	No	Yes	No	
Any breast milk	17 (43.6)	22 (56.4%)	21 (44.7)	26 (55.3%)	0.919
Any expressed breast milk	5 (12.8%)	34 (87.2%)	5 (10.6%)	41 (87.2%)	0.632
Any formula/cows' milk	31 (79.5%)	8 (20.5%)	39 (83%)	8 (17%)	0.783
Any jars	19 (48.7%)	18 (46.2%)	26 (55.3%)	18 (38.3%)	0.361
Any home made food	29 (74.4%)	10 (25.6%)	35 (74.5%)	11 (23.4%)	0.535
Any other drink (water/juice) apart from milk	22 (56.4%)	13 (33.3%)	30 (63.8%)	15 (31.9%)	0.519

Values are number (within group percentage); Chi-square test p-values

Table 6.7 presents the mean intake (g/day) of breast milk, expressed breast milk, formula milk and fish as recorded in the FFQ which referred to the 7 days prior to the 6 month visit, and also shows the infant age (in days) when solid foods were introduced into the diet. Moreover this table presents the duration of intake of infant supplements (in days) in the last 6 months. Only 2 infants in the control group and 3 in the salmon group were taking supplements at between 3 and 6 months of infant age. The between group comparisons showed no differences in the two groups, although fish intake (g/day) tended to be higher in the salmon group compared to the control (50 vs. 15 g/day).

Table 6.7: Aspects of infant diet up to 6 months of age according to study group

	Control group (n=39)			Salmon group (n=47)			p
	n	Mean	SD	n	Mean	SD	
Infant age when solids introduced (days)	39	132	40	47	147	62	0.181
Infant supplement intake (days in the last 6 mo)	2	5.5	6.4	3	15	15	0.381
Fish intake (g/week)	3	**15.4**	14.4	3	**50.0**	17.3	**0.058**
Intake of breast milk (g/week)	17	4210	1943	21	4834	2034	0.342
Intake of expressed breast milk (g/week)	5	430	369	5	92	82	0.111
Intake of formula milk (g/week)	6	4904	808	7	4960	1570	0.936

Values are mean (SD), independent t-test p-values

Table 6.8 shows in detail which solid foods were primarily introduced to the infants as recorded to the FFQ at 6 months. Most (n=56) infants were firstly introduced to baby rice as a solid food and less to vegetables, cooked fruit and baby cereals.

Table 6.8: Solid food introduction according to study group

Solid food introduction at 6 months		Control group (n=39)	Salmon group (n=47)	Total (n=86)
No solid yet introduced		2	1	3
First solid food eaten	Baby rice	25	31	56
	Baby cereal	2	2	4
	Vegetables	3	5	8
	Yogurt	2	0	2
	Cooked fruit	2	3	5
	Banana	1	1	2
	Other fresh fruit	0	1	1
	Rusks	1	1	2
	Dried vegetables	0	1	1
	Vegetable jar	1	1	2

Values are number

Table 6.9 shows the daily intake of selected macro- and micro-nutrients reflecting the 7 days prior to the administration of the FFQ at 6 months. As discussed in the methods, some nutrient intakes are estimations as the databases providing the relevant values were incomplete or the

manufacturers of the infant products did not provide such nutritional information. SFA, MUFA, PUFA, n-3 PUFA and n-6 PUFA intakes shown should be treated with caution as the SWS have declared these nutrients as incomplete. The between group comparisons showed no difference in the intake of any of the macro- or micro-nutrients with the exception of vitamin E which was significantly higher in the salmon compared to the control group.

Table 6.9: Energy and nutrient intakes in infants at 6 months of age according to study group

Nutrient	Control group (n=39)		Salmon group (n=47)		p
	Mean	SD/IR	Mean	SD/IR	
Energy (Kcal) [1]	685	173	720	154	0.330
Energy (KJ) [1]	2872	730	3016	645	0.336
Energy/kg body weight	364	96	394	90	0.144
Protein[1]	17.2	7.5	16.8	4.7	0.791
Total fat[1]	32.1	7.5	33.9	7.6	0.279
Carbohydrate (g)[1]	84.1	26.7	89.4	23.2	0.337
SFAs (g)[1]	14.0	4.0	14.7	3.4	0.393
MUFAs (g)[1]	11.2	4.2	12.6	3.3	0.095
PUFAs (g)[1]	4.5	1.9	5.0	1.2	0.123
Total n-3 PUFAs (g)[1]	0.24	0.28	0.27	0.31	0.612
Total n-6 PUFAs (g)[1]	1.26	1.43	1.53	1.62	0.421
Fibre (g)[1] (Englyst method)	4.1	3.9	4.4	3.4	0.753
Sodium (g)[1]	210	109	204	71	0.760
Potassium (g)[1]	840	361	865	261	0.716
Calcium (mg) [1]	499	242	491	169	0.869
Magnesium (mg) [1]	67.4	38.2	69.0	28.4	0.835
Phosphorus (mg) [1]	340	211	324	129	0.666
Iron (mg) [2]	4.8	1.3, 6.9	4.6	2.1, 6.5	0.886
Zinc (mg) [1]	4.5	1.9	4.6	1.3	0.835
Selenium (µg) [1,3]	11.3	3.7	11.7	2.6	0.573
Retinol equivalents (µg) [1]	578	317	571	147	0.902
Vitamin D (µg) [2]	7.6	1.0, 11.1	7.6	2.5, 11.0	0.907
Vitamin E (mg) [2]	5.5	3.3, 7.6	7.0	4.8, 8.4	**0.050**
Thiamin B1 (mg) [1]	0.63	0.45	0.61	0.30	0.856
Riboflavin B2 (mg) [1]	0.84	0.48	0.83	0.36	0.917
Niacin (mg) [1]	5.1	3.2	4.8	2.1	0.604
Vitamin B6 (mg) [1]	0.53	0.36	0.56	0.56	0.778
Vitamin B12 (mg) [1]	1.3	1.0	1.3	0.8	0.995
Folate (µg) [1]	105	53	115	45	0.319
Vitamin C (mg) [1]	73.7	35.4	77.6	26.5	0.574
EPA (mg) [2]	0.0	0.0, 0.01	0.0	0.0, 0.004	0.239
DHA (mg) [2]	0.003	0.0, 0.05	0.017	0.0, 0.047	0.332
Total EPA plus DHA (mg) [2]	0.004	0.0, 0.057	0.021	0.0, 0.056	0.302

[1] Values are mean (SD), independent t-test p-values

[2] Values are medians (interquartile range), Mann-Whitney test p-values

[3] Values completed for all foods in the FFQ - however data are limited as the content of food varies widely and depends on soil content of the region

6.3.2 Anthropometry, allergy and health status of the infants as reported in the 6 month questionnaire

Table 6.10 shows the height and weight of the infants at age 6 months; there were no differences between groups. Table 6.11 shows height and weight distribution of the infants at six months by sex.

Table 6.10: Height and weight at 6 months of age according to study group

	Control group (n=39)	**Salmon group (n=47)**	**p**	**Total (n=86)**
Height (cm)	66.9 (2.4)	67.3 (2.5)	0.438	67.13 (0.26)
Weight (kg)	7.95 (0.97)	7.72 (0.91)	0.282	7.82 (0.10)

Values are mean (SD), independent t-test p-values

Table 6.11: Height and weight at 6 months of age according to sex

	Control group (n=39)		**Salmon group (n=47)**	
	Male (n=21)	Female (n=18)	Male (n=19)	Female (n=28)
Height (cm)	67.4 (2.6)	66.4 (2.2)	68.3 (2.4)	66.7 (2.3)
Weight (kg)	8.03 (0.99)	7.85 (0.96)	8.15 (0.92)	7.43 (0.79)

Values are mean (SD)

Table 6.12 presents the atopy-associated events such as wheezing, pneumonia, chest infection, itchy skin, and dry skin, as reported by the parents. Also information on having pets at home, which could be associated with the protection or sensitization to atopy, is provided.

Table 6.12: Information on atopic phenomena and pets at home according to study group

	Control group (n=39)		Salmon group (n=47)		p
	Yes	No	Yes	No	
Wheezing	8	31	11	36	0.314
Pneumonia/bronchiolitis	1	38	1	46	0.894
Chest infection/bronchitis	2	36	1	46	0.332
Itchy skin	8	31	10	36	0.539
Dry skin	4	27	10	32	0.166
Cat at home	14	25	10	37	0.153
Dog at home	8	31	7	40	0.574

Values are number, Chi-square test p-values

Some questions in the 6 month follow up questionnaire referred to the mothers: 3/39 mothers in the control group and 1/47 in the salmon group smoked during the first 6 months of their infants' life. Moreover 13/39 mothers in the control group and 15/47 in the salmon group were taking supplements during the 3 months prior to the 6 month visit. The main supplements being used were: multivitamins, special multivitamin supplements for breastfeeding or pregnancy (with or without n-3 fatty acids), cod liver oil, vitamin C, iron, calcium and vitamin D.

6.3.3 Infant clinical outcomes at 6 months

Table 6.13 shows the presence and severity of AD and the frequencies of positive AD scores and the mean values of the AD scores for infants in both groups. Relatively few infants (19/86; 22.1%) manifested AD and severity of AD (SCORAD) in those infants with the disease was not different between groups although it tended to be higher in the control group (mean severity score = 10) compared to the salmon group (mean severity score = 7.4) (Table 6.13). Controlling for birth order, paternal atopy and maternal smoking, did not influence these findings.

Table 6.13: Atopic dermatitis at 6 months of age according to study group

Infant clinical outcomes at 6 months: SCORAD	Control group (n=38)	Salmon group (n=48)	p
Positive to SCORAD [1]	7/38 (18.4%)	12/48 (25%)	0.465
SCORAD [2]	1.84 (5.30)	1.85 (3.65)	0.990
AD severity score [2]	10.00 (8.851)	7.42 (3.450)	0.483

[1] Values are number (within group %), Chi-square test p-values
[2] Values are mean (SD), independent t-test p-values

Positive SPTs were defined as reaction to cows' milk and egg allergens. Table 6.14 presents the SPT positivity in the two groups and also the mean SPT responses to all allergens. Mean SPT responses to allergens were very low, reflecting that many infants were not sensitised (see below) and so did not show a response.

Very few infants responded positively to most of the allergens used (Table 6.15). Those that did respond were sensitized to cat hair, dog hair, hens' egg or cows' milk protein. There was no difference in pattern between control and salmon groups. No infants in the salmon group were sensitized to salmon. After examining whether SPT $\geq$ 2 mm between the 2 groups could be affected by birth order, paternal atopy and maternal smoking, birth order (p=0.032) and maternal smoking (p=0.007) proved to be confounding factors.

Table 6.14: Frequencies of any positive SPT, positive SPT to milk and egg sensitization and mean SPT responses to allergens according to study group

	Control group (n=38)	Salmon group (n=48)	p
Any SPT ≥ 2mm [1]	5/38 (13.1%)	6/48 (12.5%)	0.927
Any SPT ≥ 3mm [1]	1/38 (2.6%)	2/48 (4.2%)	0.686
SPT to cows' milk ≥ 2mm [1]	2/38 (5.3%)	2/48 (4.2%)	0.810
SPT to hens' egg ≥ 2mm [1]	4/38 (10.5%)	3/48 (6.3%)	0.471
SPT to negative control (mm) [2]	0.00 (0.00)	0.00 (0.00)	-
SPT to HDM (mm) [2]	0.00 (0.00)	0.00 (0.00)	-
SPT to cat (mm) [2]	0.05 (0.32)	0.06 (0.32)	0.888
SPT to dog (mm) [2]	0.00 (0.00)	0.08 (0.40)	0.159
SPT to grass pollen (mm) [2]	0.00 (0.00)	0.02 (0.14)	0.322
SPT to tree (mm) [2]	0.00 (0.00)	0.02 (0.14)	0.322
SPT to milk (mm) [2]	0.11 (0.45)	0.08 (0.40)	0.816
SPT to egg (mm) [2]	0.34 (1.15)	0.19 (0.76)	0.477
SPT to salmon (mm) [2]	0.00 (0.00)	0.00 (0.00)	-
SPT to histamine (positive control) (mm) [2]	3.61 (1.05)	4.10 (1.12)	0.037

[1] Values are number (within group %), Chi-square test p-values
[2] Values are mean (SD), independent t-test p-values

Table 6.15: Positive skin prick tests in infants at age 6 months according to study group

Allergen	Control group	Salmon group
Dermatophagoides pteronyssinus (House dust mite (HDM))	0	0
Felis domesticus (cat)	1	1
Canis familiaris (dog)	0	2
6 Grass mix pollen	0	0
3 Tree mix pollen	0	0
Salmon	0	0
Hens' egg	4	3
Cows' milk, raw	2	2

Values are frequency i.e. number

Table 6.16 describes total and specific IgE measured in the infant blood at 6 months of age and also the frequencies of the infants that had detectable specific IgE to hens' egg or cows' milk in the two study groups. Total IgE was not different between groups. Concentrations of specific IgE were low and at the limit of detection. Salmon-specific IgE was not different between groups. Controlling total IgE for birth order, paternal atopy and maternal smoking as potential effect modifiers did not change the findings.

Table 6.16: Total and specific IgE in infant serum at age 6 months according to study group

Infant clinical outcomes at 6 months: IgE	Control group	Salmon group	p
IgE to egg albumin detected (dichotomized) [1]	2/27 (7.40%)	2/32 (6.30%)	0.860
IgE to egg yolk detected (dichotomized) [1]	2/27 (7.4%)	0/32 (0%)	0.112
IgE to cows' milk detected (dichotomized) [1]	2/27 (7.40%)	0/32 (0%)	0.117
Total Ige (kU/L) [2]	7.98 (5.83)	8.59 (7.01)	0.701
IgE to egg albumin (kU/L) [2]	0.52 (0.62)	0.37 (0.11)	0.231
IgE to egg yolk (kU/L) [2]	0.37 (0.09)	0.35 (0.00)	0.161
IgE to cows' milk (kU/L) [2]	0.36 (0.04)	0.35 (0.00)	0.162
IgE to salmon (kU/L) [2]	0.35 (0.00)	0.35 (0.00)	0.387

[1] Values are number (within group %), Chi-square test p-values
[2] Values are mean (SD), independent t-test p-values

Table 6.17 shows the occurrence of the possible combinations between parental and infant SPT positivity/negativity. There were no differences between groups.

Table 6.17: Combination of maternal/paternal SPT with infant SPT according to study group

	Control group (n=38)	Salmon group (n=48)	p
Both maternal and paternal positive SPT and infant positive SPT	1 (2.6%)	2 (4.2%)	0.928
Maternal or paternal positive SPT and infant positive SPT	2 (5.3%)	3 (6.2%)	
No maternal/paternal positive SPT and infant positive SPT	1 (2.6%)	0 (0.0%)	
Either maternal/paternal positive SPT and infant negative SPT	17 (44.7%)	28 (58.3%)	
Maternal/paternal negative SPT and infant negative SPT	3 (7.9%)	1 (2.1%)	
Both maternal and paternal positive SPT and infant negative SPT	9 (23.7%)	13 (27.1%)	
Both maternal and paternal negative SPT and infant negative SPT	5 (13.2%)	1 (2.1%)	

Values are number (within group %), Chi-square test p-value, Positive SPT is defined as ≥ 2mm wheal diameter

6.4 Discussion

This chapter describes the feeding patterns of the babies born to the SIPS mothers at 6 months of age, the concentrations of total and specific IgE in the blood of those infants, the results of skin prick testing for sensitisation to several different allergens, and the assessment of AD in those infants all at age six months. There was no effect of maternal salmon consumption on plasma IgE, SPT positivity, and occurrence of AD or its severity. The hypotheses of the work described in this chapter are that the maternal oily fish intervention will reduce sensitization to common allergens in infants at 6 months of age (determined by SPT), reduce AD score in infants at 6 months of age (determined by SCORAD) and decrease plasma IgE concentration in infants at 6 months of age. The work described here does not provide support for any of these hypotheses. The lack of effect on IgE and on clinical outcome at 6 months is despite the differences in immune outcomes in umbilical cord blod observed between control and salmon groups (Chapter 5).

6.4.1 Clinical outcomes

The results obtained in this chapter indicate that the intervention that increased oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/week) during pregnancy, was not successful in reducing sensitization to common allergens, AD or IgE concentration. These findings will be discussed in comparison to observational and supplementation studies conducted during pregnancy (and lactation) in relation to LC n-3 PUFA exposure.

SIPS was powered according to an assumed sensitization to at least one common allergen in 45% of the infants participating in the control arm at 6 months of age, based upon the high risk nature of the pregnancies. In fact only about 13% of the infants in the control group were sensitized and so the study was underpowered to detect a difference in sensitization.

A higher than anticipated number of the SIPS infants were still breastfeeding and had not been introduced to a large amounts of solids. Breastfeeding and avoidance of solids are protective against sensitization (318;319) which might explain the low rate of sensitization seen. It will be prudent to re-examine sensitization and the other outcomes at a later age (24, 30 or 36 months). In fact a 30 month follow-up of the infants is about to begin.

As indicated above, SPT positive responses to any allergen were very low in both groups; 5 subjects in the control and 6 in the salmon group had any positive SPT response. Most of these infants were sensitized to hens' egg (4 in the control and 3 in the salmon group) or cows' milk (2 in each group), very few to cat (1 in each study group) or dog (2 in the salmon group) and none to the grass and tree pollens, or to salmon. The fact that no infant was sensitized to salmon antigen in the salmon group suggests that the increased maternal and fetal exposure to fish did not sensitize the infant immune system at least up to 6 months of life.

When combining the results of the maternal and paternal SPT responses with the infant SPT responses to the same allergens it was interestingly found that when the mother or the father or both parents had any positive SPT response, only 8/86 (3 in control and 5 in salmon group) infants had corresponding positive responses to any allergen whereas 67/86 infants had corresponding negative SPT responses to any allergen (26 in control and 41 in salmon group). Out of 7 infants who were SPT positive for egg protein, 6 had detectable anti-egg IgE. Also when combining the infant clinical results of IgE and SPT for egg and cows' milk, IgE was detected and SPT was positive to egg at the same time for 4/86 infants (2/38 in the control and 2/48 in the salmon group). For 3 infants (2 in the control group and 1 in the salmon group) the SPT to egg was positive but the specific IgE was below the detection level. Likewise, out of 4 infants who were SPT positive for cows' milk protein, 2 had detectable anti-cows' milk IgE. 2/38 infants in the control group and none in the salmon group were both positive for SPT and IgE to cows' milk protein. For 2 offspring in the salmon group the SPT was positive in response to cows' milk but no IgE was detected. It is interesting that the same pattern happened in response to both allergens: more positive responses were detected by SPT which were not confirmed by the IgE measurement.

6.4.2 Comparison to other studies investigating the effect of maternal LC n-3 PUFA intake

6.4.2.1 Observational studies

All five studies investigating the effect of maternal fish intake during pregnancy on atopic or allergic outcomes in infants/children of those pregnancies concluded protective associations (151-155). The study conducted by Salam *et al.* did not look at SPT or IgE to allergens but

reported asthma related outcomes (155). Romieu *et al.* found an increase in maternal fish intake during pregnancy from once per week to 2.5 times per week was associated with lower risk of eczema at age 1 year (OR 0.73; 95% CI 0.55–0.98; p=0.036), positive SPT for HDM at 6 years (OR 0.68; 95% CI 0.46–1.01; p= 0.058), and atopic wheeze at age 6 years (OR 0.55; 95% CI 0.31–0.96; p=0.034). Weekly maternal fish intake during pregnancy was inversely associated with eczema at 1 year (p=0.050), positive SPT for HDM at 6 years (p=0.048), atopic wheeze at 6 years (p=0.028) (151). Calvani *et al.* observed stronger and more significant beneficial effects of increased oily fish intake during pregnancy for children (mean age 5 years) of non-allergic mothers compared to those of allergic mothers for food (milk and egg) sensitizations (p for trend=0.002) but not for inhalant sensitizations. It is not clear why these findings were different (154). Willers *et al.* examined atopic eczema in children followed up to 5 years of age in a large cohort. Maternal total fish intake ≥ 1/week vs. never was associated with decreased risk of doctor diagnosed eczema (OR 0.57; 95% CI 0.35– 0.92; p-trend 0.008), current treated eczema (OR 0.58; 95% CI 0.32–1.06; p-trend 0.028), and ever having eczema (OR 0.68; 95% CI 0.43–1.10; p-trend 0.05) (153). Sausenthaler *et al*. reported eczema sensitization and IgE in infants followed up to 2 years in a large prospective cohort study. High maternal fish intake vs. low was associated with decreased doctor-diagnosed eczema risk in children at 2 years (OR 0.75; 95% CI 0.57–0.98; p<0.05) (152).

The large cohort studies conducted by Sausenthaler *et al.* and Willers *et al*. concluded similar associations: high (≥ 1 time/week) vs. low maternal fish intake during pregnancy was associated with decreased doctor-diagnosed eczema. The greater decrease in the study of Willers *et al.* compared to the study of Sausenthaler *et al.* (43% vs. 25%, respectively) could be related to the fact that the study of Willers *et al.* followed-up children for a longer period, allowing for manifestations of atopic disease to be revealed at the stage of clinical assessment.

Across these five studies, the extent of lowering atopic risk effect of maternal fish intake was highly variable. Fish intake resulted in a decrease in infant or childhood atopy which ranged between 25% and 95%. However, most decreases in atopic risk ranged between 40% and 80%.

There is one study, by Hoppu *et al.* (156) that investigated maternal fish consumption during lactation (one month after birth rather pregnancy) on AD development on the infant at 4 timepoints. The primary objective of the study was to examine the effect of breast milk fatty

acid composition on atopic dermatitis during the first year of life. Although a higher percentage of EPA in breast milk was related to lower risk of AD, fish consumption frequency during lactation was not associated with breast milk EPA content. This may be attributed to the fact that breast milk fatty acid composition is determined more by fatty acids accumulated in maternal adipose tissue during pregnancy rather than dietary intake of fatty acids during lactation (157;158;331). The authors stated that maternal fish intake during pregnancy would have been more appropriate to investigate in relation to breast milk composition.

All these studies were powered enough to show such large effects on atopic outcomes compared to SIPS that had no effect on atopic outcomes probably because of the size of the study at 6 months in relation to the low level of sensitisation. Also the infants in all these observational design studies were followed up later in infancy and childhood (follow up from 1-5 years of age) compared to SIPS (follow-up only to 6 months) so that the infant immune system would mature and develop detectable responses to allergens.

6.4.2.2 Experimental studies

Studies of maternal fish oil supplementation during pregnancy reported effects on umbilical cord blood immune markers (blood cytokine mRNA (178), plasma cytokines (35), LTB_4 production from neutrophils (176), cytokine production by mononuclear cells (132) and an altered cord blood haemopoietic progenitor phenotype (173)). These immunologic effects might be expected to impact on allergic sensitization and on the development of atopic disease. Dunstan *et al.* (132) reported beneficial effects on atopic outcomes in one year old infants as a result of maternal fish oil supplementation during pregnancy (less severe atopic dermatitis (OR 0.09) only when the SCORAD index was modified to >25, and lower risk of positive SPT to egg (OR 0.34)) in the infants in the fish oil group. However, the link between the CBMC cytokine responses measured at birth in the two groups and the development of the clinical features (AD, asthma and food allergy) at one year of age was not consistent with the assumed predisposing immunology of AD (132). This study provided women with the large dose of 3.1 g/day EPA plus DHA compared to 0.495 g/day EPA plus DHA that the women were taking from study salmon in SIPS The study of Kraus-Etschmann did not report any clinical assessments of the infants (178). Olsen *et al.* (180) identified that, compared with the olive oil control, fish oil supplementation in late pregnancy is associated with a marked

reduction in atopic manifestations in the offspring at age 16 years, suggesting a long-term effect of any immunologic changes that occurred in pregnancy and early life of those children. After 16 years, there was a lower prevalence of asthma (all types), AD or allergic rhinitis (by 57%) and of allergic asthma, AD or allergic rhinitis (by 69%) in the fish oil group compared to the olive oil control group. However in the Olsen *et al*. study there was also a no intervention group and there were no differencs in outcome between that group and the fish oil group.

A study of fish oil supplementation during both pregnancy and lactation (week 25 of gestation to 3-4 months lactation) with atopic outcomes in the offspring at 6 months and 1 year of age, showed expected effects on n-3 PUFA status and these were associated with differences in PGE_2 production by stimulated maternal blood (182). The latter might be expected to influence Th2 polarisation. Indeed, infants from mothers in the fish oil group had a reduced risk of developing allergic sensitisation to egg, IgE-associated eczema and food allergy during the first year of life (181). In detail, the prevalence of any positive SPT and of a positive SPT to egg in the infants at 1 year of age was significantly lower in the fish oil group compared to the placebo group. Eczema in the presence of detectable IgE antibodies or positive SPT towards egg, milk, or wheat and food allergy (reaction to egg or milk) during the first 12 months of life were significantly lower in the fish oil group. In a regression analysis, after controlling for confounding factors, it was found that the risk of developing any positive SPT, a positive SPT to egg, or IgE-associated eczema was three to four times less in the fish oil group compared to the placebo. The risk of developing food allergy was reduced ten times in the fish oil group in comparison to the control. These significant effects were seen in the offspring of non-allergic mothers but not of allergic mothers. This study provided women with 2.7 g/day EPA plus DHA, which is a high supplementation dose when compared with SIPS in which the dietary intake of EPA plus DHA per day was 0.495 g, according to the current recommendations (two portions of study salmon per week).

A study of maternal fish oil supplementation during lactation (179) was the only one of these studies investigating immune outcomes in the offspring beyond birth. Infants of lactating mothers who received fish oil supplements had a higher n-3 PUFA status at 4 months of age and interferon-γ production at 2.5 years of age was higher in the fish oil group, an observation that may reflect faster maturation of the immune system. This study was not powered to assess clinical outcomes such as atopic sensitization, and no differences in atopic outcomes or in plasma IgE were observed between the groups. No significant association was found between

in vitro cytokine production and plasma IgE levels and there was also no significant association between plasma IgE and eczema, wheezing, or food allergy, although both trends tended to be positive.

Each of the intervention trials described above were conducted with fish oil supplements rather that fish and the dose of LC n-3 PUFAs was much higher compared to the provision of EPA and DHA from 2 portions of salmon. Also fish oil supplements were provided on a daily basis rather than twice per week. Furthermore, the clinical outcomes in these supplementation studies were not assessed before the infant age of one year. These differences in the design of the trials besides sample size might explain why SIPS showed no effect on the atopic clinical outcomes.

6.4.3 Diet and other characteristics at 6 months

The purpose of this chapter regarding the diet and other characteristics of the offspring at 6 months of age is to describe the infants in the two groups (anthropometry, health status, reported allergies), their feeding patterns in the first 6 months of age (milk consumption, type of milk consumed, introduction of solids) and their nutrient intakes at 6 months by FFQ assessment. Because all these activities depend on the carer/s, the intervention itself and the grouping in the treatment groups might have affected the dietary choices of the 6 month population.

At 6 months 90% of the infants in each group had been breastfed; 77% and 89% in the control and salmon groups respectively were formula fed, whereas 41% (control group) and 45 % (salmon group) were still breastfeeding at 6 months. This shows that the mothers involved in SIPS had a healthy lifestyle, feeding the newborns with breastmilk according to the latest recommendation (6 months of breastfeeding) for optimum health (332). Data from the latest infant feeding survey in the UK has shown that 25% of all mothers in the UK were still breastfeeding at 6 months postnatally (333). The proportion in SIPS is higher than this.

In terms of feeding duration, breastfeeding duration was 146/180 days (almost 5 months) on average in both groups, formula feeding was 124 days in the control and 152 days in the

salmon group and exclusive breastfeeding duration was higher in the control group compared to the salmon group (44 vs. 27 days), but the difference was not significant.

Regarding feeding patterns and the focus of the study on LC n-3 PUFAs, the days of consumption of milks fortified with or without EPA plus DHA were recorded. Both groups consumed fortified formula milks for most days rather than unfortified, whereas the salmon group consumed supplemented milks for more days compared to the control group (97 vs. 71 days). It may be that being in the salmon group prompted the mothers to be more conscious about ensuring a good LC n-3 PUFA intake in their infants.

There were very similar patterns between the two groups regarding consumption frequencies of breast milk and other food groups to the 7 days prior to completing the FFQ. The only exception was the frequency of consumption of vegetable jars. Regarding quantities consumed in the past 7 days the salmon group consumed more breast milk and less expressed breast milk (the difference was not significant). Infants in the salmon group tended to have a higher intake of fish in the last week compared to control group (50 g/week vs. 15 g/week). Again, some mothers in the salmon group may have been influenced by the intervention and knowing the potential benefits of fish on reducing allergic risk, fed their infants with larger quantities of fish than the mothers in the control group.

The macro- and micro-nutrient intakes for the total diet were similar in the two groups. The intake of energy/kg of body weight was 364 kJ/kg and 394 kJ/ kg in the control and the salmon groups, respectively. Comparison of those values to the dietary reference values (DRV) (231) for formula fed infants in the UK (400 kJ/kg/day) at 6 months showed that the energy intake derived from the FFQ data is close to the DRV values especially for the salmon group.

Length and weight by gender in the control and salmon groups were compared with the DRV tables for length and height for boys and girls at 6 months. According to the DRV the average height for boys and girls is 67.9 cm and 66.3 cm respectively and the average weight is 8 kg and 7.4 kg, respectively. The average values from SIPS are very similar for both groups. Overall, mean weight and length in both groups, approximated the 50th percentiles on more recent standardized growth charts (334) showing normal growth and development for these infants.

The ability of the 6 month FFQ to rank intakes of energy and nutrients accurately is enhanced by the detail and quality of the information collected and by its short period of recall (1 week) which allows capture of the diet without much recall bias. Furthermore the FFQ records both the amount consumed and the frequency of consumption of all foods and drinks and information on brands and types of baby foods and milks used. In addition myself and L-S Kremmyda in collaboration with the SWS nutrition team found the exact nutritional content of the baby foods/milks missing from the SWS database.

A limitation of this study is that the population that was interested to take part had a high standard healthy living profile which was reflected not only in the compliance to eating salmon but in the high rate of breastfeeding in both treatment groups. It must be noted that an algorithm was used to estimate human milk intake which relates length of suckling to intake (324;335). According to the validation of this FFQ that was also used in the 6 month infant population of the SWS study, the FFQ tended to produce higher values for actual intakes of energy and nutrients (apart from sodium) when compared with the method of 4-day weighed diaries, which is a result of gradual change in infant diet over time (324). Nevertheless, the FFQ was a useful tool to assess energy and intakes in this relatively large group of infants. Overall, the 6 month follow up questionnaire gave very detailed information on the feeding and dietary patterns of the infants of both groups at 6 months of age as well as information on their growth and development.

6.5 Conclusion

This chapter described the feeding patterns of the babies born to the SIPS mothers at 6 months of age, the concentrations of total and specific IgE in the blood of those infants, the results of skin prick testing for sensitisation to several different allergens, and the assessment of AD in those infants all at age six months. There was no effect of maternal salmon consumption on plasma IgE, SPT positivity, and occurrence of AD or its severity. The work described here does not provide support for any of the hypotheses being tested. However despite the absence of effect on clinical outcomes of atopy at 6 months, the immune system of the newborn had shown differences at birth between the two groups (Chapter 5). Thus, the absence of effect might be attributed to the fact that 6 months was quite early for SIPS babies to show signs of sensitization. The future follow-up of these infants at 30 months might reveal the development of detectable responses to allergens. Other than that, all SIPS babies, in both study groups have normal and healthy growth.

7 Final Discussion

7.1 Overview and summary of the main findings of the study

Epidemiological data has shown that in the last 30-40 years the incidence of childhood atopic disease has dramatically increased especially in Western environments (68;72;74;336;337). This has happened in parallel with a decrease in infectious diseases which has led to the development of the hygiene hypothesis (72;88;111). Numerous environmental factors play an important role in determining the risk of atopic disease; these factors include diet, smoking and pollutants (338-341). One of the major dietary changes that has happened in the last 3-4 decades is the decreased intake of LC n-3 PUFAs (EPA and DHA) and a related increased intake of n-6 PUFAs (80). It is believed that this dietary change may have a causal role in increasing risk of atopic disease. Also, the decreased intake of vitamin D and of antioxidants such as selenium (200;342) may also play a causal role. It has been shown that exposure to LC n-3 PUFAs, vitamin D, vitamin E and selenium modifies fetal/neonatal immune responses (202;203;207-209;337).

Currently, in the UK it is recommended that pregnant women and women or reproductive age should consume two portions of fish per week, of each at least one should be oily in order to achieve a minimum intake of 450 mg/day of EPA plus DHA (23). There is quite strong evidence from epidemiological studies of lowered risk of infant/childhood atopy with high fish consumption during pregnancy. The evidence from the experimental studies with fish oils is not so strong, but there is some evidence supporting a protective effect towards the development of atopic disease in the infant (203). However, there are no intervention studies with fish during pregnancy. So, it remained to explore the effect of early intervention with fish (i.e. in pregnancy) on atopy-related outcomes in the infants of those pregnancies. The difference between fish and fish oil supplements, is that fish as a whole food is source not only of LC n-3 PUFAs but also source of micronutrients as vitamin D and selenium.

The Salmon in Pregnancy Study (SIPS) described here is the first randomised controlled trial of oily fish (farmed salmon) in pregnant women; the study was single-blind (investigator) and the babies of the women recruited were at high risk of developing atopic disease. SIPS specifically focuses on pregnant women whose offspring are at risk of developing atopic

disease, with one aim being to identify whether there is an effect on atopy outcomes in the offspring. The overall general hypotheses being investigated, are that: 'increased consumption of oily fish during pregnancy by women at risk of having offspring who will develop atopy will increase their LC n-3 PUFA status and antioxidant status and that of their developing baby and will ameliorate the development of atopic markers and manifestations in the infants'. The present thesis focuses on specific aspects of the SIPS.

The aims of my study are:

1. to increase oily fish consumption from a habitually low intake (≤ 2 portions/month) to the recommended maximum level of intake (two portions/week) during pregnancy, in women whose offspring are at a high risk of developing atopic disease;
2. to investigate the effect of increased maternal oily fish consumption on cord plasma and cord blood mononuclear cell (CBMC) LC n-3 PUFA status;
3. to investigate the effect of increased maternal oily fish consumption on markers of infant immunity at birth (i.e. in cord blood);
4. to investigate the effect of increased maternal oily fish consumption on atopic sensitization and atopic dermatitis in infants at 6 months of age.

The research hypotheses of my study are that increased consumption of farmed salmon during pregnancy by women with high risk of having an atopic offspring will:

1. increase maternal LC n-3 PUFA intake;
2. increase cord plasma LC n-3 PUFA status;
3. increase CBMC LC n-3 PUFA status;
4. alter immune markers in cord blood in a way that would indicate decreased atopy risk;
5. reduce sensitisation to common allergens and reduce atopic dermatitis score in infants at 6 months of age.

The data obtained allowed the first three hypotheses to be accepted, the fourth to be partially accepted as there was some impact of the salmon intervention on the infant immunity whereas the fifth cannot be accepted as there was no reduction in allergic sensitization at 6 months of infant age.

The main findings of the present thesis will be summarized below and further discussed according to the literature. In this context, the strengths and limitation of this study will be highlighted, final conclusions will be drawn and suggestions/plans for future work will be suggested.

A total of 123 women were randomly assigned to consume either 2 portions of farmed salmon per week (from week 20 until delivery) or to continue their habitual diet low in oily fish (less or equal to two portions per month) from week 20 until the end of their pregnancy. Briefly, 62 and 61 pregnant women were randomly assigned to the salmon and control groups, respectively. At birth, 107 subjects (n=53 in salmon and n=54 in control group) were enrolled in the study. After delivery, at 6 months 86 subjects completed the 6 month appointment (n=47 in salmon group and n=39 in control group). In total the drop out rate was 30%. At the 6 month appointment the parents were also assessed for atopy by skin prick test (SPT) and this was completed by 86 mothers and 76 fathers in total. The baseline characteristics of SIPS parents and babies are described in detail in Chapter 2.

Main findings of the maternal dietary and nutrient analysis during pregnancy

SIPS successfully increased oily fish intake in the salmon group to the target of 2 portions of study salmon per week. This was reported both in the FFQs and in the fish diaries although there were differences in the reporting of the intakes of the seven nutrients of interest (EPA, DHA, total n-3 PUFAs, total n-6 PUFAs, vitamin D, vitamin E and selenium) between the two methods. According to the FFQ (at 34 weeks gestation) all volunteers in the salmon group reported intake of 1-2 times per week of salmon and according to the fish diaries (between 21-38 weeks gestation) the median intake was 1.94 portions of study salmon per week. Also it was shown that 79.6% of the volunteers in the salmon group ate 2 or more portions of study salmon per week for the 81.3% of intervention period. The control group had a very low intake of oily and total fish which was significantly lower than in the salmon group. This demonstrates the compliance to the intervention in both arms of the study.

According to the FFQ, median daily intake of EPA plus DHA was 402.6 mg for the salmon group and 30 mg for the control group ($p<0.001$). The fish diary results showed higher intakes for the salmon group (490 mg/day EPA plus DHA) and similar intakes for the control group

(24 mg/day EPA plus DHA) compared to the FFQ calculations. The calculated intake of LC n-3 PUFAs in the salmon group exceeds the recommendation for minimum intake of EPA+DHA during pregnancy (109% of 450 mg/day) and the latest WHO/FAO recommendation (2008) for minimum DHA intake of 200 mg/day during pregnancy and lactation (199).

In addition, increased salmon intake increased total dietary intake of selenium (58.2 μg/d vs. 49 μg/d) and vitamin D (5.75 μg/d vs. 2.9 μg/d) in the salmon group compared to the control group. Increased salmon consumption was compensated by decreased consumption of other types of seafood (non-oily fish and fish fingers/fish dishes) and of red meat, chicken, and total meat.

Main findings from the maternal & infant fatty acid status

Fatty acids in the blood stream (plasma and cells) provide the link between the dietary intake and the bioavailability of the LC n-3 PUFAs in the mother and subsequent supply to the fetal circulation. The data obtained from the fatty acid analysis of maternal blood during pregnancy (20, 34, 38 weeks gestation) and infant blood at birth (cord blood) allowed the hypothesis that the maternal oily fish intervention will result in higher amounts of EPA and DHA in maternal PC and in neonatal (cord) plasma lipids to be supported. However, the hypothesis that high oily fish consumption will result in higher amounts of EPA and DHA in CBMCs was not proven.

The literature has shown that percentages of EPA and DHA in maternal plasma lipids decline during pregnancy (257-259). This was confirmed in the control group in SIPS, and is probably related with preferential transfer of DHA through the placenta to the growing fetus (253). SIPS, being the first randomised controlled trial with fish during pregnancy, showed that consumption of two portions of salmon per week (between week 20 till delivery) prevented the pregnancy-associated decrease in the percentages of EPA and DHA in the phospholipid (PC) fraction, and actually resulted in increased levels of these LC n-3 PUFAs in maternal PC compared to the control group. Thus, the consumption of 2 portions of salmon per week, as confirmed by two dietary assessment tools (FFQs and fish diaries) and which resulted in an intake of more than the recommended amount of EPA+DHA, was also proven biochemically by the increased status of those LC n-3 PUFAs in maternal plasma. The higher EPA and DHA

status in maternal plasma PC may indicate a better ability to supply the fetus with these structurally and functionally important fatty acids.

In the cord plasma, significant changes in the status of LC n-3 (EPA and DHA) and n-6 (ARA) PUFAs were observed in almost all lipid fractions when data were expressed as percentages rather than absolute amounts. The pattern observed was that EPA and DHA status increased significantly, whereas ARA status decreased significantly in cord blood plasma in the salmon group neonates compared to those of the control group. A similar trend was found in these LC PUFAs in immune cells (CBMCs) but the differences between groups did not reach significance. However, in the immune chapter (Chapter 5) some differences in functions of CBMCs between the two groups were seen (e.g. eicosanoid and cytokine production) suggesting that the observed differences in fatty acid composition of CBMCs were sufficient to lead to, or at least mark, changes in cell function.

Main findings from the infant immune system at birth

It was hypothesized that the increased intake of EPA plus DHA during pregnancy which resulted in higher total n-3 PUFA status in cord blood and in a tendency to a higher n-3 PUFA in immune cells (CBMCs), would have immunomodulatory effects in the developing infant immune system and predisposition to atopy in a protective way.

The results obtained indicate that maternal salmon consumption during pregnancy results in some differences in cord blood immune cell phenotypes at birth (more leukocytes, neutrophils, lymphocytes, helper T-cells, cytotoxic T-cells and T-regulatory cells), a generalised decrease in production of cytokines in response to a T cell mitogen (PHA), TLR agonists and allergens, that became significant for several cytokines in response to the mitogen PHA (IL-2, IL-4, IL-5, IL-10, and TNF-α) and the TLR-4 ligand LPS (IL-10, TNF-α), and a tendency to lower PGE_2 production in response to LPS. The cord plasma concentration of IgE was not affected by the salmon intervention. IL-13 was not detected in most cord plasmas and no other cytokines were measured in those samples.

Thus, there is evidence to support two of the hypotheses (altered immune cell phenotypes and altered T cell responses) and to support in part a third (decreased PGE_2 production) while there is no evidence in support of the fourth (decreased IgE concentration).

Main findings from the clinical assessment of atopy at 6 months

It was considered that the maternal oily fish intervention would reduce sensitization to common allergens in infants at 6 months of age (determined by SPT), reduce eczema score in infants at 6 months of age (determined by SCORAD) and decrease plasma IgE concentration in infants at 6 months of age. The results obtained did not provide support for any of these hypotheses and thus they cannot be supported from this study. The lack of effect on IgE and on clinical outcome at 6 months is despite the immune differences observed between control and salmon groups in cord blood (Chapter 5).

The data collected on infant feeding patterns showed that the babies were not different in the two study groups in this regard. 41% of the infants in the control group and 45 % of the infants in the salmon group were still being breastfed at age 6 months whereas 34% of the women in England and Wales still breastfeed at 6 months post-partum (343). Breastfeeding duration was almost 5 months on average in both groups. The salmon group consumed milks fortified with n-3 PUFAs for a longer duration compared to the control group (97 vs. 71 days), but the difference was not significant. Infants in the salmon group tended to have a higher intake of fish in the last week (prior to the 6 month clinic visit) compared to control group (50 g/week vs. 15 g/week). Most likely, mothers in the salmon group were influenced by the intervention and knowing the potential benefits of fish and n-3 PUFAs on reducing allergic risk, fed their infants with larger quantities of fish than the mothers in the control group and also used n-3 PUFA fortified milks to a greater extent.

There were no gender and anthropometric (weight, length) differences between the babies in the two groups, and the nutrient intakes (including kJ/kg body weight) from the total diet did not differ either. Overall, the growth and development measurements of the SIPS infants compared to UK growth charts (231;334) showed that the SIPS babies are developing normally.

To sum up, SIPS is the first randomised control trial to show that if pregnant women who do not habitually eat oily fish, consume two portions of oily fish (salmon) per week, from week 20 of gestation to delivery, they will:

- Increase their intake of LC n-3 PUFAs (achieving recommended intake), vitamin D and selenium
- Increase their status (maternal blood plasma phospholipid percentages) of LC n-3 PUFAs
- Increase their neonates (cord blood plasma) status of LC n-3 PUFAs
- Modify neonatal immune responses (CBMCs).

The increased maternal and neonatal LC n-3 PUFA status did not result in changes in the clinical outcomes of atopy at 6 months of baby age.

7.2 General comments, strengths & limitations of SIPS study

The SIPS intervention was conducted between mid-pregnancy to delivery. This period of intervention was chosen based on previous supplementation studies with fish oils which focused on similar periods of gestation, indicating that this is a critical window for early life intervention (132;177;180;181). Furthermore, the demand for LC n-3 PUFAs increases dramatically the last trimester of pregnancy (250;253).

- Maternal diet

The FFQ most likely underestimates the intake of salmon, since an intake of 2 portions per week falls into the FFQ category 1-2 times per week, for which the multiplication factor for all calculations was 1.5. This is a limitation of the FFQ as a dietary assessment method, because it has an ordinal scale of consumption frequency categories (1-2 times/week), rather than a scale providing a true measure. The fish diaries were important not only to prove compliance to the intervention but also as a precise measure of fish consumption frequency (this showed that the women were eating 1.94 portions of salmon per week) and subsequently nutrient intake. The use of the diaries can be considered as a strength of SIPS. The FFQs provide dietary data for the 3 months prior to the FFQ administration. This is considered to be an advantage of the FFQ, reflecting the consumption frequency over a long period of time. Another advantage of the FFQ is that it is validated on the Southampton population for use in pregnant women (228) and it includes distinct fish categories. The FFQ method has also been used in large epidemiological studies testing similar hypotheses to SIPS i.e. maternal fish intake during

pregnancy in relation to infant atopic outcomes (203), a fact enhancing the choice of FFQ as the main dietary assessment method in SIPS.

FFQs might not be the ideal tool to investigate nutrient-disease associations (222). However, the SWS FFQ used by SIPS has been validated for calculating nutrient intakes compared to a 4 day prospective food diary (222). A limitation of this FFQ is that has not been validated to calculate total diet EPA and DHA. However the main sources of EPA and DHA are fish and fish oil supplements or functional foods. The FFQ indicated that no subject was consuming fish oil supplements during pregnancy and a minority of subjects was consuming functional foods that contained fish oils. This consumption did not differ significantly between the two groups. The calculations of 'total diet' EPA and DHA intake included fish oils in functional foods and between the two groups no substantial difference was observed in EPA and DHA intake from 'total fish'. So, it seems that the intake of EPA and DHA from total fish is a good estimation of EPA and DHA from total diet. Recent studies have reviewed the use of FFQs as a validated tool to estimate LC n-3 PUFA and micronutrient intakes during pregnancy (344;345). However one disadvantage of FFQs is that they may not be able to detect small differences in nutrient intake compared to the fish diaries as the consumption frequencies were not categorised, but measured in an exact and precise way rather than to an ordinal scale. However, the fish diaries have two limitations: they are not validated, as the FFQs are, and also are influenced by individual variability in the way in which they are completed by the subjects. The FFQ was a very useful tool but at the same time it was long and time consuming to complete. In this way, an alternative shorter and more specific FFQ on LC n-3 PUFAs could be more practical to use and maybe the drop-out rate of reduced.

The increase in intake of key nutrients other than LC n-3 PUFAs in the salmon group clearly indicates that salmon delivers more than just EPA and DHA and in this respect salmon may be superior to supplementation with fish oil capsules. From the 2 portions of study salmon, the requirements in EPA and DHA can be met (109% of minimum recommended intake), and the fish can make a major contribution in the daily intake of vitamin D (40% of the recommended intake) and Se (20% of the recommended intake) amongst other nutrients. This could be considered as an additional strength of the study compared to similar design supplementation trials.

- Maternal and infant fatty acid status

The results of the maternal plasma PC fatty acid analysis provide biochemical proof that the intervention (i.e. increased salmon consumption) had been successful and that the subjects had complied to it. Regarding the maternal plasma PC fatty acid data, at baseline (20 weeks gestation) the salmon group had higher percentages of EPA, DHA and total n-3 PUFAs and a lower percentage of ARA compared to the control group. This baseline difference in LC n-3 PUFA status matches with the baseline difference in fish and LC n-3 PUFA consumption reported in chapter 3. Although the subjects were randomly assigned to the two groups at study entry, this baseline difference is a limitation of SIPS study. However, this baseline difference validates the SWS FFQ as a dietary assessment method of n-3 PUFA intakes, and this is a novel validation for this FFQ.

The results from plasma fatty acid analysis were presented in this thesis; red blood cells (RBCs) better to capture longer term characteristics of the diet. Analysis of RBCs from SIPS have been performed (group of Angel Gil, University of Granada, Spain) and these show an increase in EPA and DHA in the salmon group. The findings on maternal fatty acids in plasma PC suggest that the mother is progressively depleted in these fatty acids (especially DHA and ARA), as they are transported to the fetus to meet the demands for growth and development. This decline in maternal LC PUFA status over the course of pregnancy has been demonstrated before and the extent of the decline in both DHA and ARA seen here is consistent with this earlier work (258;259).

In contrast to the control group, which had a mean daily intake of about 30 mg of EPA plus DHA from the total diet, the salmon group had a mean daily EPA+DHA intake of over 400 mg from the total diet. In accordance with this difference in dietary intake, the maternal status of EPA, DPA and DHA (expressed as % of fatty acids) in the salmon group was higher than in the control group. This is the first time that this effect of maternal salmon consumption in pregnancy has been demonstrated. A higher LC n-3 PUFA status in maternal blood may mean that the mother can supply increased amounts of these important fatty acids to the fetus. This may be important for fetal growth and development and also might play a key role in the development of the fetal immune system (202).

The salmon intervention enhanced the pregnancy associated decline in ARA in maternal plasma PC. This effect is consistent with other observations where LC n-3 PUFAs are

incoporated into lipids at the expejse of ARA. This may not be a desirable effect as ARA too has important roles in fetal growth and develolment (10;243;263).

The inclusion of both expressions of fatty acid results (percentages and absolute concentrations) in the current thesis is a strength of the study because the combination provides complete information of the differences between the groups. In general, greater and more significant effects were observed when the percentage data were used.

This is the first study to show that a dietary intervention with fish during pregnancy increases LC n-3 PUFA status of the fetus/neonate. Higher cord RBC LC n-3 PUFAs has been reported following fish oil supplementation in pregnant women (174). In the current study DHA and ARA in the cord plasma PC were much higher than in the maternal plasma PC (at 38 weeks). DHA was on average 57% higher and ARA 219% higher in cord plasma PC. These findings agree with those from previous studies that found that relative amounts of ARA and DHA in umbilical cord blood were significantly higher than in maternal blood (258;264). This might point to a high neonatal need for ARA and DHA which are the most abundant n-6 and n-3 PUFA in neural and retinal tissue, respectively (and also in immune cells).

Unfortunately, salmon intervention did not have a significant effect on the LC n-3 PUFA composition of the cord blood immune cells (CBMCs). No previous supplementation study has measured the fatty acid status of the immune cells in the cord blood. Another novel finding of SIPS is the positive correlations between the DHA status in the cord blood (plasma (expressed either as percentage and absolute concentration) and mononuclear cells) with the gestational length. This result is supported by evidence in the literature that relates maternal dietary intake of DHA (and ARA) with gestational length and infant characteristics at birth (birth weight, head circumference) (39;243;248;263), indicating the importance of DHA supply to the fetus to support *in utero* brain growth.

- Infant immunity

Immune cell subset analysis in combination with the measurement of immune cell numbers showed that the numbers of T helper ($CD3^+CD4^+$) cells, cytotoxic T ($CD3^+CD8^+$) cells and T regulatory ($CD4^+CD25^+CD127^{low}$) cells were higher in the salmon group compared to the control group. The percentages of these cells in the lymphocyte and total leukocyte

populations did not differ between the two groups, but the numbers of lymphocytes and total leukocytes (and also neutrophils) were higher in the salmon group. Dunstan *et al.* (35) observed no significant difference in the frequency of various lymphocyte subsets (% and absolute concentration) between the fish oil supplemented and the control group. It is noteworthy that the method used to identify the T regulatory cells in SIPS was not optimal; one antibody stain (to the FOXP3 transcription factor), which according to the literature (306) would definitely determine $CD4^{+}CD25^{+}CD127^{low}$ cells as T regulatory was not used. The reason for this omission is that the flow cytometer used for the SIPS analysis could only detect three antibody stains at the same time, and the use of a fourth stain was not possible. One limitation of SIPS is that the cord blood immune cell subsets were characterised by extracellular surface markers and not intracellular markers which describe the functional properties of these cells. However, the production of Th1 and Th2 cytokines by unstimulated and stimulated CBMCs was investigated as a measure to obtain information about the functional phenotype of these cells.

A strength of the current study is the use of different (ex vivo) stimulants in order to investigate different aspects of the immune system including both innate and adaptive immunity, using allergens, a mitogen and TLR ligands. Also an advantage was the method of cytokine determination by using a flow cytometry based assay, termed cytometric bead array, which measures at the same time a range of cytokines characteristic of different responses: Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-5), and T regulatory (IL-10). In general, cytokine responses were lower from CBMCs in the salmon group compared to the control, although not always significantly. The only cytokine measured in plasma, IL-13, was detectable in less than 10% of the samples. Dunstan *et al.* could detect IL-13 in 64% of plasma samples in the control group and in 45% of plasma samples in the fish oil group (35). The observation of generally lower cytokine responses seen in SIPS agrees with the findings of Dunstan *et al.*, although these are some differences in the detail (132). Kraus-Etschmann *et al.* measured cytokine expression patterns in the cord blood by real time RT-PCR method looking at the mRNA levels of Th2 associated molecules CCR4, IL-13 and IL-4, Th1 related molecules IL-1 and IFN-γ, and T regulatory related molecule TGF-β. Fish oil supplementation decreased mRNA levels of the Th2 related molecules compared with placebo, whereas levels of mRNA for Th1 cytokines remained unaffected. TGF-β mRNA expression, which has shown to be involved in tolerance towards allergens (346), was significantly higher in the fish oil group (178). So this European study agrees with the Australian study in that the Th2 related molecules

(chemokines and cytokines) are reduced with fish oil. In general the current results from SIPS are in agreement with this: production of the Th2 cytokines (IL-4 and IL-5) was lower in the fish oil group while production of the prototypical Th1 cytokine IFN-γ although lower in the fish oil group was not significantly different between groups. Thus it appears that fish oil decreases the Th2 type response with less of an impact on the Th1 type response. Indeed, although there was no significant effect on the IFN-γ to IL-4 ratio (Th1/Th2) this tended to be higher in the salmon group.

PGE_2 production from unstimulated and PHA-stimulated CBMCs did not differ between the two study groups, but PGE_2 production from LPS-stimulated CBMCs tended to be lower in the salmon group compared to the control group (p-value=0.056). Krauss-Etschmann *et al.* did not report PGE_2 production in infant immune cells at birth. Neither did Dunstan *et al.* although Prescott and Dunstan (176) showed that fish oil supplementation during pregnancy resulted in lower LTB_4 production and higher LTB_5 production by cord blood neutrophils. It could be speculated that increased provision of EPA and DHA from the mother to the fetus and subsequent decreased incorporation of ARA into CB immune cells and lower production of ARA lipid mediators (PGE_2, LTB_4) as well as increased production of the less potent EPA and DHA lipid mediators (PGE_3, LTB_5) from the neonatal immune cells might occur. This could possibly affect the offsprings' T-cell and B-cell function and predisposition to a less inflammatory environment in early life so that there is less risk of developing atopic disease in infancy and later in life.

- Clinical atopy outcomes

A limitation of the current study is the initial reliance on reported atopy by the parents (at study entry, 13 weeks gestation) to determine high risk pregnant women of having an atopic offspring. This was based on self-reported clinical signs of atopy in the past 5 years (prior to the screening of eligibility to take part in SIPS; 13 weeks of gestation) from the mother, the father or sibling. Maternal and paternal atopy was also examined using SPT, but only after the end of the intervention, when this action had been approved by the local ethics committee. The SPT results showed that the majority of subjects who reported atopy also had positive SPT to at least one allergen, despite the fact that at the end of the study it was not possible to examine all subjects, as some of them had already dropped out or they declined to participate in this

aspect of the study. It would be better if the examination of the SPT happened at study entry, so as to be included as an eligibility criterion.

SIPS was powered according to an assumed sensitization to at least one common allergen in 45% of the infants participating in the control arm at 6 months of age, based upon the high risk nature of the pregnancies. In this event only 13% of the infants in the control group were sensitized (i.e. had a positive SPT) and so the study was underpowered to detect a difference in sensitization. Furthermore, a higher than anticipated number of the SIPS infants were still breastfeeding and had not been introduced to a large amount of solids. Breastfeeding and avoidance of solids are protective against sensitization (318;319) which might explain the low rate of sensitization seen. It will be prudent to re-examine sensitization and the other outcomes at a later age (24, 30 or 36 months).

When combining the results of the maternal and paternal SPT responses with the offsping SPT responses to the same allergens it was interestingly found that when the mother or the father or both parents had any positive SPT response, only 8/86 (3 in control and 5 in salmon group) infants had corresponding positive responses to any allergen whereas 67/86 infants had corresponding negative SPT responses to any allergen (26 in control and 41 in salmon group). Out of 7 infants who were SPT positive for egg protein, 6 had detectable anti-egg IgE. Likewise, out of 4 infants who were SPT positive for cows' milk protein, 2 had detectable anti-cows' milk IgE. It is interesting that the same pattern happened in response to both allergens: more positive responses were detected by SPT which were not confirmed by the IgE measurement.

Epidemiological studies (151-153;156) were sufficiently powered to show associations with atopic outcomes compared to SIPS. Also the infants studied in observational studies were followed up later in infancy and childhood (follow up from 1-5 years of age) compared to SIPS (follow-up only to 6 months) so that the infant immune system would mature and develop detectable responses to allergic antigens.

Supplementation trials (35;132;173;176;178;180-182) were conducted in pregnancy with fish oil supplements rather that fish and the dose of LC n-3 PUFAs was much higher compared to the provision of EPA and DHA from 2 portions of salmon. Also fish oil supplements were provided on a daily basis rather than twice per week. Furthermore, the clinical outcomes in

these supplementation studies were not assessed before the infant age of one year. These differences of the design of the trials besides the power issue might explain why SIPS showed no effect on the atopic clinical outcomes at 6 months of infant age.

One general limitation of the results presented within this thesis is that statistical analyses were not corrected for multiple comparisons. Thus some of the findings, particularly those with weak significance would likely disappear following such correction. However, quite a number of highly significant effects were observed, especially in relation to LC n-3 PUFA intake and status and these will likely remain significant after correction for multiple testing.

7.3 Conclusions

SIPS is the first randomized controlled trial that succeeded to increase the intake of oily fish during pregnancy from low (≤ portions per month) to the recommended intake (2 portions per week). Subsequent to the increased intake of oily fish, significantly increased the intake of LC n-3 PUFAs, vitamin D and selenium in the salmon group compared to the control group. The salmon intervention not only prevented the pregnancy associated decrease in percentages of EPA and DHA in the PC fraction, but also resulted in increased levels of these LC n-3 PUFAs in maternal PC compared to the control group. In cord blood plasma, EPA and DHA status was increased significantly whereas the ARA status was decreased significantly in the salmon group infants compared to those of the control group. A similar trend was found in those LC PUFAs measured in the immune cells (CBMCs) but the differences were not statistically significant. Nevertheless higher cord plasma PC EPA and DHA were associated with higher CBMC EPA and DHA, suggesting that as supply of these fatty acids to the fetus increases (by increased oily fish consumption) then there is enhanced incorporation into immune cells. In this way the maternal salmon intake during pregnancy resulted in some differences in cord blood immune cell phenotypes at birth (more leukocytes, neutrophils, lymphocytes, helper T-cells, cytotoxic T-cells and T-regulatory cells), a generalised decrease in production of cytokines in response to a T cell mitogen, TLR agonists and allergens, that became significant for several cytokines in response to the mitogen PHA and the TLR-4 ligand LPS, and a tendency to lower PGE_2 production in response to LPS. The cord plasma concentration of IgE was not affected by the salmon intervention and IL-13 was not detected in most cord plasmas.
At the 6 months post-partum clinical assessment, there was no effect of maternal salmon consumption on plasma IgE, SPT positivity, and occurrence of atopic dermatitis or its severity.

However despite the absence of effect on clinical outcomes of atopy at 6 months, the immune system of the newborn had shown differences at birth between the two groups. In this way, the absence of effect might be attributed to the fact that 6 months was quite early (immune system immaturation) for SIPS babies to show signs of sensitization. The future follow-up of these infants at 30 months might reveal the development of detectable responses to allergic antigens. Other than that, at 6 months, all SIPS babies, in both study groups were growing in a normal and healthy way.

7.4 Future work

The SIPS design and results presented in this thesis add valuable knowledge to the existing literature in the exciting field of maternal nutrition (in this case LC n-3 PUFAs in oily fish) in early life and how this may affect health and disease (atopy) development later in life (infancy and childhood). Findings from the maternal fatty acid status and changes in the immune system in both groups during pregnancy have been reported by Lefkothea-Stella Kremmyda in her thesis. Future follow up of this cohort would be appropriate since the clinical outcomes of atopy at 6 months did not show any effects of the salmon intervention. Clinical manifestations of atopy might be more pronounced later on in life (early and/or later childhood) and signs of the development of atopic disease might be more evident between the two groups. The principal investigator of SIPS, Professor Philip Calder, has already received approval from the ethics committee responsible to follow up the SIPS infants at 30 months of age (starting in July 2010) so as to assess clinical signs of atopy. This timepoint would also be a great opportunity to explore the effect of the maternal LC n-3 PUFA intake on neurodevelopment and visual development of the children, since there are few intervention studies with oily fish in this field and also taking into consideration that such a study (with fish and not supplements) is quite laborious and expensive. As for most RCTs, SIPS was fairly small in size (n=123 mothers at study entry and n=86 infants at 6 months) and this fact limited the ability to detect differences in atopic outcomes between the two groups. Ideally, the same study design with bigger sample size (for example n=150 infants at 6 months) would be more appropriate to identify more clear responses of the infant immune system at birth and to assess clinical signs of atopy and probable differences between the two groups at 6 months or 1 year of age. Furthermore it would be novel to have multiple sites of intervention (different cities or countries), so as to explore regional and ethnic variability and other factors but this would require even more resources and funding than available to SIPS. Also it would be more

appropriate to assess maternal/paternal/sibling atopy by SPT at study entry rather than the end of the intervention, so as have better selection criterion for study entry; self reported atopy could simply be a screening criterion.

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APPENDIX

Appendix-Clinical materials and methods

1. Initial information sheet for pregnant women

Salmon In Pregnancy Study (SIPS): The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies

Ethical approval number 07/Q1704/43
Co-ordinator: Dr Liz Miles

Chief Investigator: Prof Philip Calder

Telephone: 023 8079 6925 or 023 8079 5252 or 07864 831283
Email: sips@soton.ac.uk

Version 2, 8 May 2007

We would like to invite you to participate in a research study about a mother's nutrition and her baby's development of allergies and growth. Before you agree to participate it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1 What is the purpose of this study?

The purpose is to study how a mother's nutrition may influence their baby's development in the womb and the likelihood of allergies in later childhood. We wish to select a group of pregnant women who would be suitable to help us in research into the possible protective effects of eating oily fish (salmon) in pregnancy on development of allergies in babies.

2 Why have I been chosen?

We are looking for women aged from 18-40 years who are in the early stages of pregnancy (about 14 weeks or less) who may be able to assist us in our research.

3 Do I have to take part?

You are under no obligation to take part. If you take part you are still free to withdraw at any time and without giving a reason. If you wish to withdraw you can request that your data and samples be destroyed. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you are interested in having more information about the study please complete the 'interested in study' form (found at reception) and give it to the receptionist at your 13 week ultrasound scan appointment. Alternatively you can contact us on the telephone numbers or email address shown in this leaflet. A member of the research team will then discuss the study with you and answer any questions you may have. If you are satisfied with the explanation and would like to help us with our research we will ask you to sign a consent form. We will give you a copy of the consent form and this information leaflet to keep.

4 What will happen to me if I take part?

If you are happy to take part in this first stage of the study we wish to gather 3 pieces of information about you.

Firstly, we would like you to complete a few questions about your health and any allergies which you or your family may have.

Secondly, we would like to assess the growth and development of your baby in pregnancy. This will mean that during your routine ultrasound scans (13 & 20 weeks) a few extra measurements will be made of the baby's size and of the size and blood flow in some of its organs, including the heart and liver.

Thirdly, we would like to gather some information on how your body adapts to the pregnancy. To do this we would like to measure your height, your weight and your body composition (i.e. amount of muscle and fat). We use specialised weighing scales, which measure your weight and use a tiny electrical current to assess your body composition. This is a harmless procedure and has

been used to assess the body composition of pregnant mothers in previous studies.
When we have gathered this information we would like to use it to select mothers who are suitable to help with assessing the possible benefits of eating salmon in pregnancy. If you were suitable for this, we would provide more details for you and invite you to take part. Once again you would be under no obligation to take part, and a decision to withdraw at any time, or a decision not to take part, would not affect the standard of care you receive.

5 What is an ultrasound scan?

Ultrasound uses sound waves to produce pictures of your body and baby. Ultrasound has been used for many years to help us know if the baby is developing normally and growing properly.

6 What do I need to do for the scan?

A moderately full bladder is needed to allow us to obtain good views of your pregnancy. Please drink a pint of water, squash or 3 cups of tea / coffee about an hour before your scan appointment. Do not empty your bladder until after your scan. This is a medical examination and an important antenatal screening test. For your comfort, health and safety please ensure only one adult accompanies you for your scan. We would advise you not to bring young children as this can cause delays and distraction to the medical team. However if it is unavoidable we ask that any young children you wish to bring with you are supervised in the waiting area by another adult while the scan is in progress.

7 What are the possible benefits of taking part?

By participating in this study you may be able to help other children in the future from developing allergies. Suitable mothers will be offered the additional scan and will be given scan pictures of their baby in the womb.

8 What are the possible disadvantages and risks of taking part?

None, other than the giving up of your time to help us with our research. This will include an extra ultrasound scan appointment at the Princess Anne Hospital which will take no longer than 1 hour and some extra measurements and questions asked at your routine NHS 13 and 20 week ultrasound appointments which will take approximately 1 hour.

9 What will happen if anything goes wrong?

If you have a concern about any aspect of the study you should speak to the researchers who will do their best to answer your questions (telephone 023 8079 6925). If you remain unhappy and wish to complain formally you should contact the Wellcome Trust Clinical Research Facility in the first instance (telephone 023 8079 4989). Any complaint about the way that you have been dealt with during the study or any possible harm you might suffer will be addressed. In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

10 Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential.

11 What will happen to the study results?

The results from this first part of the study will be used to identify pregnant women suitable to help us with assessing the possible benefits of eating salmon in pregnancy. The results may be submitted for publication in medical literature. If you wish to see copies of any reports and publications, please contact the researchers listed and they will be happy to supply these for you.

12 Who is funding the study?

This study is funded by the European Commission (website: http://ec.europa.eu/index_en.htm).

13 Who has reviewed the study?

The Southampton and South West Hampshire Local Research Ethics Committees.

14 Contact for further information

If you have any questions then please contact the SIPS Project Team on Tel: 023 8079 6925 or 023 8079 5252 or 07864 831283. There is an answer phone available and all calls will be returned. Alternatively email us on sips@soton.ac.uk

Thank you for taking the time to read this

2 Volunteer initial consent form

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Title of study: **The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies**
Local ethical committee submission number……
Chief Investigator: Professor Philip Calder, Tel: 023 8079 5250
Contact details for research team: Dr Liz Miles, Tel: 023 8079 6925 or 023 80 79 5252

*PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:*

1. *I am not taking part in any other study (i.e. Southampton Women's Survey).* ☐
2. *I have read the initial information sheet dated 8/05/07 (version 2) for the above study and have been given a copy to keep. I have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me.* ☐
3. *I understand that my participation in this study is voluntary and that I am free to decline entry into the study, and that during the study I am free to withdraw at any time without giving a reason and that withdrawal will not affect any future medical care.* ☐
4. *Data to be collected. I agree to have my weight, body composition and height measured for research in this project (delete as appropriate).* ☐
5. *I agree to have additional measurements made of the growth and development of my baby and allow a sample of my afterbirth (placenta) to be collected.* ☐
6. *Information to be collected. I agree to complete a short questionnaire on my health and I give permission for someone from the research team to look at my medical notes to get information pertinent to the study. I understand that the information will be kept confidential.* ☐
7. *I agree to take part in the above study* ☐

_____	_____	_____
Name of Patient	*Date*	*Signature*
_____	_____	_____
Name of Person taking consent	*Date*	*Signature*

1 for patient, 1 for researcher, 1 to be kept with hospital notes.
Version 2, 08 May 2007 Ethics Number: 07/Q1704/43

3 Initial questionnaire (<14 weeks gestation)

Volunteer Number **P**

Date informed consent given

Initial visit check list (please tick when sample/data collected):

a	Ultrasound scan	☐
b	Body composition	☐
c	Initial questionnaire	☐

Name and Contact Details

Name

Address

Telephone – home/work

Telephone – mobile

Other contact

Personal Details

Date of birth

Age

Hospital number

Date of last menstrual period

Expected date of delivery

Consultant

Community midwife & clinic

Health visitor & clinic

Doctor & surgery

Eligible for SIPS?

	Yes	No
Atopy (1° relative of baby affected by atopy)		
Habitual oily fish intake ≤ 2 portions per month?		
Aged 18-40 year?		
Not consuming fish oil supplements?		
Yes to all above question = eligible for SIPS		

General Health

1. Height

2. Weight

3. Mothers' history of any ailments or diseases

Bioelectrical impedance

(Note – individuals with indwelling medical devices must not have bioelectrical impedance measurements taken

4. Do you have any indwelling electrical devices (i.e. pacemakers)?

☐ **Yes – DO NOT TAKE ELECTRICAL IMPEDANCE MEASUREMENT**

☐ **No**

Ask volunteer to empty bladder. Take measurements and attach print off securely to this page.

IF INELIGIBLE FOR SIPS – END QUESTIONNAIRE HERE
(signature still required at end of this questionnaire)

5. Medication/supplements/vitamins (taken in the last 4 weeks)

Therapy	Dose route and regimen	Date started/stopped

6. Number of pregnancies (including this pregnancy)

7. Number of children

8. History of smoking Never / Past smoker / current smoker

If current or past smoker please give details (i.e. number of cigarettes)

9. Current alcohol intake in drinks per week (1 beer or 1 glass of wine or 1 shot of spirits).

Drink per week	
0	
1-7	
8-14	

Family History of Atopy (within the last 5 years)

10. Have any of the following persons had asthma?

Mother			Father			Any sibling		
Yes	No	Not sure	Yes	No	Not sure	Yes	No	Not sure

If yes please give details **(i.e. diagnosed by clinician, onset, duration etc.)**

.....

.....

.....

11. Have any of the following persons had hayfever?

Mother			Father			Any sibling		
Yes	No	Not sure	Yes	No	Not sure	Yes	No	Not sure

If yes please give details **(i.e. diagnosed by clinician, onset, duration etc.)**

.....

.....

.....

12. Have any of the following persons had an itchy rash persisting (even sporadically) over 6 months?

Mother			Father			Any sibling		
Yes	No	Not sure	Yes	No	Not sure	Yes	No	Not sure

If yes please give details **(i.e. diagnosed by clinician, onset, duration etc.)**

.....

.....

13. Have any of the following persons had wheezing when breathing?

Mother			Father			Any sibling		
Yes	No	Not sure	Yes	No	Not sure	Yes	No	Not sure

If yes please give details **(i.e. diagnosed by clinician, onset, duration etc.)**

14. Have any of the following persons had itchy stuffy nose or swollen, itchy eyes when not with a cold?

Mother			Father			Any sibling		
Yes	No	Not sure	Yes	No	Not sure	Yes	No	Not sure

If yes please give details **(i.e. diagnosed by clinician, onset, duration etc.)**

15. Do you have pets in the home?

	Yes	No	Details
Cat			
Dog			
Other			

16. Do you regularly visit a home where there are pets?

	Yes	No	Details
Cat			
Dog			
Other			

Habitual intake of fish

17. Do you normally eat fish?

18. Which types of fish do you eat (i.e. cod, mackerel, sole, kipper, halibut, bass, bream etc.)?

19. How often do you eat each of these (i.e. number of time per week or month or year)?

Thank you for giving us this information

Information collected by:
Investigator Name……..

Investigator Signature……

Date…….

4 Information sheet for pregnant women

Salmon in Pregnancy Study (SIPS):
The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies

Ethics Number: 07/Q1704/43

Co-ordinator: Dr Liz Miles

Chief Investigator: Prof Philip Calder

Telephone: 023 8079 5250 or 023 8079 6925
or 023 8079 5252
(Mobile - 07864 831283)
Email sips@soton.ac.uk

Version 4, 7th May 2008

We would like to invite you to participate in research studying whether eating salmon in pregnancy can prevent allergy in children. Before you agree to participate it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

1 What is the purpose of this study?

The reason for the large increase in allergies in the last 30 years is not understood. One suggestion is that changes in the fats we eat are making us more susceptible to allergies. People in the UK with a typical 'modern' diet eat very little oily fish. We are interested in studying possible benefits of eating oily fish in pregnancy on development of allergies in children. We know that babies who later get allergies show some slight changes in their immune response at birth. These changes do not allow us to accurately predict which babies will get allergies, but do tell us that a tendency to do so may partly originate before the baby is born. Therefore we wish to study the effect of eating oily fish during pregnancy on signs of allergy in the baby. We will start our study in 2007 and it will finish in 2010.

2 Why have I been chosen?

We are looking for women aged 18-40 who are in the early stages of pregnancy and who have allergies themselves or whose partner, or any other children, suffer from allergies (i.e. hayfever, eczema, asthma or food allergies). This increases your baby's risk of developing allergies him/herself. We wish to investigate the effect of eating oily fish (salmon) in pregnancy and need a group of mothers who are happy to eat 2 portions of salmon per week but who usually do not eat oily fish more than twice per month. We will provide this salmon for you and your partner. However, we need to compare mothers who have eaten salmon with a group of mothers who have not (i.e. mothers who carry on eating their normal diet). Thus we will allocate those happy to join our study into the salmon or no salmon group randomly (i.e. by chance). Current government guidelines advise that we should all eat more oily fish but that pregnant women should not eat more than 2 oily fish meals per week [www.eatwell.gov.uk/healthydiet/nutritionessentials/fishandshellfish/]. Our salmon group women will be eating the amount of oily fish recommended by the government but the no salmon group, who will continue with their normal diet, will be eating less oily fish than is currently recommended. We are hoping that 100 women will agree to take part.

3 Do I have to take part?

You are under no obligation to take part. If you take part you are still free to withdraw at any time and without giving a reason. If you wish to withdraw you can request that your data and samples be destroyed. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive. If you decide to take part you will be given this information sheet to keep and will be asked to sign a consent form.

4 What will happen to me if I take part?

You and (ideally) your partner will be invited to attend the Southampton Women's Survey Unit at the Princess Anne Hospital at about 20 weeks of pregnancy to meet a member of the research team. The research study will be explained fully and you will be given the opportunity to ask questions.

We will ask both of you some questions about your health, lifestyle and allergies. We will take some blood (30 ml, about 2 tablespoonfuls from you and ask you to provide a urine sample. We will measure your weight and your body composition (i.e. amount of muscle and fat). We use specialised weighing scales which measure your weight and by sending a very small electrical current through your body can assess your body composition. This is a harmless procedure and has been used to assess the body composition of pregnant mothers in previous studies.

The sonographer will perform an ultrasound scan (this will replace your normal 20 week NHS ultrasound scan) and give you pictures of your baby from the scan to keep. On this morning we will ask you not to eat or drink (except water) before your appointment and we will give you breakfast at the appointment. We will complete a questionnaire with you about your normal diet. This appointment will take approximately 1 hour. At this time we will allocate you to either the group of mothers asked to eat salmon or to the no salmon group. If you are in the salmon eating group we will supply you (and your partner) with frozen salmon portions sufficient for you to have 2 portions per week. We will give you one month's supply every month from when you join the study until your baby is born. We will ask mothers in the no-salmon group not to eat more than 2 portions of oily fish (salmon, tuna, pilchards, sardines) per month whilst on this study (tinned tuna is OK).

We would like to see you for 2 more appointments during your pregnancy (weeks 34 and 38). On each visit we will ask you some questions about your health, take a blood sample, measure body composition and ask you to provide a urine

sample. We will provide breakfast and also offer you an ultrasound scan of your baby at 34 weeks. These appointments will take approximately 1-1½ hours.
At week 38 we would like you to collect a small stool sample for us. This is to compare with the development of 'friendly bacteria' in your baby's bowel. We are interested in this because it has been suggested that some 'friendly bacteria' normally found in babies bowels may be protective against the development of allergies.
At the birth of your baby we will collect a blood sample from the umbilical cord. This is a harmless procedure and does not involve the newborn baby at all and is carried out by the midwife present at the birth or a researcher. We will also collect a sample of umbilical cord and of placenta (which are normally disposed of). A research nurse will measure the size of your baby whilst you are in hospital
If you decide to breast feed your baby we would like you to provide us with a sample (5 ml, about 1 teaspoonful) of your breast milk on the first day of breast feeding and then days 5, 14 and 28 after your baby is born (if you are still breastfeeding your baby at this time).
We would like you to measure your body composition at home daily for the first 2 weeks after your baby is born with specialised weighing scales which we will provide. This will take less than 5 minutes each day.
We would like to measure your body composition as before and your bone mineral density by DEXA scan within 2 weeks of you giving birth. The appointment will last, at most, 45 minutes. During this time we will measure your height and weight and perform a bone density scan.
The bone density scan does not work if you are wearing any metal objects like buckles, zips or rings, so we do ask you to wear tracksuit bottoms and a T-shirt, if possible. The bone density scan involves lying on a table and a small scanning arm will pass over, about two feet in the air; it does not touch you. The dose of x-rays is equivalent to about three day's natural background x-rays (or a day trip to Cornwall). The scan will not cause any pain or harm.
We would like to follow your baby for his/her first 6 months of life. You will receive a separate information sheet about this and you will have the opportunity to discuss this further and to ask any questions that you might have before deciding if you wish to help with this research.
At the follow up appointment (or at a mutually convenient time) we would like to perform some skin prick testing on you and your partner. This is to confirm the allergies you told us about when we first met you. Skin prick testing is performed by placing a drop of clear solution on the arm and pricking the skin through the solution. From your partner we would like to take a blood sample (10mls, about 2 teaspoonfuls).
All blood and breast milk samples donated for this research study will be frozen and stored for analyses related to the development of allergy.

5 What are the possible benefits of taking part?

By participating in this study you may be able to help other children in the future from developing allergies.

6 What are the possible disadvantages and risks of taking part?

Taking blood can occasionally cause some discomfort and bruising. Skin prick testing can cause some local itching which may last for about 15-30 minutes and can be relieved by using some calamine lotion. You will be giving up your time to help us with research including a longer appointment at your routine 20 week NHS ultrasound scan appointment and 2 extra appointments at the Princess Anne Hospital at 34 and 38 weeks of pregnancy (approximately 1-1½ hours). In addition, we would like you to record your consumption of fish, provide breast milk samples (on 4 days over the first month), collect your baby's stools (on 4 days over the first three months), measure your own body composition at home by standing on a specialised weighing scale (daily for the first two weeks after you give birth – each weighing will take no more than 5 minutes), and if possible allow your bone density to be measured within two weeks of giving birth (this will require a visit to the Princess Anne Hospital for a 45 minute appointment).

7 What will happen if anything goes wrong?

If you have a concern about any aspect of the study you should speak to the researchers who will do their best to answer your questions (telephone 023 8079 6925). If you remain unhappy and wish to complain formally you should contact the Wellcome Trust Clinical Research Facility in the first instance (telephone 023 8079 4989). Any complaint about the way that you have been dealt with during the study or any possible harm you might suffer will be addressed.

In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

8 Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. We will inform your GP that we have approached you and will notify them of your participation.

9 What will happen to the study results?

The results will be submitted for publication in medical literature. If you wish to see copies of any reports and publications, please contact the researchers listed and they will be happy to supply these for you. Once the study is complete, an open meeting will be arranged so that participants may discuss outcomes and get a summary of the results.

10 Will I be reimbursed?

We will reimburse you your travel expenses and parking fee at each appointment.

11 Who is funding the study?

This study is funded by the European Commission (website: http://ec.europa.eu/index_en.htm).

12 Who has reviewed the study?

The Southampton and South West Hampshire Local Research Ethics Committees.

13 Contact for further information

If you have any questions then please contact the SIPS Project Research Team on the numbers/email given on the front of this leaflet.

Thank you for taking the time to read this

5 Consent form for pregnant women

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Title of study: **The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies**

Study ID Number……..

Chief Investigator: Professor Philip Calder, Tel: 023 8079 5250

Contact details for research team: Dr Liz Miles, Tel: 023 8079 6925 or 023 8079 5252

*PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:*

8. *I have read the information sheet dated 15/05/07 (version 2) for the above study and have been given a copy to keep. I have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me.* ☐

9. *I understand that my participation in this study is voluntary and that I am free to decline entry into the study, and that during the study I am free to withdraw at any time without giving a reason and that withdrawal will not affect any future medical care.* ☐

10. *Samples to be collected. I agree to give a sample of my blood, urine, umbilical cord blood, umbilical cord, placenta, stool and breast milk (if applicable) for research in this project (delete as appropriate). I agree to be skin prick tested. I understand how the samples will be collected and how these procedures will be performed and that they will be used for the purposes if this investigation and will then be disposed of. I understand that my name will not be linked to these samples.* ☐

11. *I agree to have my body composition measured by bioelectrical impedence and bone mineral density measured by DEXA scan.* ☐

12. *Information to be collected. I give permission for someone from the research team to look at my medical notes to get information pertinent to the study. I understand that the information will be kept confidential.* ☐

13. *I understand that my Doctor will be informed of my participation in the study.* ☐

7. *I agree to take part in the above study* ☐

_____	_____	_____
Name of Patient	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

Version 3, 12th October 2007 *Ethics Number: 07/Q1704/43*

6 SIPS recruitment questionnaire (19-20 weeks gestation)

Volunteer Number

Date informed consent given

19 week visit check list (please tick when sample/data collected):

a	Urine sample	☐
b	Blood sample	☐
c	Food frequency questionnaire	☐
d	Ultrasound scan	☐
e	Body composition	☐

Name and Contact Details

Name

Address

Telephone – home/work

Telephone – mobile

Other contact

Personal Details

Date of Birth

Hospital Number

Expected date of delivery

Consultant

Community midwife & clinic

Health visitor & clinic

Doctor & surgery

General Health

1. Height

2.
Weight……..

3. Mothers' history of any ailments or diseases

4. Medication/supplements/vitamins (taken in the last 4 weeks)

Therapy	Dose route and regimen	Date started/stopped

5. Number of pregnancies (including this pregnancy)

6. Number of children

7. History of smoking Never / Past smoker / current smoker

If current or past smoker please give details (i.e. number of cigarettes)

8. Current alcohol intake in drinks per week (1 beer or 1 glass of wine or 1 shot of spirits).

Drink per week	
0	
1-7	
8-14	
>14	

Activity and Exercise

Can I firstly ask you about your activity and exercise patterns over the last three months? As before, we would like you to divide up a "typical" day into three types of activity. These are:
(1) sleeping or lying, (2) sitting, (3) standing or walking.

10.1 Over a typical 24 hour day how many hours have you generally spent sleeping or lying with your feet up?

☐☐ hrs ☐☐ mins

(*ask time usually go to bed & wake up, including any at work!*)

This would indicate xx hours sitting or on your feet.

10.2 Of those hours how many on a typical day have you spent sitting down? (*e.g. includes sitting at work, mealtimes, driving, reading, watching TV*)

☐☐ hrs ☐☐ mins

10.3 This would mean that you have spent about xx hours a day on your feet. Does this sound about right?

☐☐ hrs ☐☐ mins

10.4 Out of these xx hours spent on your feet, about how much of the time were you **actively on the move** (rather than standing fairly still)?

1.	Very little	10%
2.	Some	30%
3.	About half	50%
4.	Most	70%
5.	Almost all	90%

☐

10.5 During the past three months, how often have you done the following kinds of exercise or activities?

a) **strenuous exercise** which made your heart beat rapidly **AND** left you breathless e.g. jogging, vigorous swimming or cycling, aerobics.

FFQ categories 1-7 ☐ >x1 ☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

b) **moderate exercise** which left you exhausted but not breathless, e.g. brisk walking, dancing, easy swimming or cycling, badminton, sailing.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

c) gentle exercise which left you tired but not exhausted, e.g. walking, heavy housework (including washing windows and polishing), gardening, DIY, golf.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average **about how long did** each period of activity last?

☐ hrs ☐☐ mins

10.6 Which of the following best describes your walking speed at present?

1. Very slow
2. Stroll at an easy pace
3. Normal speed
4. Fairly brisk
5. Fast

Family Social History

18. Mothers' occupation

19. Fathers' occupation

20. Mothers highest level of education

School	
Further	
Higher	

21. Fathers highest level of education

School	
Further	
Higher	

Bioelectrical impedance

(Note – individuals with indwelling medical devices must not have bioelectrical impedance measurements taken

22. Do you have any indwelling electrical devices (i.e. pacemakers)?

☐ **Yes – DO NOT TAKE ELECTRICAL IMPEDANCE MEASUREMENT**

☐ **No**

Ask volunteer to empty bladder. Take measurements and attach print off securely to this page.

Thank you for giving us this information

Information collected by:
Investigator Name……..

Investigator Signature……

Date…….

7 SIPS Food Frequency Questionnaire for Women (to be completed at 19-20 weeks, 32-34 weeks of pregnancy and 3 months postpartum)

Time point:

Volunteer Number
Name
Date of Birth
Date

2: DIETARY QUESTIONS

2.1 Now I am going to ask you about the foods you have eaten over the past 3 months. To do this I have a list of foods and I would like you to tell me how often you have eaten each food. As before the list may include foods you never ate or you may find foods which you eat a lot are missing. These can be added on at the end. *(Define the 3 month period)*

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
1	White Bread	1	2	3	4	5	6	7	☐
	When you ate bread/toast/sandwiches, how many slices/rolls did you eat at a typical meal? ☐☐.☐ *Rolls (count as 2 slices)* *French bread (2" counts as 1 slice)*								
2	Brown and wholemeal bread/rolls	1	2	3	4	5	6	7	☐
	How many slices/rolls did you eat at a typical meal? ☐☐.☐ *Rolls (count as 2 slices)*								
3	Crackers and cheese biscuits	1	2	3	4	5	6	7	☐
4	Wholemeal and rye crackers	1	2	3	4	5	6	7	☐
5	'Bran' breakfast cereals	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
6	Other breakfast cereals	1	2	3	4	5	6	7	☐
7	Added bran to foods	1	2	3	4	5	6	7	☐
8	Cakes and gateaux	1	2	3	4	5	6	7	☐
9	Buns	1	2	3	4	5	6	7	☐
10	Pastries	1	2	3	4	5	6	7	☐
11	Biscuits-chocolate, digestive and ginger	1	2	3	4	5	6	7	☐☐
12	Other biscuits	1	2	3	4	5	6	7	☐☐
13	Fruit puddings	1	2	3	4	5	6	7	☐
14	Milk based puddings and sauces	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
15	Other puddings	1	2	3	4	5	6	7	☐
16	Yogurt and fruit fools	1	2	3	4	5	6	7	☐
17	Potatoes – boiled and jacket	1	2	3	4	5	6	7	☐
	When you ate these how many potatoes did you eat at a typical meal? ☐☐.☐ *Large baking (count as 3)/new (count as 0.5)*								
18	Roast potatoes and chips	1	2	3	4	5	6	7	☐
	When you ate these how many potatoes did you eat at a typical meal? ☐☐.☐								
19	Yorkshire puddings and savoury pancakes	1	2	3	4	5	6	7	☐
20	Brown and white rice	1	2	3	4	5	6	7	☐
21	Pasta and dumplings	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
22	Tinned vegetables	1	2	3	4	5	6	7	☐
23	Peas and green beans	1	2	3	4	5	6	7	☐
24	Carrots	1	2	3	4	5	6	7	☐
25	Parsnips, swede and turnip	1	2	3	4	5	6	7	☐
26	Sweetcorn and mixed veg	1	2	3	4	5	6	7	☐
27	Beans and pulses	1	2	3	4	5	6	7	☐
28	Tomatoes	1	2	3	4	5	6	7	☐
29	Spinach	1	2	3	4	5	6	7	☐
30	Broccoli, Brussels sprouts and spring greens	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
31	Cabbage and cauliflower	1	2	3	4	5	6	7	☐
32	Peppers and watercress	1	2	3	4	5	6	7	☐
33	Onion	1	2	3	4	5	6	7	☐
34	Green salad	1	2	3	4	5	6	7	☐
35	Side salads in dressing	1	2	3	4	5	6	7	☐
36	Courgettes, marrow and leeks	1	2	3	4	5	6	7	☐
37	Mushrooms	1	2	3	4	5	6	7	☐
38	Vegetable dishes	1	2	3	4	5	6	7	☐
39	Vegetarian foods	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
40	Tinned fruit not including grapefruit, prunes, figs or blackcurrants	1	2	3	4	5	6	7	☐
41	Cooked fruit not including blackcurrants	1	2	3	4	5	6	7	☐
42	Dried fruit	1	2	3	4	5	6	7	☐
43	Fresh apples and pears	1	2	3	4	5	6	7	☐
44	Fresh oranges and orange juice	1	2	3	4	5	6	7	☐☐
45	Grapefruit and grapefruit juice	1	2	3	4	5	6	7	☐☐
46	Blackcurrants, ribena and hi-juice blackcurrant drinks	1	2	3	4	5	6	7	☐☐
47	Other fruit juices (not squashes)	1	2	3	4	5	6	7	☐☐
48	Diet Coke and Pepsi not including caffeine free	1	2	3	4	5	6	7	☐☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
49	Coke and Pepsi	1	2	3	4	5	6	7	☐☐
50	Soft drinks not including diet drinks (low calorie or low sugar)	1	2	3	4	5	6	7	☐☐
51	Bananas	1	2	3	4	5	6	7	☐
52	Fresh peaches, plums, cherries and grapes	1	2	3	4	5	6	7	☐
53	Strawberries and raspberries	1	2	3	4	5	6	7	☐
54	Fresh pineapple, melon, kiwi and other tropical fruits	1	2	3	4	5	6	7	☐
55	Nuts	1	2	3	4	5	6	7	☐
56	Bacon and gammon	1	2	3	4	5	6	7	☐
57	Pork	1	2	3	4	5	6	7	☐
58	Chicken and turkey	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
59	Lamb	1	2	3	4	5	6	7	☐
60	Beef	1	2	3	4	5	6	7	☐
61	Minced meat dishes	1	2	3	4	5	6	7	☐
62	Meat Pies	1	2	3	4	5	6	7	☐
63	Liver and kidney	1	2	3	4	5	6	7	☐
64	Paté and liver sausage	1	2	3	4	5	6	7	☐
65	Faggots and black pudding	1	2	3	4	5	6	7	☐
66	Sausages	1	2	3	4	5	6	7	☐
67	Ham and luncheon meat	1	2	3	4	5	6	7	☐
68	White fish	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
69	Fish fingers and fish dishes	1	2	3	4	5	6	7	☐
70	Oily fish	1	2	3	4	5	6	7	☐
71	Shellfish	1	2	3	4	5	6	7	☐
72	Boiled and poached eggs	1	2	3	4	5	6	7	☐
73	Omelette and fried eggs	1	2	3	4	5	6	7	☐
74	Cottage Cheese	1	2	3	4	5	6	7	☐
75	Cheese	1	2	3	4	5	6	7	☐
76	Pizza, quiches and cheese flans	1	2	3	4	5	6	7	☐
77	Soup	1	2	3	4	5	6	7	☐
78	Mayonnaise and salad cream	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
79	Pickles, chutney, tomato ketchup and brown sauce	1	2	3	4	5	6	7	☐
80	Chocolate	1	2	3	4	5	6	7	☐☐
81	Other sweets	1	2	3	4	5	6	7	☐☐
82	Ice cream and chocolate desserts	1	2	3	4	5	6	7	☐
83	Cream	1	2	3	4	5	6	7	☐
84	Crisps and savoury snacks	1	2	3	4	5	6	7	☐☐
85	Sweet spreads	1	2	3	4	5	6	7	☐
86A	Gravy granules and powders	1	2	3	4	5	6	7	☐
86B	Stock cubes and Marmite	1	2	3	4	5	6	7	☐

	FOOD DESCRIPTION	FREQUENCY EATEN							
FOOD CODE		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
87	Drinking chocolate and milk shakes not including McDonald style milkshakes	1	2	3	4	5	6	7	☐
88	Decaffeinated coffee and tea	1	2	3	4	5	6	7	☐☐
89	Tea	1	2	3	4	5	6	7	☐☐
90	Coffee	1	2	3	4	5	6	7	☐☐
93	Spreading fat (1) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐
94	Spreading fat (2) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐
95	Spreading fat (3) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐
96	Frying fat or oil (1) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐
97	Frying fat or oil (2) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐
98	Frying fat or oil (3) _____ F☐☐☐☐☐	1	2	3	4	5	6	7	☐

FOOD CODE	FOOD DESCRIPTION	FREQUENCY EATEN							
		Never	Once every 2-3 Months	Once a Month	Once a Fortnight	1-2 Times per Week	3-6 Times per Week	Once a day	More than once a day
99	Other vegetable oil (1) e.g. salad dressings, marinades _____ F	1	2	3	4	5	6	7	☐
100	Other vegetable oil (2) e.g. salad dressings, marinades _____ F	1	2	3	4	5	6	7	☐

2.2 Are there food or drinks which you have eaten or drunk **once a week or more** which are not on the list? Include breakfast bars such as Nutrigrain and Kellogg's

0.No/1. Yes ☐

If Yes

Name of food/drink	1-2 times per week	3-6 times per week	Once a day	More than once a day
☐☐☐☐☐				☐
☐☐☐☐☐				☐
☐☐☐☐☐				☐
☐☐☐☐☐				☐
☐☐☐☐☐				☐

Now I would like to ask in more detail about some specific foods

2.3: Which types of milk have you used regularly in drinks and added to breakfast cereals over the last 3 months?

0. None
1. Whole pasteurised
2. Semi-skimmed pasteurised
3. Skimmed pasteurised
4. Whole UHT
5. Semi-skimmed UHT
6. Skimmed UHT
7. Other

Milk 1 ☐ Other (specify)_____

Milk 2 ☐ Other (specify)_____

Milk 3 ☐ Other (specify)_____

2.4 * On average over the last 3 months how much of each milk have you consumed per day?

Milk 1 ☐ . ☐☐ pints

Milk 2 ☐ . ☐☐ pints

Milk 3 ☐ . ☐☐ pints

2.5 Have you added sugar to breakfast cereals, tea & coffee, puddings etc.?

0. No *go to 2.7* ☐
1. Yes

2.6 Approximately how many teaspoons of sugar have you added each day? ☐☐

2.7 When you eat meat, how much of the fat have you usually cut off (including chicken skin)?

1. all 100%
2. most 60%
3. some 30% ☐
4. none 0%
9. not applicable

2.8 Just thinking about the **past week** how many servings did you eat of:

Vegetables and vegetable-containing dishes (excluding potatoes)?	
fruit and pure fruit juices?	
meat and fish and their dishes?	

3: FOOD SUPPLEMENTS & DIETARY CHANGES

3.1 During the past three months have you taken any pills, tonics or tablets to supplement your diet? (e.g. vitamins, minerals, iron tablets, folic acid, fish oils etc.) ☐
0. No 1. Yes
If yes, please state which:
(for number per day, record number of tablets/capsules/teaspoons per day, as appropriate)

Supplement	Number per day	How many days in the last 90?	Did you start taking this: 1: Less than 1 month ago 2: 1-2 months ago 3: More than 2 months ago
☐☐☐☐			
☐☐☐☐			
☐☐☐☐			
☐☐☐☐			
☐☐☐☐			

5: ALCOHOL CONSUMPTION

I'd like to ask you a few questions about your drinking and smoking habits.

5.1 Do you ever drink alcohol? ☐
0. No *go to section 6*
1. Yes

5.2 During the past three months:

a) How often have you drunk
Shandy or Low Alcohol Beer/Lager/Cider? FFQ 1-7 ☐ >x1 ☐☐
(don't include alcohol **free** lager etc)

b) When you drank these how many <u>pints</u> did you normally have? ☐☐ . ☐☐
(if range given code mid-point)

5.3 a) How often have you drunk
Beer/Stout/Lager/Cider/Alcopops? FFQ 1-7 ☐ >x1 ☐☐

b) When you drank these how many <u>pints</u> did you normally have?
(if range given code mid-point) ☐☐ . ☐☐

5.4 a) How often have you drunk
Low alcohol wine? FFQ 1-7 ☐ >x1 ☐☐

b) When you drank this how many <u>glasses</u> did you normally have?
(if range given code mid-point) ☐☐ . ☐☐

5.5 a) How often have you drunk
Wine/Sherry/Martini/Cinzano? FFQ 1-7 ☐ > x1 ☐☐

b) When you drank these how many <u>glasses</u> did you normally have?
(if range given code mid-point) ☐☐ . ☐☐

5.5 a) How often have you drunk
Spirits/Liqueurs? FFQ 1-7 ☐ > x1 ☐☐

b) When you drank these how many <u>measures</u> did you normally have?
(if range given code mid-point) ☐☐ . ☐☐

DIETARY QUESTIONS ON FOOD ITEMS FORTIFIED WITH ESSENTIAL FATTY ACIDS, PROBIOTICS, PREBIOTICS

We are particularly interested in your consumption of food items with added **omega-3, omega-6, probiotics or prebiotics.** I have a list of food items that have these things added to help you remember the specific products you may consume. Are there foods or drinks with added **omega-3, omega-6, probiotics or prebiotics** that you have eaten or drunk **once a week or more**?

0.No/1. Yes ☐

If Yes

Name of food/drink	Code	**Portion**	**Number of days per week**							**More than once a day**
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐
			1	2	3	4	5	6	7	☐

8 SIPS Salmon consumption diary during pregnancy (salmon group)

Volunteer Number

Volunteer Name

Please tick the days on which you eat your salmon. This should be twice per week. Could you also include any other fish you eat (which type and how it is cooked). Thank you. SIPS research team (tel: 02380 796925 or 02380 795252).

Month 5 / date started…….	**Fish eaten**
Week 21 – day 1	
Week 21 – day 2	
Week 21 – day 3	
Week 21 – day 4	
Week 21 – day 5	
Week 21 – day 6	
Week 21 – day 7	
Week 22 – day 1	
Week 22 – day 2	
Week 22 – day 3	
Week 22 – day 4	
Week 22 – day 5	
Week 22 – day 6	
Week 22 – day 7	
Week 23 – day 1	
Week 23 – day 2	
Week 23 – day 3	
Week 23 – day 4	
Week 23 – day 5	
Week 23 – day 6	
Week 23 – day 7	
Week 24 – day 1	
Week 24 – day 2	
Week 24 – day 3	
Week 24 – day 4	
Week 24 – day 5	
Week 24 – day 6	
Week 24 – day 7	

9 SIPS Fish consumption diary during pregnancy (control group)

Please tick the days on which you eat fish and write what type of fish it is and how it is cooked, i.e., baked cod in crumbs; smoked haddock, poached; fresh tuna, fried; tinned tuna; fresh trout, grilled.

During your pregnancy, while you are on the study (i.e. from the 20th week until delivery) please could you avoid eating more than 1 portion of oily fish per month. We have listed some examples of oily fish below. You can eat as much non-oily fish as you usually like to. We have listed some examples of non-oily fish below. Thank you. SIPS research team (tel: 023 8079 6925 or 023 8079 5252).

Oily Fish	**Non-oily/white fish**
Please eat no more than once per month	**Eat as much as usual**
Salmon (fresh or canned) Trout Mackerel Herring Tuna (fresh only) Sardines Pilchards Kipper Eel Anchovies Swordfish Bloater Carp Sprats Whitebait	Cod Tinned tuna Haddock Plaice Coley Whiting Skate Hake Hoki Flounder John dory Ling Red and grey mullet Dogfish/rock salmon Turbot Tilapia Marlin Sea bream Lemon sole Halibut Shark Pollack Monk fish Cat fish Red snapper Seabass Dover sole Crab

Volunteer Number

Volunteer Name

Please tick the days on which you eat fish and write which type and how it is cooked. Thank you. SIPS research team (tel: 023 8079 6925 or 023 8079 5252).

Month 5 / date started…….	**Fish eaten**
Week 21 – day 1	
Week 21 – day 2	
Week 21 – day 3	
Week 21 – day 4	
Week 21 – day 5	
Week 21 – day 6	
Week 21 – day 7	
Week 22 – day 1	
Week 22 – day 2	
Week 22 – day 3	
Week 22 – day 4	
Week 22 – day 5	
Week 22 – day 6	
Week 22 – day 7	
Week 23 – day 1	
Week 23 – day 2	
Week 23 – day 3	
Week 23 – day 4	
Week 23 – day 5	
Week 23 – day 6	
Week 23 – day 7	
Week 24 – day 1	
Week 24 – day 2	
Week 24 – day 3	
Week 24 – day 4	
Week 24 – day 5	
Week 24 – day 6	
Week 24 – day 7	

10 SIPS 32-34 week of pregnancy appointment questionnaire

Volunteer Number .....

34 week visit check list (please tick when sample/data collected):

a	Urine sample	☐
b	Blood sample	☐
c	Food frequency questionnaire	☐
d	Ultrasound scan	☐
e	Body composition	☐

General Health

1. Weight .....

3. Mother's history of any ailments or diseases

.....

.....

.....

.....

4. Medication/supplements/vitamins (taken in the last 4 weeks)

Therapy	Dose route and regimen	Date started/stopped

5. Do you currently smoke? Yes / No

If 'yes' please give details (i.e. number of cigarettes)

6. How many alcoholic beverages per week do you drink at the moment (1 beer or 1 glass of wine or 1 shot of spirits).

Drink per week	
0	
1-7	
8-14	
>14	

Activity and Exercise

Can I firstly ask you about your activity and exercise patterns over the last three months? As before, we would like you to divide up a "typical" day into three types of activity. These are:

(1) sleeping or lying, (2) sitting, (3) standing or walking.

10.1 Over a typical 24 hour day how many hours have you generally spent sleeping or lying with your feet up?

☐☐ hrs ☐☐ mins

(*ask time usually go to bed & wake up, including any at work!*)

This would indicate xx hours sitting or on your feet.

10.2 Of those hours how many on a typical day have you spent sitting down? (*e.g. includes sitting at work, mealtimes, driving, reading, watching TV*)

☐☐ hrs ☐☐ mins

10.3 This would mean that you have spent about xx hours a day on your feet.

Does this sound about right?

hrs ☐☐ mins ☐☐

10.4 Out of these xx hours spent on your feet, about how much of the time were you **actively on the move** (rather than standing fairly still)?

1.	Very little	10%	☐
2.	Some	30%	
3.	About half	50%	
4.	Most	70%	
5.	Almost all	90%	

10.5 During the past three months, how often have you done the following kinds of exercise or activities?

a) **strenuous exercise** which made your heart beat rapidly **AND** left you breathless e.g. jogging, vigorous swimming or cycling, aerobics.

FFQ categories 1-7 ☐ >x1 ☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

b) **moderate exercise** which left you exhausted but not breathless, e.g. brisk walking, dancing, easy swimming or cycling, badminton, sailing.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

c) **gentle exercise** which left you tired but not exhausted, e.g. walking, heavy housework (including washing windows and polishing), gardening, DIY, golf.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average **about how long did** each period of activity last?

☐ hrs ☐☐ mins

10.6 Which of the following best describes your walking speed at present?

1. Very slow
2. Stroll at an easy pace
3. Normal speed
4. Fairly brisk
5. Fast

☐

8. If in the intervention group have you had any problem eating your salmon?

<u>Bioelectrical impedance</u>

(Note – individuals with indwelling medical devices must not have bioelectrical impedance measurements taken

9. Do you have any indwelling electrical devices (i.e. pacemakers)?

☐ **Yes – DO NOT TAKE ELECTRICAL IMPEDANCE MEASUREMENT**

☐ **No**

Ask volunteer to empty bladder. Take measurements and attach print off securely to this page.

Thank you for giving us this information

Information collected by:
Investigator Name……..

Investigator Signature……

Date…….

11 SIPS 38 week of pregnancy appointment questionnaire

Volunteer Number .....

38 week visit check list (please tick when sample/data collected):

a Urine sample ☐

b Blood sample ☐

c Stool sample ☐

General Health

2. Have you had any health problems since last appointment

.....

.....

.....

4. Medication/supplements/vitamins (taken in the last 4 weeks)

Therapy	Dose route and regimen	Date started/stopped

5. Do you currently smoke? Yes / No

If 'yes' please give details (i.e. number of cigarettes)

6. How many alcoholic beverages per week do you drink at the moment (1 beer or 1 glass of wine or 1 shot of spirits).

Drink per week	
0	
1-7	
8-14	
>14	

Activity and Exercise

Can I firstly ask you about your activity and exercise patterns over the last three months? As before, we would like you to divide up a "typical" day into three types of activity. These are:

(1) sleeping or lying, (2) sitting, (3) standing or walking.

10.1 Over a typical 24 hour day how many hours have you generally spent sleeping or lying with your feet up?

☐☐ hrs ☐☐ mins

(*ask time usually go to bed & wake up, including any at work!*)

This would indicate xx hours sitting or on your feet.

10.2 Of those hours how many on a typical day have you spent sitting down? (*e.g. includes sitting at work, mealtimes, driving, reading, watching TV*)

☐☐ hrs ☐☐ mins

10.3 This would mean that you have spent about xx hours a day on your feet.

Does this sound about right?

hrs ☐☐ mins ☐☐

10.4 Out of these xx hours spent on your feet, about how much of the time were you **actively on the move** (rather than standing fairly still)?

1.	Very little	10%	☐
2.	Some	30%	
3.	About half	50%	
4.	Most	70%	
5.	Almost all	90%	

10.5 During the past three months, how often have you done the following kinds of exercise or activities?

a) **strenuous exercise** which made your heart beat rapidly **AND** left you breathless e.g. jogging, vigorous swimming or cycling, aerobics.

FFQ categories 1-7 ☐ >x1 ☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

b) **moderate exercise** which left you exhausted but not breathless, e.g. brisk walking, dancing, easy swimming or cycling, badminton, sailing.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average about how long did each period of activity last?

☐ hrs ☐☐ mins

d) **gentle exercise** which left you tired but not exhausted, e.g. walking, heavy housework (including washing windows and polishing), gardening, DIY, golf.

FFQ categories 1-7 ☐ >x1 ☐☐

and on average **about how long did** each period of activity last?

☐ hrs ☐☐ mins

10.6 Which of the following best describes your walking speed at present?

1. Very slow
2. Stroll at an easy pace
3. Normal speed
4. Fairly brisk
5. Fast

9. If in the intervention group have you had any problem eating your salmon?

Thank you for giving us this information

Information collected by:
Investigator Name……..

Investigator Signature……

Date…….

12 Baby feeding and health diary

Please keep this daily record of your baby's feeds and any illness he or she may have for the first 6 months after birth. If you need to write more than you can fit on the form please write it on the reverse of the page. If you have any questions about this form please call us on 023 8079 6925 or 023 8079 5252

1 Feeding

Please mark (B) for breastfeeding and (F) for formula feeding or (B&F) for both. If your baby is having formula milk please note which brand (i.e. Cow & Gate, Farley, Milupa, SMA) and the type (i.e. Premium, First, Aptamil First, Gold). If you have not changed anything from the day before just tick the day to tell us that it is the same as above.

2 Weaning

Please write on the diary any foods and approximate quantities (i.e. 1 teaspoon, 3 teaspoons) that your baby has when he/she starts to eat some more solid food. This will probably be at around 6 months of age (check with your midwife, health visitor or doctor for advice).

3 Health

Please write on the diary any illnesses that your baby may have during the first 6 months. This includes sicknesses such as colds, coughs, vomiting, diarrhoea, wheezes, rashes etc. Please also note any medicines your baby has and immunizations that he/she has.

Example
Baby Name.....*Arthur Salmon*

Date	1 Feed – Breast or Formula (& type)	2 Weaning foods	3 Health
1	B		
2	B		
3	B		
4	B		Cold
5	B		Cold
6	B & F (SMA Gold)		Cold, vomited
7	✓		Cold
8	✓		
9	✓		
10	✓		

Month 1 Baby Name……Date of Birth……..

Study ID……

Date	1 Feed – Breast or Formula (& type)	2 Weaning foods	3 Health
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
17			
18			
19			
20			
21			
22			
23			
24			
25			
26			
27			
28			
29			
30			
31			

13 Information Sheet for infant visit at 6 months

We would like to invite you and your baby to participate in the next stage of a research study about the development of allergy in children. Before you agree to participate it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your baby to take part.

1 What is the purpose of this study?

The reason for the large increase in the numbers of people with allergies in the last 30 years is not understood. One suggestion is that changes in the fats we eat are making us more susceptible to allergies. Notably, people in the UK with a typical 'modern' diet eat very little oily fish. We (at the Division of Developmental Origins of Health and Disease) are interested in studying possible benefits of eating oily fish in pregnancy on development of allergies in children. We know that babies who later get allergies show some slight changes in their immune response at birth. These changes do not allow us to accurately predict which babies will get allergies, but do tell us that the mechanisms for developing allergies probably start before the baby is born. Therefore we wish to investigate the effect of increasing the amount of oily fish eaten by women during pregnancy on signs of allergy in the newborn babies. We will start our study in 2007 and it will finish in 2010.

2 Why have I been chosen?

We are very grateful for your participation in the first stage of this study (from 20 weeks of your pregnancy until the birth of your baby) and we would like to collect some information about your baby during the first 6 months of his/her life.

3 Do I have to take part?

You are under no obligation to take part. If you take part you are still free to withdraw at any time and without giving a reason. If you wish to withdraw, you can request that your baby's data and samples be destroyed. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you or your baby receives. If you decide to take part you will be given this information sheet to keep and will be asked to sign a consent form.

4 What will happen to me if I take part?

We wish to follow the progress of your baby in the first 6 months of his/her life. We would like you to fill out a brief diary for the first 6 months of your baby's feeding habits and any illnesses your baby has.

We would like you to collect a small sample of your baby's stool on days 1 (or 2), 5, 14, 28 and at the end of his/her first and second months. This is to look at the development of 'friendly bacteria' in your baby's bowel. We are interested in this because it has been suggested that some 'friendly bacteria' normally found in babies bowels may be protective against the development of allergies.

We would like to see you and your baby for two appointments in this time, the first when your baby is less than 2 weeks of age and the second when he/she is 6 months old. At both of these appointments we will explain all the checks which we wish to perform and ask you to give written consent if you are happy with the explanation.

We want to assess your baby's growth with a bone density test (DEXA scan) when your baby is less than 2 weeks old. You will be invited to bring your baby to Burley Ward at the Princess Anne Hospital. The appointment will last, at most, 45 minutes. During this time we will measure your child's height and weight, arm size and perform a bone density scan for your child. Please bring your baby wearing easily removable clothing if possible as we undress them and wrap them in a blanket for the scan.

Later, at 6 months of age, we would like to see your baby for a clinical check at the Wellcome Trust Clinical Research Facility at Southampton General Hospital. At this appointment we wish to assess your baby for any symptoms of allergy and perform skin tests to some common allergens. This will involve placing 8 - 10 drops of clear solutions on the back or forearm and pricking the skin through the solutions. These are placed in an area about one-third of the area of this information sheet. If your baby is sensitised to one of the substances used, there will be a small reaction on the skin around the area of application. We would like to take a small sample of blood (3-5mls, no more than 1 teaspoonful). We will use lignocaine cream that will numb the area before taking the sample. It will be taken by a Paediatric trained Doctor or nurse. We will collect the diaries which you have completed (from the first stage of the study) on your baby's feeding and health in the first 6 months. This appointment will take approximately 1 hour.

5 What are the possible benefits of taking part?

Your baby will have an assessment of bone density. We will of course provide any medical advice necessary if the bone mineral density values are found to be low.

By participating in this study you may be able to help other children in the future from developing allergies.

6 What are the possible disadvantages and risks of taking part?

The bone density scan will not cause any pain or harm. It involves your child lying on a table and a small scanning arm will pass over, about two feet in the air; it does not touch your child. The dose of x-rays is equivalent to about three day's natural background x-rays (or a day trip to Cornwall).

Skin prick testing will cause itching due to the reaction to the positive control (histamine); this is similar to a "nettle rash". The itching may last between 15 – 30 minutes and calamine lotion will be offered to relieve this. Taking blood can sometimes cause the baby to cry as we will ask you to hold your baby firmly and some bruising may occur.

7 What will happen if anything goes wrong?

If you have a concern about any aspect of the study you should speak to the researchers who will do their best to answer your questions (telephone 023 8079 6925). If you remain unhappy and wish to complain formally you should contact the Wellcome Trust Clinical Research Facility in the first instance (telephone 023 8079 4989). Any complaint about the way that you have been dealt with during the study or any possible harm you might suffer will be addressed.

In the unlikely event that you are harmed due to someone's negligence, then you may have grounds for a legal action for compensation but you may have to pay your legal costs.

8 Will my taking part in this study be kept confidential?

All information which is collected about you and your baby during the course of the research will be kept strictly confidential. We will inform your GP that we have approached you and will notify them of your participation.

9 What will happen to the study results?

The results will be submitted for publication in medical literature. If you wish to see copies of any reports and publications, please contact the researchers listed and they will be happy to supply these for you. Once the study is complete, an open meeting will be arranged so that participants may discuss outcomes and get a summary of the results.

10 Will I be reimbursed?

We will reimburse you your travel expenses and parking fee at each appointment.

11 Who is funding the study?

This study is funded by the European Commission (website: http://ec.europa.eu/index_en.htm).

12 Who has reviewed the study?

The Southampton and South West Hampshire Local Research Ethics Committees.

13. Contact for further information

If you have any questions then please contact the SIPS Project Research Team, Tel: 023 8079 6925 or 023 8079 5252. There is an answer phone available and all calls will be returned. Alternatively email us on sips@soton.ac.uk

Thank you for taking the time to read this.

UNIVERSITY OF Southampton
School of Medicine

The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies

Salmon in Pregnancy Study Information Sheet for Parents of Babies

Ethics Number: 07/Q1704/43

Co-ordinator: Dr Liz Miles

Chief Investigator: Prof Philip Calder

Telephone: 023 8079 6925 or 023 8079 5252 or 07864 831283

Email sips@soton.ac.uk

Version 3, 14th February 2008

14 Consent form for follow-up infant at 6 months

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Title of study: **The effects of oily fish in pregnancy on signs of allergic diseases in infants at high risk of developing allergies**

Ethics number: 07/Q1704/43 Study ID No……

Chief Investigator: Professor Philip Calder, Tel: 023 8079 5250

Contact details for research team: Dr Liz Miles, Tel: 023 8079 6925 or 023 8079 5252

*PLEASE **INITIAL** THE BOXES IF YOU AGREE WITH EACH SECTION:*

I have read the information sheet dated 14th February (version 3) for the above study and have been given a copy to keep. I have had the opportunity to ask questions, understand why the research is being done and any possible risks which have been explained to me. ☐

I give permission for my child to be skin prick tested to common allergens, to have a sample of blood taken and for a clinical check (delete as appropriate). I understand how the skin test will be done, and how the blood sample will be collected and that this is voluntary and that I am free to withdraw my approval at any time without my or my baby's medical treatment or legal rights being affected. ☐

I give permission for someone from the research team to look at my child's medical notes to get information pertinent to the study. I understand that the information will be kept confidential. ☐

I understand that my Doctor and/or I may be informed of any of the results of tests done as part of the research. ☐

I understand that the research may not directly benefit my child's health. ☐

_____ _____ _____

Name of Patient *Date* *Signature*

_____ _____ _____

Name of Person taking consent *Date* *Signature*

1 for patient, 1 for researcher, 1 to be kept with hospital notes.

15 Body composition post-natal measurements at home

Volunteer Number

Volunteer Name

Height

Please read through the following notes and instructions for measuring your body composition with the Tanita UM-014S daily for your first 14 days after delivery. Thank you. If you have any questions please call the SIPS research team (tel: 023 8079 6925 or 023 8079 5252 or 07864 831283).

To make these measurements as accurate as you can please could you:

1. Undress (or wear as few clothes as possible) before stepping on the Tanita machine.

2. Try to take the readings at the **same time of day** (about 3 hours after rising or eating or exercise).

3. Try to keep body water levels the same for each measurement i.e. try **to drink the same amount before** the measurement and try to **empty your bladder just before** you step on the Tanita machine.

Instructions for using the Tanita UM-014S

1. Turn on the Power
Press the Set button to turn on the machine. It will beep to confirm activation, the Personal data number will be displayed, and the display will flash.

2. Get pen and form
Get your form for writing down your measurements and a pen and place them close to the Tanita machine

3. Select a Personal data Number
Press the Up/Down buttons to select a Personal data number. Once you reach the Personal data number you wish to use, press the Set button. The machine will beep once to confirm.

4. Set Age
Use the Up/Down buttons to scroll through numbers. When you reach your age, press the Set button. The machine will beep once to confirm.

5. Select Female or Male
Use the Up/Down buttons to scroll through Female (F), Male (M) settings, then press the Set button. The machine will beep once to confirm.

6. Specify the Height
Use the Up/Down buttons to specify Height (in centimetres) and then press the Set button (this is written on your body weight and composition recording form). The machine will beep once to confirm. The machine will beep twice and the display will flash all data (Male/Female, Age, Height) three times to confirm the programming.

7. Step on

After programming your personal data you are ready to take a reading. The machine will beep again and the display will show "0.0". Now step onto the platform. **Note:** Once the scales are programmed you can turn the machine on with the memory button and press set when the machine displays your personal data number (normally 1). The machine will then display your data then 0.0 and is ready for you to step on.

8. Write down and keep record of your readings

Your weight will be shown first. Continue to stand on the platform. "00000" will appear on the display and disappear one by one from left to right. Your body fat percentage will appear on the display screen. Do not step off until body fat percentage is shown. The display will then flash your weight and body fat percentage alternately three times. The unit will then shut down automatically. Please write your weight and body composition in the table below.

Date started…….	**Weight (Kg)**	**Body fat (%)**
day 1		
day 2		
day 3		
day 4		
day 5		
day 6		
day 7		
day 8		
day 9		
day 10		
day 11		
day 12		
day 13		
day 14		

16 Infant clinical assessment: skin prick testing (standard operating procedure (SOP))

1. Expose the flexor aspect of the forearm.

2. Do not sterilise the skin in any way since antiseptics or alcohol temporarily impair the ability of the skin to react.

3. Starting 2 cm from the antecubital fossa, mark out sites about 2.5 cm apart with a Soluprick pen, with different symbols for the different allergens.

4. Place one drop of each of the allergen solutions on the forearm skin. A positive control (histamine) and a negative control (the allergen diluent), must also be applied on the same arm.

5. Push a lancet through the drop of allergen or control solution at 90° to the skin, without drawing blood.

6. Repeat the procedure for each allergen and control using a new lancet each time.

7. Carefully remove the surplus fluid from all sites simultaneously by placing a paper tissue over the forearm. Take care not to cross contaminate the sites with other allergen solutions.

8. The size of the wheal and flare should be measured at the time of peak response. This will be at 15 minutes post skin puncture.

9. Measurement should be made with a skin test reaction gauge and the maximum wheal diameters should be noted along with the perpendicular diameters.

10. A wheal diameter of at least 3 mm greater than the negative control is considered to be positive.

Clear records of the skin test should be made including the following information:

-The date
-The name of the patient/subject
-The name of the investigator
-The size of the response in millimetres

The following may also be recorded:

-Any recent medication (please note that certain medications e.g. anti-histamines, affect skin reactivity and any results obtained under these conditions should be interpreted with this in mind).

17 SOP containing additional information for paediatric SPT.

Background: SPT is performed by introducing a small amount of allergen, approximately 0.1µl of extract, into the epidermis. The immediate, type one, response wheal and flare reaction is a consequence of allergen-specific IgE molecules being cross-linked by allergen. This results in signalling via Fcε receptors on mast cells which leads to degranulation and release of histamine, proteases, proteoglycans, prostaglandin D2 (PGD2), leukotriene C4 (LTC4) and platelet-activating factor causing a wheal and flare reaction. Histamine is considered to be the major mediator in the allergic wheal reaction. The wheal is caused by extravasation of serum from capillaries in the skin, which results from a direct effect of histamine. Pruritis and a larger erythematous flare accompany the wheal.

Skin prick testing is a reliable test to determine sensitivity and subsequent conformation of allergy in susceptible individuals.

Purpose: To ensure that this procedure is carried out in a consistent fashion.

Scope: To ensure that skin prick testing is performed in a consistent manner using the same solutions (ALK Abello) and equipment. This SOP applies to any members of the SIPS team or SWS team who may undertake skin prick testing on infants.

Responsibility: It is the responsibility of the individual members of the SIPS team to read and ensue they have received the necessary training to undertake skin prick testing on infants on the SIPS.

Procedure: The procedure will be carried out as detailed on the SUHT guideline for skin prick testing

Supporting Documents:
Protocol for SIPS Version 1, dated 20/02/07
SOP for taking Informed Consent (No 001C)
SUHT Policy and Procedure for Hand Washing
DoH Consent – what you have a right to expect. A guide for Adults
SUHT Guideline on SPT

Consumables:

NB: ALL SKIN PRICK TEST SOLUTIONS <u>MUST</u> BE ORDERED VIA PHARMACY AS THEY ARE CLASSED AS A "PATIENT NAMED TREATMENT"

Company Name & Address:
UniDrug Distribution Ltd
Amber Park
Berristow Lane
South Normanton
Derbyshire, DE55 2FH

Skin Prick Test Solution	Concentration	Cost (2ml vials)
Negative Control	Saline	£8.00
Dermatophagoides pteronyssinus	Soluprick standard quality (SQ) 10 HEP ((Histamine Equivalent in Prick testing)	£27.00
Felix domesticus	SQ 10 HEP	£27.00
Dog	SQ 10 HEP	£27.00
Grass pollen mix	SQ 10 HEP	£27.00
Tree pollen mix	SQ 10 HEP	£27.00
Cow's milk raw	1:20 w/v	£27.00
Whole hen's egg	1:100 w/v	£27.00
Histamine (positive) control	10 mg/ml	£8.00

Lancets: Via Linda Jackson (IIR Research Division) IDT for supplies (Cases 1000/ 5 boxes 200)

18 SOP for SCORAD

Background

The severity of atopic dermatitis can be quantified by using a clinical measuring tool Scoring Atopic Dermatitis (SCORAD). SCORAD has been previously validated in other studies SCORAD can be completed in 10 minutes and uses a body map proforma found in the six-month questionnaire.

Purpose

To ensure that correct and standardised scoring for eczema severity is undertaken. The validated SCORAD system is to be used.

Scope

SCORAD is an international validated tool used for the assessment of eczema. The following SOP is to be used in conjunction with the validated tool and has been written to assist the novice in understanding the terms and to give knowledge on how to perform the calculation in determining the total score. This standard operating procedure is to be used for Salmon in Pregnancy Study, LREC No: 07/1704/43 and used in conjunction with the protocol. This SOP applies to all individuals undertaking the assessment of SCORAD.

No advice is to be given to parent / carer if an infant has eczema. If required, suggest they contact their General Practitioner or health Visitor for further advice and treatment options.

Responsibilities

It is the responsibility of the individual members of the study team to read and ensure they have received sufficient training and have been deemed competent to undertake the assessment of SCORAD by the Clinical Co-Investigator (Dr Mich Lajeunesse). If any new

member should join the study team they will need to be trained and validated against an existent user to exclude Intra-Observer Variability (IOV).

Procedure

1. Explain procedure to parents.

2. Obtain informed written consent using supporting documentation.

3. Wash hands in accordance with SUHT guideline on hand washing.

4. Undress infant and examine for signs of eczema. If present, evaluate eczema using SCORAD using the proforma in the six month questionnaire.

5. Start the SCORAD assessment with evaluation of the extent (see definition below). Draw the involved areas on the evaluation sheet.

Extent the atopic dermatitis covers,
SCORAD is based on the rule of nine's. This principle was first used for determining the severity of burns to the human body. Each arm and the head constitute 9% of the body surface area. The front and back of the torso and each leg constitutes 18% of body surface area thus giving a total body surface area expressed as a percentage. Within the first domain the extent of body surface is expressed by indicating the total area involved. The size of the volunteer's palm is equivalent to 1% of body surface area.

1. Continue the SCORAD assessment with evaluation of the intensity (see definitions and grades below). Place figures in the boxes in the table on part B of the form. Add up the figures and place in box B.

Intensity (severity),
a. Erythema – A reddening of the skin that disappears under pressure.
b. Oedema/papules – Raised bumps which can be felt under the skin. This element cannot be evaluated only by the eye, the rash needs to be felt to establish correct graduation.
c. Oozing/crust – oozing lesions due to skin oedema and vesiculation.
d. Excoriation/scratch marks - Abrasions, signs of scratching and/or rubbing caused by mechanical trauma.
e. Lichenification- thickened and coarse appearance in chronic rashes. Accentuation of normal wrinkling which separates into thinner rhomboidal areas. Normally grayish or brownish in colour. This is generally seen in patients over the age of 2 years of age.
f. Skin dryness – Dry skin should be better appraised away from the inflamed areas and without any application of emollients or moisturizers. The dry, scaly skin coming from the healing of previous rashes should not be considered. Palpation is important in evaluating the roughness of the skin. The presence of cracked skin is usually associated with marked dryness in the hands and feet.

Choose a representative area to calculate the intensity of eczema. Place your figures in the appropriate sections on the sheet provided. These parameters are graded on a scale of 0 – 3.

0 = Absent
1 = Mild
2 = Moderate
3 = Severe

The following table of grades can be used to define intensity:

Erythema	1. Mild	Faintly detectable, very light pink
	2. Moderate	Dull red, clearly distinguishable
	3. Seve re	Deep dark red
Papules	1. Mild	Barely perceptible elevation
	2. Moderate	Clearly perceptible elevation
	3. Severe	Marked and extensive
Excoriations	1. Mild	Scant evidence with no signs of deeper skin change.
	2. Moderate	Several linear marks of skin with some showing evidence of deeper skin injury.
	3. Severe	Many erosive or crusty lesions.
Lichenification	1. Mild	Slight thickening of the skin discernable only by touch and with skin markings minimally exaggerated.
	2. Moderate	Definite thickening of the skin with skin markings exaggerated so that they form visible criss-cross pattern
	3. Severe	Thickened indurated skin with skin markings visibly portraying an exaggerated criss-cross pattern

1. Continue the SCORAD assessment with evaluation of the subjective criteria (see definition below). Draw on the visual analogue scale on the evaluation sheet. Place figure in box C.

Subjective symptoms of pruritis and sleep loss.

Subjective criteria

The third domain is the subjective scores of **pruritus** and **sleep loss**. The size of the participants' palm is equivalent to 1% of body surface area. The subjective symptoms are measured on a visual analogue scale between zero and ten and are an average for the last three days or nights. These figures are used and calculated to give a final figure expressed as a percentage (Kunz et al.)

1.Calculate the proportion of involved surface area segment by segment (extent). Place figure in box A.

2.Complete SCORAD calculations as post visit administration.

3.Post Visit Administration

1.Complete SCORAD as follows. Total the results for all segments.

2.Make calculations using the body map form (use example cited below), and a calculator if needed and record on form.

SCORAD Calculation

The calculation is derived in the following way:

Extent / 5 + Intensity x 7 / 2 + Subjective Score = total

Example – if extent (A) - 5, intensity (B) - 10 and subjective score (C) - 3, total would equal 39.

A/5 = 1

7B/2 = 35

C = 3

Total = 39

19 SIPS Neonatal Anthropometry

Maternal Volunteer Number
Date

Date informed consent given .....

Name and Contact Details

Baby Name .....

Mothers' Name .....

Address .....

.....

.....

Telephone – home/work .....

Telephone – mobile .....

Other contact .....

Personal Details

Date of Birth Gender .....

Infant Hospital Number .....

Anthropometry

12 INFANT EXAMINATION

12.1 Measurement Date d d m m y y

12.2 Time (24 hr clock)

12.3 Measurer

12.4 Helpers (Parent 90)

12.5 Occipito-frontal circumference ☐☐.☐ cm

☐☐.☐ cm

☐☐.☐ cm

Crying
0. No
1. Yes ☐

12.6 Crown-heel length (left leg) ☐☐.☐ cm

☐☐.☐ cm

☐☐.☐ cm

Crying
0. No
1. Yes ☐

12.7 Minimum carriage reading ☐☐☐ mm

12.8 Anthropometer used ☐

12.9 Baby weight (preferably nude) ☐.☐☐ kg

12.10 Weight of any clothes / nappy ☐.☐☐ kg

Information collected by:
Investigator Name……..

Investigator Signature……

Date……

20 Infant health and food frequency questionnaire at 6 Months of Age

Volunteer Number	**B**
Infant's Name (Forename, Surname)	
Mother's Name (Forename, Surname)	

Date informed consent given

Check clinical cover is in place (tick if confirmed) ☐

[Clinical cover Dr Mich Lajeunesse or Dr Woolf Walker confirm by calling PA Chris Dyche xt 4335 **2 hours prior to appointment time** (if unable to get an answer xt 4829 or 8973)]

6 month visit check list (please tick when sample/data collected):

a Completed baby feeding and health diary ☐

b Skin test ☐

c Blood sample ☐

Note to administrators. Questions 1 to 4 are the FFQ question and must be asked by appropriately trained staff (Maria V and Stella K). The front sheet and questions 4 to 9 are to be completed by designated CRF nurses or SIPS research team staff. Clinical procedures to be carried out by designated CRF nurses.

Infant's date of birth d d m m y y ☐☐ ☐☐ ☐☐

Sex M=Male F=Female ☐

Date of interview d d m m y y ☐☐ ☐☐ ☐☐

Interviewer ☐☐

I would firstly like to ask you about your baby's feeding. I'll start with questions about the type of milk or formula he/she has had.

1. Milk or Formula Feeding

1.1 ***Did you ever put your baby to the breast, even for a single feed?***

0. No
1. Yes *go to 1.3* ☐

1.2 *Was this? :

1. Personal choice
2. Because you were unwell
3. Because the baby was too small or unwell
8. Any other reason (e.g. advised not to; problems with previous baby)

☐

Then go to 1.10

1.3 Did you ever require antibiotic injections, tablets or surgery for mastitis or a breast abscess?

0. No
1. Yes ☐

1.4 Are you still breast feeding?

0. No
1. Yes*go to 1.6* ☐

1.5 How old was your baby when he/she last had a breast feed? *Note: refer to volunteer's infant feeding diary if possible.*

☐ mths ☐ ks ☐ s

or

On what date did he/she last have a breast feed? d d m m y y ☐☐ ☐☐ ☐☐

Go to 1.8

1.6 *What is the *main* ***reason for continuing to breast feed?***

1 Enjoyment
2 Best for baby / prevention of allergies
3 Cheaper
4 Baby prefers it or refuses other feeds
5 Convenience
6 Any other / multiple reasons, e.g. help lose weight, so sleeps through night

☐

1.7 Except for breast milk, has he/she ever had any other milk or formula in a bottle or cup or for mixing with solid foods, eg rusks?

0. No *go to section 2*
1. Yes ☐

1.8 How old was your baby when he/she started regularly having other milk or formula feeds from a bottle or cup or for mixing with solid foods? *Note: refer to volunteer's infant feeding diary if possible.*

☐ mths ☐ wks ☐ days

or

On what date did he/she start regularly having milk or formula feeds?

d d m m y y

☐☐ ☐☐ ☐☐

1.9 *What was the *main* ***reason for starting other milk or formula feeds?*** ☐☐

1. Return to work
2. Breastfeeding took too long or was too tiring
3. Convenience or to allow others in the family to feed
4. To try and get him/her to sleep through the night
5. The right time/age to change
6. Insufficient milk to satisfy the baby
7. Baby wouldn't suck because unwell or low birth weight
8. Baby wouldn't suck or not interested – no apparent reason
9. Baby irritable or colicky, or not gaining weight
10. Painful breasts / sore nipples / mastitis / breast abscess
11. Milk dried up
12. To mix with cereals/dried foods

88. Any other reason / multiple reasons
    e.g. mother unwell, inverted nipples, not enjoying it, bitten by baby

1.10*Please tell me which types of milk or formula you have used and what age your baby was when it was started and stopped or on what date it was started and stopped. *Note: refer to volunteer's infant feeding diary if possible.*

Use 88's if still using

Name of formula	**Formula code**	**Age started**			**Age stopped (month/day)**			**Date started**	**Date stopped**
		Mths	**wks**	**days**	**mths**	**wks**	**days**		

2. Food Frequency Questionnaire

I am now going to ask you about the milk or formula your baby has had in the **past week**.

If no breast feeding in the past 7 days go to 2.5

2.1 Not including expressed breast milk, can you tell me how many days out of the past 7 days he/she was breast fed? On average, how many feeds per day did he/she receive on these days? How long on average was he/she actively sucking** per day **on these days?
Note: refer to volunteer's infant feeding diary if possible.

Number of days in the past 7 days	Number of feeds per day	Average time actively sucking per day
		hrs ns

2.2 In the** past 7 days **did he/she have any expressed breast milk?

0. No *go to 2.4*
1. Yes

2.3 How many days out of the past 7 days did he/she have expressed breast milk? On average, how many times per day did he/she have expressed breast milk on these days? What was the average amount of milk** per day **on these days?

How many days out of the past 7	How many times per day	Volume per day								
		oz					mls			
				.						

2.4 In the past week did he/she have any other milk or formula except breast milk? Include any milk used for mixing with food.
Note: refer to volunteer's infant feeding diary if possible.

0. No *go to 2.6*
1. Yes

***2.5*Can you tell me the types of milk or formula he/she has had in the past 7 days? How many days out of the past 7 days was* type of milk *given? How many times per day was* type of milk *given? What was the average amount of* type of milk *per day on these days?** *Note: refer to volunteer's infant feeding diary if possible.* **Repeat for any other types of milk used.**

Name of formula	Formula code	How many days out of the past 7	How many times per day	Volume per day								
				oz					mls			
						.						
						.						
						.						
						.						

Have you included any milk used for mixing with food? *If no, adjust table above.*

***2.6 Now I am going to ask you about the** foods **your baby has eaten in the** past week**. I will ask you how many times he/she has eaten certain foods and also the amount of food eaten. You should only include food actually eaten, do not include food that was left over or spilled. I have a list of foods, many of which may not have been eaten in the past week or ever. You may also find that foods your baby has eaten in the past week are missing and these will be added on at the end.** *Note: refer to volunteer's infant feeding diary if possible.*
*

Food code	Food	No. of times in past wk	Brand codes	No. of times/ brand	Average amount on each occasion				
1	Pure baby rice, not including fruit flavoured rice				No. of tablespn dried		.		
							.		
							.		
2	Other dried baby cereals				No. of tablespn dried		.		
							.		
							.		
3	Rusks				No. of rusks		.		
							.		
							.		
	Were they 1. Original 3. Savoury 2. Reduced sugar 4. Gluten free								

****2.7 Did your baby eat any other dried baby foods in the past week? 0. No*** **go to 2.9**

1. Yes ☐

2.8

Food code	Food	No. of times in past wk	Brand codes	No. of times/ brand	Average no. of tablespoons dried on each occasion
4	Dried meat or fish based meals				.
					.
					.
5	Dried vegetable, pasta or rice based meals				.
					.
					.
6	Dried desserts				.
					.
					.

2.9*Did your baby eat any jars, tins or pots of baby foods in the past week?

0. No *go to 2.11*
1. Yes ☐

2.10

Food code	Food	No. of times in past wk	Brand code	No. of times/ brand	Size of jar/tinA	Average number of jars on each occasion
7	Breakfast meals such as porridge					.
						.
						.
8	Meat or fish based meals					.
						.
						.
9	Vegetable, pasta or rice based savoury meals					.
						.
						.
10	Milk or cereal based desserts					.
						.
						.
11	Fruit based desserts, not including pure fruit puree					.
						.
						.
12	Pure fruit puree					.
						.
						.

1 = Small jars (100-150 g) Usually from 4 months
2 = Medium jars (160-200 g) Usually from 7 months
3 = Large jars (220-250 g) Usually from 12 months (toddlers)

2.11*Did your baby eat anything else apart from these ready made baby foods in the past week?

0. No *go to 2.13*
1. Yes ☐

2.12

Food code	Food	No. of times in past wk	Average amount on each occasion
13	Weetabix or other wheat bisks		No. of biscuits ☐ ☐☐
14	Other cereals, not including Weetabix or baby cereals		No. of tbsp ☐ ☐☐

2.13

Food code	Food	No. of times in past wk	Average number of tablespoons on each occasion
15	Potatoes		.
16	Rice		.
17	Pasta including tinned spaghetti		.
18	Meat		.
19	Fish		.
20	Beans and pulses, including baked beans, kidney beans, chick peas and lentils		.
21	Other vegetables		.

2.14

Food code	Food	No. of times in past wk	Average amount on each occasion
22	Yogurt and fromage frais		Weight (grams) ☐☐☐ small pot approx 50g avge pot approx 100g
	1) Ordinary wholemilk 2) Low fat 3) Danone baby fromage frais with follow on milk 4) Onky Blok fromage frais with Added vitamins 5) Tescos fromage frais with added vitamins 88) Other (specify) …… *for multiple types use 77*		☐☐
23	Cooked fruit		No. of tbsp ☐ ☐☐
24	Banana		No. of bananas ☐ ☐☐
25	Other fresh fruit:(*1 serving = 1 apple/peach/pear 2 apricot/plum, 10 strawberry, 25 cherry/ grape)*		No. of servings ☐ ☐☐
26	Bread or toast		No. of slices ☐ ☐☐
27	Crackers or breadsticks (Ritz size = 1 Cream cracker size = 2) *(Baby breadstick = 0.3 Adult b'stick = 2)*		No. of crackers or breadsticks ☐ ☐☐
28	Biscuits		No. of biscuits ☐ ☐☐

2.15*Did your baby have anything else to** drink **apart from milk or formula in the past week?

0. No *go to 2.15*
1. Yes ☐

2.16

Food code	Food	No. of times in past wk	Brand code	No. of times/ brand	Volume (oz) on each occasion
29	Baby fruit juice *(for concentrate, use vol. After standard dilution))*				.
					.
					.
30	Baby herbal drinks				.
					.
					.
31	Squash, not including diet drinks labelled low calorie or low sugar				.
	Was it 1. Ribena or hi juice blackcurrant 8. Other				☐
32	Diet squash labelled low calorie or low sugar				.
	Was it ~~1. Low sugar Ribena~~ 8. Other 2. Really Light Ribena				☐
33	Tea *Volume of tea not including milk*				.
34	Water *Not including water used to mix with food or drink which must have water added, such as squash and dried baby food*				.

2.17*Is there anything else he/she has had to eat or drink in the past week that we have not already included? Here is a list to help remind you about foods which he/she may have had but if there are any others which are not on the list please let me know what these are.

0. No *go to section 3*
1. Yes ☐

2.18

Brand/Description	No. of times in past wk	Portion size	Code	Weight

2.19 If fish consumed in question 2.12, record details of types and frequencies of fish below.

Note: Check if number of times in past week entered below agrees with previous answer for this question i.e. question 2.12 food code 19.

NAME OF FOOD/DRINK	**CODE**	**Portion size**	**Number of times in past week**
			☐
			☐
			☐

2.20 DIETARY QUESTIONS ON FOOD ITEMS FORTIFIED WITH ESSENTIAL FATTY ACIDS, PROBIOTICS, PREBIOTICS

We are particularly interested in your baby's consumption of food items with added omega-3, omega-6, probiotics or prebiotics. I have a list of food items that have these things added to help you remember the specific products the baby might have consumed. Are any of there foods or drinks with added omega-3, omega-6, probiotics or prebiotics that the baby has eaten or drunk in the past week?

0.No/1. Yes ☐

If Yes

NAME OF FOOD/DRINK	CODE	Portion	Number of times in last week
			☐
			☐
			☐
			☐
			☐
			☐

3. Introduction of foods and supplement use

Now I'd like to ask you about when various foods were first introduced to your baby. **Note: refer to volunteer's infant feeding diary if possible.**

3.1 How old was he/she when solids were first regularly introduced?

Mths ***wks*** ☐ ***days*** ☐ ☐

or

On what date were solids first regularly introduced? ☐☐ ☐☐ ☐☐

D d m m y y

3.2What was the first solid food he/she regularly ate?

Use the FFQ list to prompt and code this
Use 88 for multiple foods

☐☐

3.3During the past 3 months have you given him/her any vitamins or mineral drops, including iron and fluoride drops?

0. No *go to 3.5*
1. Yes

☐

3.4 If yes, please state which:

Supplement Name	**Code**	**How many days in the last 90?**	**No. of drops per day**	**Did he/she start this:** 1: Less than 1 mth ago 2: 1-2 months ago 3: More than 2 mths ago

3.5 During the past three months have you taken any pills, tonics or tablets to supplement your diet? (e.g. vitamins, minerals, iron tablets, folic acid, fish oils etc.)

0. No *go to section 4*
1. Yes

☐

3.6 If yes, please state which:

Supplement Name	**Code**	**How many days in the last 90**	**Number per day**	**Did you start this:** 1: Less than 1 mth ago 2: 1-2 months ago 3: More than 2 mths ago

4. BABY'S ILLNESSES

I would just like to ask a few questions about any illnesses the baby might have had **at any stage since he/she was born**. *(Prolonged period with <1 week break between bouts - enter 88)* **Note: refer to volunteer's infant health diary if possible.**

4.1 Has he/she had any episodes of chestiness associated with wheezing or whistling in his/her chest? (includes wheezy bronchitis, asthma)

No 0. *go to 4.3*
Yes - number of times ☐☐

4.2 How old was he/she at the start of the first episode? ***mths*** ☐ ***wks*** ☐ ☐

4.3 Other than during the first week of a cold has he/she ever woken at night with coughing for 3 or more nights in a row?

0. No
1. Yes ☐

4.4 *Over the last 3 months, (90 days) on roughly how many *days* ***has he/she had a cough, cold or runny nose?***

0. None
1. 1-15 days ☐
2. 16-30 days
3. 31-45 days
4. 45 days or more (more than half the time)

4.5 Has he/she ever been diagnosed by a doctor as having had pneumonia or bronchiolitis? (don't include bronchitis or "chest infection")

No 0.
Yes – number of times ☐☐

4.6 Has he/she ever been diagnosed by a doctor as having had a chest infection or bronchitis ? (includes wheezy bronchitis)

No 0.
Yes – number of times ☐☐

4.7 Has he/she had any episodes of croup or a croupy cough ?

(i.e. a barking cough worse at night)
No 0.
Yes – number of episodes ☐☐

4. 8 Has he/she had any bouts of vomiting lasting 2 days or longer ?

(do not include possetting or regurgitation)
No 0.
Yes – number of bouts ☐☐

4.9 Has he/she had any bouts of diarrhoea lasting 2 days or longer?

(probe; diarrhoea=frequent unformed stools)
No 0.
Yes – number of bouts ☐☐

4.10 Has he/she ever been diagnosed by a doctor as having an ear infection?

No 0.
Yes – number of times ☐☐

4.11 Has he/she had an itchy skin condition *at any time since birth* ***- by itchy we mean scratching or rubbing the skin a lot? (exclude chicken pox)***

0. No *go to 4.14*
1. Yes ☐

4.12 Has this skin condition ever affected *the cheeks, the outer arms or legs,* ***or the*** *skin creases* ***in the past - by skin creases we mean the folds of the elbows, behind the knees, the fronts of the ankles, or around the eyes?***

0. No
1. Yes ☐

4.13 How old was he/she when the rash *first* ***appeared?*** ***mths*** ☐ ***wks*** ☐ ***ys*** ☐

4.14 Has he/she suffered from a generally dry skin?

(do not include a dry skin in the immediate postnatal period)

0. No
1. Yes ☐
8. To a minor degree

4.15*Has he/she had a** scaly, or red and weeping **skin rash affecting any of the following areas** at any time **since birth:

A) the scalp or behind the ears (including "cradle cap")

0. No
1. Yes ☐

B) around the neck

0. No
1. Yes ☐

C) the cheeks or forehead

0. No
1. Yes ☐

D) either the folds of the elbows or behind the knees

0. No
1. Yes ☐

E) the forearms, wrists, shins or ankles

0. No
1. Yes ☐

F) the shoulders, chest, tummy or back

0. No
1. Yes ☐

G) in the armpits

0. No
1. Yes ☐

H) the nappy area (including nappy rash)

0. No
1. Yes ☐

If **yes to (C), (D), (E), (F) or (G)**,
have you ever been able to clearly link a rash on his/her face, trunk or limbs with teething

0. No
1. Yes ☐

or with specific foods

0. No *go to 4.17*
1. Yes ☐

4.16 If yes, which foods? _____

4.17 Was he/she <u>born</u> with any health problems or abnormalities?

0. No *go to section 5*
1. Yes ☐

4.18 What is the problem? _____ ☐☐

_____ ☐☐

_____ ☐☐

2. Only outside the house

5. ALLERGIES

One of the things we are trying to determine is why some children become allergic to cats & dogs whereas many others don't.

5.1I would first like to ask whether you have *kept a cat* ***at home at any time since your baby was born***

0. No *go to 5.3* ☐
1. Yes

5.2 If yes, and offered, is the cat kept: **if yes & not offered, go to 5.5**

1. Only in a separate room *go to 5.5* ☐
2. Only outside the house *go to 5.5*

5.3 *How often has he/she *visited homes that keep a cat or cats* ***over the last 6 months.***

0. Never
1. Infrequently (once a month or less) ☐
2. Fairly frequently (several times a month)
3. Frequently (several times a week)
4. Every day or almost every day

5.4 If yes, and offered, is the cat kept:

1. Only in a separate room ☐
2. Only outside the house

5.5 And similarly, have you *kept a dog* ***at home at any time since your baby was born?***

0. No *go to 5.7* ☐
1. Yes

If yes, and offered, is the dog kept: **if yes & not offered, go to section 6**

1. Only in a separate room *go to section 6* ☐
2. Only outside the house *go to section 6*

5.6*How often has he/she *visited homes that keep a dog or dogs* ***over the last 6 months.***

0. Never
1. Infrequently (once a month or less) ☐
2. Fairly frequently (several times month)
3. Frequently (several times a week)
4. Every day or almost every day

5.7If yes, and offered, is the dog kept:

1. Only in a separate room ☐

6. HOUSEHOLD HEATING

6.1*How is your flat/house principally heated? ☐☐

1. Gas central heating
2. Ducted central heating
3. Under floor heating
4. Night storage heaters
5. Coal/wood open fires
6. Coal/wood burners
7. Gas fires
8. Electric fires/heaters
9. Paraffin/kerosene heaters
10. Oil central heating
11. Other, *specify*

_____

6.2 Is the room where your child usually sleeps heated in this way? ☐

0. No
1. Yes *go to section 7*

6.3 ***How is the room heated where your child usually sleeps? ☐☐

1. Gas central heating
2. Ducted central heating
3. Under floor heating
4. Night storage heaters
5. Coal/wood open fires
6. Coal/wood burners
7. Gas fires
8. Electric fires/heaters
9. Paraffin/kerosene heaters
10. Oil central heating
11. Other, *specify*

_____

7. SLEEPING ARRANGEMENTS

7.1*Does he/she sleep mainly

1. in the same bedroom as brothers or sisters ☐
2. in the same bedroom as parents
3. in his/her own bedroom
8. other, *specify*

_____

7.2 How many times** per night **(between midnight and 6am) does he/she generally wake for feeding or any other reason?Please answer this in relation to the last 2 weeks? . ☐☐

per night

8.MOTHER'S SMOKING

8.1 Are you currently smoking?

0. No *go to 8.5*
1. Yes

☐

If yes, and offered, is it:

1. Only in a separate room
2. Only outside the house

☐

How many per day? ☐☐

8.2What is your current brand?

_____

8.3Does anyone else smoke in the flat/house, or is he/she ever looked after more

than once a week by anyone who smokes ?

0. No
1. Yes

☐

8.4 If yes, and offered, is it:

1. Only in a separate room
2. Only outside the house

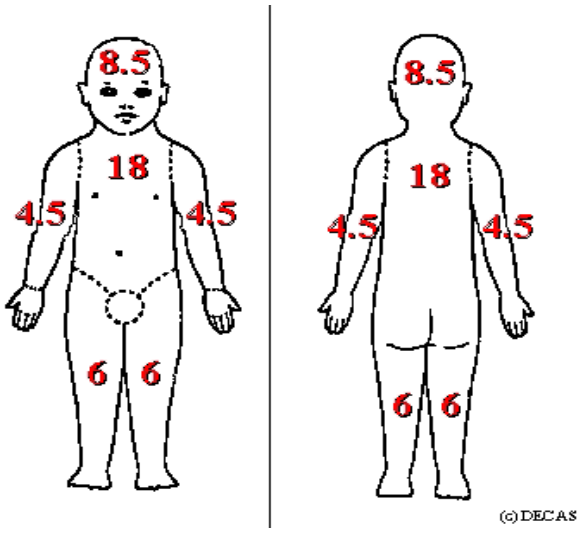
☐

Examiner:

……

9. INFANT CLINICAL EXAMINATION

9.1 SCORAD Results



Topical Steroid use:
Potency (brand name)……..

Amount / month……..

Number of flares / month ……

A: Extent. Please indicate area involved: ☐

B: Intensity ☐

CRITERIA	INTENSITY
Erythema	
Oedema/Papulation	
Oozing/Crust	
Excoriation	
Lichenification	
Dryness (evaluated on uninvolved areas)	

Means of calculation
Intensity Items
(average representative area)
0 = absence
1 = mild
2 = moderate
3 = severe

C: Subjective symptoms. Pruritis + sleep loss ☐

Visual analogue scale (average for the last 3 days or nights)

Pruritis (1-10) ◆·····◆
0 10
Sleep Loss (1-10) ◆·····◆

SCORAD A/5 + 7B/2 + C

☐

9.2 Baby length: ..... cm

9.3 Baby weight : ..... kg

9.4 Has your baby been given any medication, prescription (i.e. antibiotics) or non-prescription (i.e. infacol, Calpol) used in the last 4 weeks?

0 No

1 Yes ☐

If yes to **9.4** please give details below

Therapy	Dose route and regimen	Date started	Date stopped

9.5 Has your baby had any injections or immunisations?

0 No

1 Yes ☐

If yes **to 9.5** please give details below

	1st Immunisations	2nd Immunisations	3rd Immunisations
Polio			
HIB/Diphtheria/Tetanus			
Whooping cough			
Meningitis			
Other – Name……..			
Other – Name……..			

Skin Test Results

Attach tape of wheal read at 15 mins.

Positive (histamine)	Dog
Negative	House dust mite (D. Pteronyssinus)
Tree	Cow's Milk
Grass	Hen's Egg
Cat	Fish

FFQ Administrator

Name:……
……..

Signature:……
……...

Paediatric Nurse

Name:……
……..

Signature:……
……...

Other Administrator (if used)

Name:……
……..

Signature:……
……...

Thank you for giving us this information